

PROGRAM

All sessions will take place in Sundial I/II
Speakers – please arrive ½ hour before you session begins to connect your laptop.

FRIDAY, JANUARY 20

6:00 – 7:00 pm **Registration, Outside of Sundial I/II**

7:00 – 10:00 pm **Conference Opening, Sundial I/II**

7:00 – 7:10 pm **Introductory Remarks,**

7:10 – 8:10 pm **H. Jane Dyson, Scripps Research Institute**
Proteins Utilize Motion and Unfolded States to Expand Their Functional Repertoire

8:10 – 10:00 pm Reception, *Osprey/Flamingo*
Bring Drink Ticket

SATURDAY, JANUARY 21

8:30 am Setup posters, *Sandpiper/Pelican/Osprey/Flamingo*

8:00 – 8:45 am **Continental Breakfast, Sundial I/II**

8:45 am – 12:15 pm **Session I: Protein Structure and Dynamics in Solution Probed by Hydrogen Exchange**
Igor Kaltashov, Session Chair
each talk includes 10 minutes of discussion

9:00-9:15 am Introductory Remarks

9:15-10:15 **S.W. Englander, University of Pennsylvania**
Protein Folding and Misfolding Studied by Hydrogen Exchange, NMR, and MS

10:15-11:00 **C.V. Robinson, Cambridge University**
Protein Folding, Misfolding and Aggregation with HDX MS

11:00-11:15 **Coffee Break, Sundial I/II**

11:15-12:00 **P. Prevelige, University of Alabama**
Viral Particle Structure and Assembly by Chemical Cross-Linking and HDX MS

12:15-1:15 pm **Lunch for Conferees, Poolside**
Sorry no guests, name badge is required

1:15 pm – 4:45 pm **Session II: Chemical Cross-Linking and Selective Covalent Modification**
Elizabeth Komives, Session Chair
each talk includes 10 minutes of discussion

1:15-2:00 pm **B. Gibson, University of California, San Francisco**
Protein Cross-Linking for Tertiary Structure Assignment

2:00-2:45 pm **K. Tomer, NIH/NIEHS**
Probing Protein Interactions of HIV by Surface Modifications and MS

2:45-3:00 **Coffee Break & Snack, Sundial I/II**

- 3:00-3:45 **D. Fabris**, *University of Maryland Baltimore County*
MS3D Methods for the Structural Investigation of Retroviral RNA and Protein-RNA Complexes
- 3:45-4:45 Poster "Advertisements"
- 8:00-10:00 pm** **Poster Session and Dessert**, *Sandpiper/Pelican/Osprey/Flamingo*

SUNDAY, JANUARY 22

8:00 – 8:45 am **Continental Breakfast**, *Sundial I/II*

9:00 am – 1:00 pm **Session III: HDX MS: Experimental Techniques and Emerging Methods**
John Engen, Session Chair
Each talk includes 10 minutes of discussion

- 9:00-9:45 am **E. Komives**, *University of California San Diego*
Mapping Protein Interactions with HDX MALDI MS
- 9:45-10:30 am **M. Deinzer**, *Oregon State University*
Hydrogen/Deuterium Exchange in Combination with Computational Methods to Model Protein Structures
- 10:30-10:45 am** **Coffee Break**, *Sundial I/II*
- 10:45-11:30 am **I.A. Kaltashov**, *University of Massachusetts-Amherst*
Local Exchange Measurements without Proteolysis: HDX/CAD MS
- 11:30 am-12:15 pm **L. Konermann**, *University of Western Ontario*
Time-Resolved Electrospray Mass Spectrometry with On-Line HDX
- 12:15-1:00 pm **E. Forest**, *Institut de Biologie Structurale*
HDX MS of Large Proteins: How to Improve Spatial Resolution
- Afternoon** **Free time**

7:00 – 10:00 pm **Session IV: Radical Labeling Methods and Mass Spectrometry**
Dan Fabris, Session Chair
Each talk includes 10 minutes of discussion

- 7:00-7:45 pm **M. Chance**, *Case Western Reserve University*
Structural Proteomics of Macromolecular Complexes
- 7:45-8:30 pm **R.L. Hettich**, *Oak Ridge National Laboratory*
Protein Surface Mapping by Photochemical Oxidation to Monitor Protein Conformation
- 8:30-9:15 pm **R.W. Vachet**, *University of Massachusetts-Amherst*
Copper-Protein Interactions Studied by Metal-Catalyzed Oxidation Reactions
- 9:15-10:00 pm** **"Night cap"**, *Sandpiper/Pelican/Osprey/Flamingo*
Bring Drink Ticket
Remove all posters after 10 pm

MONDAY, JANUARY 23

8:00 – 8:45 am **Continental Breakfast, Sundial I/II**

9:00 am – 1:00 pm **Session V: HDX MS: Selected Applications**

Lars Konermann, Session Chair

Each talk includes 10 minutes of discussion

9:00-9:45 am **M.L. Gross, Washington University**

Recent Developments and Applications of PLIMSTEX and Related Methods for Protein Structure and Affinity

9:45-10:30 am **A. Heck, Utrecht University**

Structure-Function Relationships of cGMP-Dependent Protein Kinase G

10:30-10:45 am **Coffee Break, Sundial I/II**

10:45-11:30 am **J. Engen, University of New Mexico**

What HDX MS Can Reveal about Protein Function: The Case of Src-Family Kinases

11:30 am -12:15 pm **M. Fitzgerald, Duke University**

Analysis of Protein Folding and Function by SUPREX

12:15-1:00 pm **V.L. Woods, University of California San Diego**

Applications of Enhanced Amide Hydrogen Deuterium Exchange LC-MS (DXMS): From Single Amide-Resolution of Protein Dynamics to Determination of Protein 3-D Structure,

POSTERS

1. **Towards Conformer-Specific Characterization of Protein Structure and Dynamics: Combination of Hydrogen Exchange and Tandem Mass Spectrometry**, Rinat R. Abzalimov and Igor A. Kaltashov, *Department of Chemistry, University of Massachusetts at Amherst*
2. **ESI-MS Studies on Ligand Binding to Peculiar DNA and RNA Structures**, Dorothee Balbeur, Valérie Gabelica, Frédéric Rosu, Dominique Baiwir, Edwin De Pauw, *Laboratoire de Spectrométrie de Masse, Université de Liège, Institut de Chimie, Bat. B6c, B-4000 Liège, Belgique*
3. **Preliminary Deuterium Exchange Mass Spectrometry Studies of Group IA PLA2 Utilizing Novel Methods for Highly Disulfide Bonded Proteins**, John Burke, Mark Karbarz, Ray Deems, Virgil Woods, and Edward A. Dennis, *Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA*
4. **Dissecting the Mechanism Underlining the Pharmacodynamics of Partial Versus Full Agonists of PPAR α** , Scott A. Busby, Swati Prasad, Michael J. Chalmers, Bruce D. Pascal and Patrick R. Griffin, *The Scripps Research Institute, 5353 Parkside Dr. Jupiter FL*
5. **Development of H/D Exchange Mass Spectrometry as a Biophysical Assay for the Classification Nuclear Receptor Modulators**, Michael J. Chalmers, Scott A. Busby, Alessandra Cervino, Bruce D. Pascal, Swati Prasad, and Patrick R. Griffin, *The Scripps Research Institute, 5353 Parkside Drive (RF-1), Jupiter, Florida*
6. **Solution Phase H/D Exchange Coupled with Mass Spectrometry to Probe Conformation Changes between Dark State and Signaling State of Photoactive Yellow Protein from *Halorhodospira halophila***, Guilong (Charles) Cheng,¹ Michael A. Cusanovich,² Vicki H. Wysocki^{1,2} ¹*Department of Chemistry, University of Arizona*, ²*Department of Biochemistry and Molecular Biophysics, University of Arizona*
7. **Observing Intramolecular Hydrogen/Deuterium Scrambling through Monitoring the Distribution of Deuterium Incorporation in Test Peptides**, John K. Chik and David C. Schriemer, *Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta*

8. **SUPREX Behavior of Some “Non-Ideal” Proteins**, Susie Y. Dai and Michael C. Fitzgerald, *Department of Chemistry, Duke University, Durham, NC*
9. **Hydrogen Exchange and FT-ICR Mass Spectrometry Reveal Structural Differences between Ovine Prion Protein Monomer and Oligomers**, T. Daubenfeld¹, H. Rezaei², J. Grosclaude², G. van der Rest¹ and A.-P. Bouin¹ ¹ *Laboratoire des Mécanismes Réactionnels, CNRS UMR 7651, Ecole Polytechnique, 91128 Palaiseau cedex, France,* ² *Unité Virologie et Immunologie Moléculaires, INRA, Jouy-en-Josas, France*
10. **Hydrogen/Deuterium (H/D) Exchange is a Versatile Tool to Determine Regions of Destabilization in ALS-Causing SOD1 Mutant Proteins**, Armando Durazo; Bryan F. Shaw; Kym F. Faull; Julian P. Whitelegge; Edith B. Gralla; Joan S. Valentine; *University of California, Los Angeles*
11. **Proton-Deuteron Amide Exchange Studies by Mass Spectrometry for Protein Resonance Assignment in Nuclear Magnetic Resonance**, Lianmei Feng, James H. Prestegard, Ron Orlando, *Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602*
12. **The Determination of High-Affinity Protein/Inhibitor Binding Constants by ESI/HXMS**, Lee E. Frego and Walter C. Davidson, *Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT*
13. **Phosphoinositide Binding Regulates α -actinin CH2 Domain Structure: Analysis by Hydrogen/Deuterium Exchange Mass Spectrometry**, S. J. Full, J. Watson, M.L. Deinzer, P.S. Ho, and J.A. Greenwood, *Departments of Biochemistry and Biophysics and Chemistry, Oregon State University, Corvallis, OR*
14. **Exploring Folding Intermediates of Indole-3-glycerol Phosphate Synthase (α IGPS), a (β/α)₈ barrel protein, by Hydrogen Exchange Mass Spectrometry**, Zhenyu Gu & C. Robert Matthews, *Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA*
15. **Microsecond Oxidation by Laser Flash Photolysis Differentiates between the Apo and Holo Forms of Myoglobin**, David M. Hambly and Michael L. Gross, *Department of Biochemistry, Department of Chemistry, Washington University in St. Louis, Box 1134, One Brookings Dr, St. Louis, MO*
16. **The Use of Hydrogen-Deuterium Exchange to Investigate the Role of Structural Conformation and Protein Folding of the Amyloidogenic Protein beta-2-Microglobulin**, John P. Hodkinson, Antoni J. H. Borysik, Sheena E. Radford, Alison E. Ashcroft, *Astbury Centre for Structural Molecular Biology, The University of Leeds, Leeds, UK*
17. **Quantitative Mapping of Native Protein Conformational Landscapes by HXMS**, Sheila S. Jaswal¹, Amy Ruschak², Judith Frydman¹, Andrew Miranker² ¹ *Department of Biological Sciences, Stanford University* ² *Department of Molecular Biophysics and Biochemistry, Yale University*
18. **Electron Capture Dissociation Proceeds with a Low Degree of Intramolecular Migration of Amide Hydrogens**, T.J.D. Jorgensen¹, J.V. Olsen^{1,2}, M. Mann^{1,2}, P. Roepstorff¹, H. Gardsvoll³, M. Ploug³ ¹ *Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark* ² *Department of Proteomics and Signal Transduction, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany* ³ *Finsen Laboratory, Rigshospitalet, Strandboulevarden 49, DK-2100 Copenhagen Ø, Denmark*
19. **Characterization of Inter-subunit Interactions in Bacteriophage P22 Portal Complexes using Mass Spectrometry Based Hydrogen/deuterium Exchange**, Sebyung Kang¹, Anton Poliakov² and Peter E. Prevelige Jr.² *Department of Biochemistry & Molecular Genetics*¹ and *Department of Microbiology*² *University of Alabama at Birmingham*
20. **An Aptly Positioned Azide Group Enables Facile MS Detection of Cross-Linked Peptides**, Piotr T. Kasper[a], JaapWillem Back[a], Géraldine Masson[b], Aloysius F. Hartog[b], Leo J. de Koning[a], Jan H. van Maarseveen[b], Anton O. Muijsers[a], Luitzen de Jong[a] and Chris G. de Koster[a] [a] *Swammerdam Institute for Life Sciences (SILS), University of Amsterdam, Nieuwe Achtergracht 166, 1018WV Amsterdam, The Netherlands* [b] *Van 't Hoff Institute of Molecular Sciences (HIMS), University of Amsterdam, Nieuwe Achtergracht 129, 1018WS Amsterdam, The Netherlands*
21. **Site-Specific Amide Hydrogen Exchange in Melittin Probed by Electron Capture Dissociation Fourier Transform Ion Cyclotron Resonance Mass Spectrometry**, Hye Kyong Kweon and Kristina Håkansson*, *Department of Chemistry, University of Michigan, 930 North University Avenue, Ann Arbor, MI 48109-1055, USA*

22. **Mass Spectrometry Assisted Assignment of NMR Resonances in Reductively ¹³C-Methylated Proteins**, Megan A. Macnaughtan and James H. Prestegard, *Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia*
23. **Amide Hydrogen Exchange Reveals Conformational Changes in Hsp70 Chaperones Important for Allosteric Regulation**, Wolfgang Rist, Christian Graf, Bernd Bukau and Matthias P. Mayer, *Zentrum für Molekulare Biologie Heidelberg (ZMBH), Universität Heidelberg, Germany*
24. **Cross-Linking of a Small Molecule to the Hcv Ns5b Polymerase**, Graham A. McGibbon¹ and George Kukolj² ¹ *Departments of Biochemistry and Biomedical Sciences and of Chemistry, McMaster University, 1200 Main St. W., Hamilton, ON, L8N 3Z5 and 2 Boehringer Ingelheim Canada Ltd., Laval, QC*
25. **Free Radical Driven Isotopic Scrambling in the Electron Capture Dissociation of Linear Peptides**, Cheng Lin, Jason J. Cournoyer, Jason L. Pittman, Marina Belyayev, Bogdan A. Budnik, Peter B. O'Connor, *Mass Spectrometry Resource, Department of Biochemistry, Boston University School of Medicine, 670 Albany Street, Boston, MA 02118*
26. **Determination of the Oligomeric State of Phage Portal Protein Complexes by Electrospray Mass Spectrometry**, ¹Anton Poliakov, ²Esther van Duijn, ¹Chi-Yu Fu, ²Albert Heck, ¹Peter Prevelige, ¹*Depts of Microbiology and Biochemistry, University of Alabama at Birmingham, Birmingham, AL. USA;* ²*Dept of Biomolecular Mass Spectrometry, Utrecht University, Utrecht, The Netherlands.*
27. **Identification and Structure Determination of Tyrosine Nitration in Human Eosinophils Using Fticr Mass Spectrometry in Combination with Immunoanalytical and Isotope- Labelling Procedures**, Michael Przybylski^{1*}, Alina Petre¹, Reinhold Weber¹, Markus Bachschmid², Volker Ullrich², Martina Ulrich³ and Gerd Doering³ ¹*Laboratories of Analytical Chemistry and* ²*Biological Chemistry, University of Konstanz, Germany;* and ³*Institut für Allgemeine Hygiene, University of Tübingen, Germany*
28. **The Allosteric Activation of Coagulation Factor VIIa Visualized by Hydrogen Exchange**, Kasper Rand^{1†}, Thomas J. D. Jørgensen[†], Ole H. Olsen¹, Egon Persson¹, Ole N. Jensen[†], Henning R. Stennicke¹. & Mette D. Andersen¹ ¹ *Dept. of Haemostasis Biochemistry, Novo Nordisk A/S, Novo Nordisk Park, DK-2760 Måløv, Denmark* [†] *Dept. of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark*
29. **Determining the Mechanism of Bacterial Fibre Assembly using Non-Covalent Electrospray Ionisation- Mass Spectrometry**, Rebecca J. Rose^{*}, Han Remaut[†], Gabriel Waksman[†], Sheena E. Radford^{*}, Alison E. Ashcroft^{*} ^{*}*Astbury Centre for Structural Molecular Biology, University of Leeds, LS2 9JT, UK* [†]*Institute of Structural Molecular Biology, School of Crystallography, Birkbeck College, Malet Street, London, WC1E 7HX, UK*
30. **The Collaboratory for MS3D**, Carmen Pancerella,¹ Dan Fabris,² Julian Eaton,² Irwin D. Kuntz,³ Larry A. Rahn,¹ Ken Sale,¹ Christine L. Yang,¹ Malin M. Young^{1†} ¹*Sandia National Laboratories, Livermore, CA 94551-0969,* ²*University of Maryland, Baltimore County, Baltimore, MD 21250,* ³*University of California, San Francisco, San Francisco, CA 94143*
31. **Investigating Hyaluronan-Protein Interactions using Hydrogen/Deuterium Amide Exchange Mass Spectrometry**, Nicholas T. Seyfried^a, James A. Atwood III^a, Andrew Almond^b Anthony J. Day^b, Ron Orlando^a and Robert J. Woods^a ^a*Complex Carbohydrate Research Center, University of Georgia, 330 Riverbend Road, Athens, GA 30602.* ^b*Faculty of Life Sciences, University of Manchester, Manchester, M60 1QD, U.K.*
32. **The Role of Oxidative Protein Damage and Localized Conformational Change in Sporulation—A Case for Oxidative Stress Signaling**, Joshua S. Sharp¹, Daniel M. Sullivan², John Cavanagh², and Kenneth B. Tomer¹ ¹ *Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC 27709,* ² *Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC 27695*
33. **Structural Insights of Human Centrin 2 Revealed by H/D Exchange, Pepsin Digestion, and Correlation Studies**, Justin Sperry¹, Don Rempel¹, Ilan Vidavsky¹, Rajiv Kumar², Michael L. Gross¹ ¹ *Department of Chemistry, Resource for Biomedical Mass Spectrometry, Washington University in St. Louis, St. Louis, MO 63130* ² *Department of Internal Medicine, Biochemistry, and Molecular Biology, Mayo Clinic, Rochester, MN 55905*
34. **Structural Flexibility of Membrane-Fusogenic Peptides Examined by D/H-Exchange Reactions**, W. Stelzer^{*}, H. Stalz, A. J. R. Heck[†] and D. Langosch^{*} ^{*}*Chair of Biopolymer Chemistry, Technische Universität München, Weihenstephaner Berg 3, D-85354 Freising-*

Weihenstephan, Germany, ¹Department of Biomolecular Mass Spectrometry, Utrecht University, The Netherlands

35. **DXMS: Software for Automated Analysis of H-D Exchange Mass Spectral Data**, David D. Stranz¹ and Virgil L. Woods, Jr.² ¹Sierra Analytics, Inc., Modesto, CA and ²University of California San Diego, La Jolla, CA
36. **Amide ¹H/²H Exchange Reveals Changes in IκBα Conformational Flexibility Upon Binding to NF-κB**, Stephanie M. E. Truhlar and Elizabeth A. Komives, *Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California, United States.*
37. **Plans for Improved DXMS Software for Automated Analysis of H-D Exchange Lc-Mass Spectral Data**, Virgil L. Woods, Jr.¹ and David D. Stranz² ¹University of California San Diego, La Jolla, CA and ²Sierra Analytics, Inc., Modesto, CA
38. **Structural Changes of Microtubules Induced by Taxol**, Hui Xiao, Pascal Verdier-Pinard, Narcis Fernandez-Fuentes, Andras Fiser, Ruth H Angeletti, George A. Orr, Susan B Horwitz, *Albert Einstein College of Medicine, Bronx, NY*
39. **Hydrogen-Deuterium Exchange Mapping of the Arp2/3 Complex Using LC-ESI-MS**, Wendy Zencheck and Hui Xiao, PhD, *Albert Einstein College of Medicine, Department of Biochemistry*

Poster 1

Towards Conformer-Specific Characterization of Protein Structure and Dynamics: Combination of Hydrogen Exchange and Tandem Mass Spectrometry

Rinat R. Abzalimov and Igor A. Kaltashov

Department of Chemistry, University of Massachusetts at Amherst

Combination of hydrogen exchange in solution (HDX) and protein ion fragmentation in the gas phase followed by MS analysis has become a powerful tool to probe biopolymer structure and dynamics.¹ One of the most significant advantages offered by this “top down” methodology is the possibility to carry out the experimental measurements on-line, *i.e.* without the need to quench the exchange in solution prior to protein fragmentation. This eliminates back-exchange, a major factor that adversely affects the resolution afforded by HDX MS measurements carried out using a classical “bottom-up” HDX MS scheme.²

Another potential advantage offered by the top-down HDX MS measurements is a possibility to characterize protein behavior in solution in a conformation-specific fashion. Indeed, correlated or semi-correlated exchange patterns allow various conformational states of a protein to be distinguished from one another, and each conformer can be mass-selected and fragmented to produce amide protection maps specific for this particular conformer. However, this advantage has not been realized in practice as yet, since top-down HDX MS measurements are usually carried by activating the entire population of protein ions (without mass selection) to maximize the fragmentation yield. Our previous attempt to use SORI CAD as a means of producing fragment ions from mass-selected precursors in HDX MS experiments resulted in extensive hydrogen scrambling³ due to a slow heating nature of this ion activation method.⁴

In the present work we explore the utility of CAD in a collision cell of hybrid quadrupole-time-of-flight mass spectrometer as a means of obtaining information on protein structure and dynamics in a conformer-specific fashion. HDX CAD MS measurements are carried out under conditions favoring semi-correlated exchange using a set of proteins related to a well-characterized Cellular Retinoic Acid Binding Protein I (CRABP I).⁵ The experimental results indicate that hydrogen scrambling is minimal and does not compromise the quality of the protein dynamics measurements when the highly charged precursor ions are subjected to collisional activation in RF-only quadrupole cell. The incidence of hydrogen scrambling increases dramatically when multi-stage fragmentation (MS³) is used to generate fragment ions. These experiments pave the way towards implementation of conformer-specific characterization of protein behavior in solution and define the limitations of a top-down HDX MS experimental strategy.

Acknowledgements. This work was supported by a grant from the National Institutes of Health R01 GM061666.

(1) Kaltashov, I. A.; Eyles, S. J. *J. Mass Spectrom.* Crossing the phase boundary to study protein dynamics and function: combination of amide hydrogen exchange in solution and ion fragmentation in the gas phase. **2002**, *37*, 557-565

(2) Engen, J. R.; Smith, D. L. *Anal. Chem.* Investigating protein structure and dynamics by hydrogen exchange MS. **2001**, *73*, 256A-265A

(3) Hoerner, J. K.; Xiao, H.; Dobo, A.; Kaltashov, I. A. *J. Am. Chem. Soc.* Is there hydrogen scrambling in the gas phase? Energetic and structural determinants of proton mobility within protein ions. **2004**, *126*, 7709-7717

(4) McLuckey, S. A.; Goeringer, D. E. *J. Mass Spectrom.* Slow heating methods in tandem mass spectrometry. **1997**, *32*, 461-474

(5) Xiao, H.; Kaltashov, I. A. *J. Am. Soc. Mass Spectrom.* Transient structural disorder as a facilitator of protein-ligand binding: native H/D exchange-mass spectrometry study of cellular retinoic Acid binding protein I. **2005**, *16*, 869-879.

Poster 2

ESI-MS Studies on Ligand Binding to Peculiar DNA and RNA Structures

Dorothee Balbeur, Valérie Gabelica, Frédéric Rosu, Dominique Baiwir, Edwin De Pauw
*Laboratoire de Spectrométrie de Masse, Université de Liège, Institut de Chimie, Bat. B6c,
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Several ligands that bind to nucleic acids are capable of inducing a structural change in their target. A first example is aptamers: aptamers are RNA or DNA sequences specifically designed to bind a molecular target. In most cases of aptamers designed against small molecules, ligand binding induces a structural change in the aptamer. Another example is the case of DNA G-quadruplex structures. G-quadruplexes are formed by Hoogsteen hydrogen bonding between four guanines that form the so-called G-quartets. Single strands comprising several stretches of guanines can form intramolecular G-quadruplex structures which can vary in strand orientation and loop position. Some ligands that have the potential to selectively bind to G-quadruplex DNAs have been reported to induce structural changes in the G-quadruplexes. We used electrospray ionization mass spectrometry for the study of several nucleic acid-ligands systems. The ligand-induced structural changes were probed by traditional spectroscopic methods in solution. In the future, we would like to explore the possibilities offered by mass spectrometric techniques to study these structural changes in the gas phase.

The binding of neomycin and streptomycin to their RNA aptamers (23 and 46 bases respectively) has been characterized in the absence and presence of Mg^{2+} ions by ESI-MS. In the case of the streptomycin aptamer, solution studies suggest that three Mg^{2+} cations are needed for ligand binding. Mass spectrometry results suggest that only one magnesium cation is essential for ligand binding. UV-vis spectroscopy results suggest that both Mg^{2+} and streptomycin ligand contribute to the aptamer stability. In the case of the neomycin aptamer, up to three ligands bound were detected, and Mg^{2+} is not required for ligand binding.

Several ligand complexes with different G-quadruplex structures (parallel or antiparallel strands) were characterized by ESI-MS. Some ligands show a high selectivity for one or the other structure, suggesting that specific binding sites allow the differentiation between the possible conformations. Specific cation binding (NH_4^+) inside the G-quadruplex has also been detected. Preliminary results on gas-phase H/D exchange showed very fast exchange in the case of quadruplexes that include these specific cations. Future work will be focused on the elucidation of the mechanisms of this fast exchange, and on the characterization of H/D exchange rates in the presence/absence of ligands.

Poster 3

Preliminary Deuterium Exchange Mass Spectrometry Studies of Group IA PLA₂ Utilizing Novel Methods for Highly Disulfide Bonded Proteins

John Burke, Mark Karbarz, Ray Deems, Virgil Woods, and Edward A. Dennis

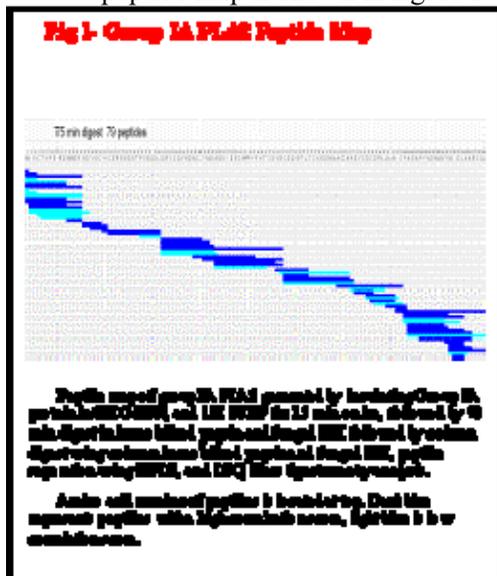
Department of Chemistry and Biochemistry, University of California San Diego, La Jolla, CA, 92037

Abstract:

Group IA Phospholipase A₂ (PLA₂) is a 119 amino acid protein that catalyzes the hydrolysis of 2-acyl fatty acids from phospholipids. *Naja naja naja* Cobra venom PLA₂ has a high degree of structural similarity to other secreted phospholipases and has been used as a model to understand the actions of the more complicated human secreted PLA₂s. Our aims in studying this enzyme are to explore how this enzyme interacts with the lipid water interface and to determine what lipid interfacial sites exist on the enzyme.

We employed an online HPLC digestion system developed by Dr. Virgil Woods at UCSD (*Proc Natl Acad Sci U S A.* 2003 June 10; 100(12): 7057–7062.) This system employs an immobilized pepsin, fungal XIII protease, and a C18 column to digest and separate peptides before analyzing them using LCQ Mass Spectrometry. This entire setup is run at 2° Celsius to minimize back exchange. Previously our laboratory has crystallized Group IA showing a tightly packed structure with seven disulfide bonds in only 119 amino acids. This basic system proved inadequate in digesting the highly disulfide bonded Group IA PLA₂. We have developed a novel immobilized protease digestion system that is capable of producing peptide maps for Group IA PLA₂.

This system employs a preliminary treatment with immobilized Fungal XIII and pepsin before HPLC injection. The protein is incubated with guanidine hydrochloride and TCEP at maximal concentrations of 8M and 1 M respectively on ice for 15 min and then incubated with the immobilized proteases for 60 minutes also on ice. The peptides are then added to the online system. This procedure has tripled the number of peptides obtained compared to the peptide maps obtained using purely column based digestion methods. The peptide maps that have been generated give full coverage over the entire protein and have significant overlap in all regions of the protein. The total peptide count is around eighty peptides for the 119 amino acid protein. The peptide map is shown in figure 1.



Control experiments testing for both back and on exchange during the digestion process show that both are within acceptable ranges for generating deuterium exchange mass spectrometry data. Deuterium exchange on-exchange studies have been done and show variable regions of exchange throughout the proteins. We are currently testing deuterium exchange patterns in varying conditions of temperature, denaturing conditions, and ligand concentrations.

Poster 4

Dissecting the Mechanism Underlining the Pharmacodynamics of Partial Versus Full Agonists of PPAR γ

Scott A. Busby, Swati Prasad, Michael J. Chalmers, Bruce D. Pascal and Patrick R. Griffin
The Scripps Research Institute, 5353 Parkside Dr. Jupiter FL 33458

Type 2 Diabetes Mellitus (DM) is characterized by insulin resistance and impaired insulin secretion. Enhancing insulin sensitivity has been a leading strategy in the treatment of Type 2 DM and PPAR γ , a member of the nuclear receptor superfamily, is known to play a major role in the modulation of insulin sensitivity by forming a heterodimer with RXR α and functioning as a transcription factor in a ligand-dependent fashion to activate insulin response genes. Current therapeutics that target PPAR γ to increase insulin sensitivity, such as the full agonists pioglitazone and rosiglitazone, lead to a number of unwanted side effects that limit their clinical utility. Research strategies have now focused on the development of PPAR γ partial agonists that have demonstrated the ability to enhance insulin sensitivity without the unwanted side effects in preclinical studies. Our goal is to use hydrogen/deuterium exchange mass spectrometry (H/D exchange) to probe the dynamics of the PPAR/RXR interaction upon binding to full and partial agonists in an effort to determine whether partial agonists differentially stabilize the PPAR/RXR complex and perhaps lead to changes in coactivator selectivity.

To determine the differences in activation of the PPAR/RXR complex upon binding of full and partial agonists, PPAR/RXR LBD heterodimer was exposed to four known PPAR γ activators. Using transient transfection assays and a biochemical FRET assay, we have shown that the ligands GW1929 and MRL 20 are full agonists, while nTZDpa and MRL 24 are partial agonists. Following incubation with each of the ligands, the PPAR/RXR dimer was exposed to deuterium and analyzed by H/D exchange. Perturbation maps were generated by subtracting the H/D exchange map of each complex from the H/D exchange map of the apo receptor for both PPAR and RXR. The results of these analyses demonstrate that the H/D exchange profiles for the PPAR portion of the heterodimer bound to the two partial agonists are nearly identical and differ significantly from the profiles obtained for the PPAR portion bound to the two full agonists. The exchange profiles for the RXR part of the heterodimer under the same conditions show very little difference in exchange regardless of the binding of a full or partial agonist with the heterodimer.

To determine whether the changes in dynamics of the PPAR/RXR heterodimer induced by the binding of partial agonists involved a change in coactivator selectivity, we screened the same four PPAR γ ligands against a co-activator gene library using a Gal4 trap assay. Our results confirm the previous observation that PPAR γ full agonists are known to robustly recruit the co-activator SRC1a. Interestingly, the partial agonists we screened prefer recruitment of a different co-activator suggesting there is a difference in coactivator selectivity upon binding the PPAR/RXR heterodimer with partial agonists. This difference in selectivity may be the result of a formation of a novel epitope for coactivators or via allosteric control of RXR .

Development of H/D Exchange Mass Spectrometry as a Biophysical Assay for the Classification Nuclear Receptor Modulators

Michael J. Chalmers, Scott A. Busby, Alessandra Cervino, Bruce D. Pascal, Swati Prasad, and Patrick R. Griffin
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Introduction

In recent years there has been intensive research to aid in the understanding of the mechanism of ligand mediated activation of nuclear receptors (NR).[1] NRs are the molecular targets of drugs for the treatment of a wide range of diseases such as type II diabetes, cancer, cardiovascular disease, and hormone replacement therapy.[1-3] The ability to alter transcriptional activity through ligand binding induced conformational changes to the receptor provides opportunities for pharmaceutical intervention. Amide H/D exchange is an ideal technique for studying these changes in NR conformation. Peroxisome proliferator-activated receptor gamma (PPAR γ) is a member of the NR superfamily and the molecular target of the glitazones, drugs used for treatment of insulin resistance associated with type II diabetes. Recently it has been shown that partial agonists of PPAR γ have insulin sensitization properties while lacking several adverse effects associated with full agonist drugs.[4] We have demonstrated that H/D exchange MS could discriminate between a full (GW1929) and partial (nTZDpa) PPAR γ agonist, however the experiments remain time consuming despite continual advances in automation (Chalmers *et al*, in press).

This work describes an experimental strategy that will allow rapid characterization of PPAR γ ligands as full or partial agonists based upon the collection of the minimum data required for a statistically valid classification. Comprehensive H/D exchange experiments have been performed with a set of three full (Rosiglitazone, GW1929 and MRL20) and four partial (MRL 24, nTZDpa, MCC555, MK886) PPAR γ agonists to determine regions yielding the most statistically significant diagnostic information (for example, residues 288-295 located in Helix 12). To increase precision (and minimize random and systematic errors) in the H/D exchange rate measurement, PPAR + ligand and apo PPAR samples are analyzed simultaneously in a single, fully automated experiment in which the order of the analysis has been randomized. All experiments comprise four replicate analyses. For the classification of uncharacterized ligands, the experimental method can now be optimized to follow the H/D exchange kinetics of a select few peptides. Data collection and analysis time will be reduced, yielding a significant reduction in the time required to classify the degree of PPAR γ modulation.

1. Gronemeyer, H., J.A. Gustafsson, and V. Laudet, 2004. **3**(11): p. 950-964.
2. Kiec-Wilk, B., A. Dembinska-Kiec, A. Olszanecka, M. Bodzioch, and K. Kawecka-Jaszcz. 2005. **56**(2): p. 149-62.
3. Vamecq, J. and N. Latruffe. Lancet, 1999. **354**(9173): p. 141-8.
4. Berger, J.P., A.E. Petro, K.L. Macnaul, L.J. Kelly, B.B. Zhang, K. Richards, A. Elbrecht, B.A. Johnson, G. Zhou, T.W. Doebber, C. Biswas, M. Parikh, N. Sharma, M.R. Tanen, G.M. Thompson, J. Ventre, A.D. Adams, R. Mosley, R.S. Surwit, and D.E. Moller. Mol Endocrinol, 2003. **17**(4): p. 662-76.

Poster 6

Solution Phase H/D Exchange Coupled with Mass Spectrometry to Probe Conformation Changes between Dark State and Signaling State of Photoactive Yellow Protein from *Halorhodospira halophila*

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Photoactive yellow protein (PYP) from halophilic purple phototrophic bacterium, *Halorhodospira halophila*, is a small 14 kDa chromophore containing protein, and is the structural prototype for a large family of signaling proteins with a common motif called a PAS domain. The chromophore, p-hydroxycinnamic acid is photoisomerized from the *trans* to *cis* form in blue light, driving the structural changes that lead to the formation of the signaling state.

Solution H/D exchange coupled with MS is a powerful technique to study protein structure and dynamics. The rates at which amide hydrogens undergo H/D exchange when exposed to D₂O depend on the participation of these amide hydrogens in intramolecular hydrogen bonding and their solvent accessibility. We are applying this technique to interrogate the protein conformation changes between dark state and the signaling state of PYP.

Following photoisomerization the signaling state forms in ~2 ms and PYP returns spontaneously to the dark state in less than one second, thus the equilibrium lies far to the dark state making characterization of the signaling state difficult. However, the mutation of methionine 100 to alanine (M100A) results in a long lived signaling state (half-life ~7 minutes), and under steady state illumination with blue light results in ~90% of M100A in the signaling state at any time. We have determined the H/D exchange time course profiles for the wild-type in the dark, and M100A in both the dark and in the signaling states (see Fig. 1) for the intact proteins. Clearly M100A exchanges substantially faster in the signaling state than in the dark state, which is consistent with a substantial structural change in the signaling state. Subsequently, the proteins were digested by pepsin online, and the resulting peptides were analyzed by LC-MS. The 30s exchange data for peptides from wild-type PYP and M100A in both the light and dark are shown in Fig. 2. These exchange data, and the time course of H/D exchange for all three states studied (PYP, M100A, M100A dark), can be analyzed in terms of specific structural domains (N-terminal cap, central beta sheet, sensing domain, connecting helix and C-terminus) to develop a model describing the structural changes that occur on the conversion from the dark to the signaling state.

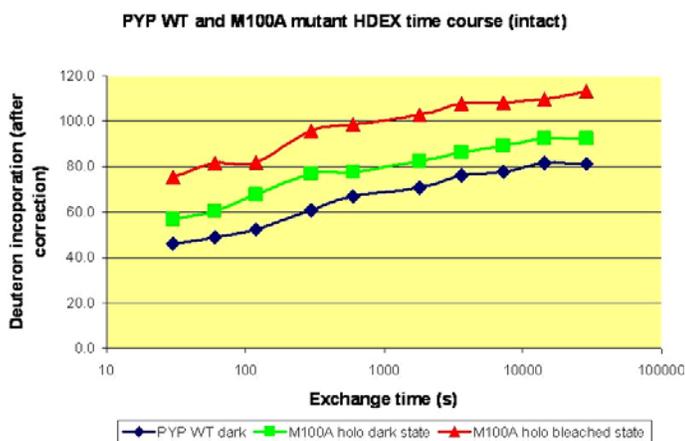


Fig. 1. Intact protein exchange time course

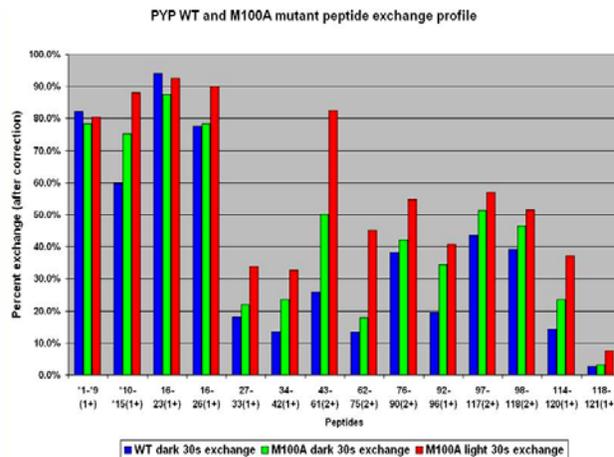


Fig. 2. 30s protein exchange, peptic Peptides deuterium uptake comparison

Poster 7

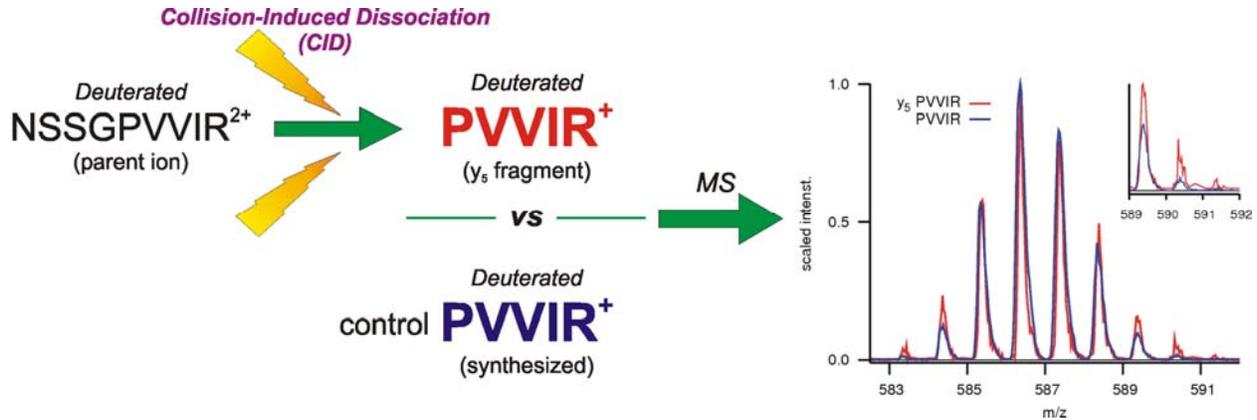
Observing Intramolecular Hydrogen/Deuterium Scrambling Through Monitoring the Distribution of Deuterium Incorporation in Test Peptides

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Hydrogen/deuterium exchange mass spectrometry (HDX MS) opens new and exciting opportunities for studying the inner-workings of biomolecular machines. However reliance on enzymatic pepsin digestion imposes limits on the ability of HDX MS to resolve locations of deuteration. In-machine fragmentation of these deuterated peptides would be a powerful means of increasing the resolving power of HDX MS. However questions on the degree to which tandem mass spectrometry methods alter the initial deuterium localization dogs its potential application. The literature is divided on the magnitude of this *scrambling*, at minimum suggesting strong context dependences. These published studies use a wide variety of different systems and methods which complicates comparisons and, for the most part, are difficult to apply in general. *We propose that intramolecular scrambling can be observed by comparing the distribution of deuterium incorporation of a fragment originating from a deuterated parent peptide to that of a synthesized and labeled copy of the same fragment.*

A series of deuterated synthetic test peptides (NSSFGP_nVVIR for n=1, 3 and 8) was subjected to collision-induced dissociation (CID) while undergoing back-exchange. Particular attention was paid to the P_nVVIR y_{n+4} ion fragments from their respective NSSFGP_nVVIR parent. At similar average deuteration, the variance in incorporation of these CID fragments was noticeably greater than that from synthesized copies. This was a results of “extra” deuterium(s) being transferred from the N-terminal NSSFG amino acids. Furthermore it was possible to estimate the potential number of transfers (~2 deuteriums) using binomial distribution model. This method can be used for validating tandem HDX MS data across a wide variety of systems and is further demonstration of the value of determining the distribution of deuterium incorporation in HDX MS experiments.



SUPREX Behavior of Some “Non-Ideal” Proteins

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SUPREX is an H/D exchange- and MALDI-MS-based technique for studying the thermodynamics of protein folding reactions. The SUPREX technique is analogous to more conventional spectroscopy-based techniques that have been widely used to evaluate protein folding free energies (ΔG_f values) and so-called m -values (i.e. $\delta\Delta G_f/\delta[\text{Denaturant}]$). Like conventional spectroscopy-based techniques, SUPREX requires an assumption of reversible “two-state” folding for the accurate evaluation of ΔG_f and m -values. An additional assumption of EX2 exchange kinetics is also important for SUPREX. Here we report on the SUPREX behavior of several model protein systems that are known to be “non-ideal” in the SUPREX experiment (i.e. non-two-state folding or under EX1/EX2 exchange kinetics).

The model systems in this study include several protein systems (α -lactalbumin, cytochrome *c*, intestinal fatty acid binding protein (IFABP), and myoglobin) in which partially folded equilibrium intermediate states have been previously identified in other biophysical studies. Theoretical and experimental results are also presented on an additional protein, ubiquitin, that exhibits EX1/EX2 exchange behavior (i.e. $k_{\text{int}} \approx k_{\text{cl}}$) at the denaturant concentrations used in the SUPREX experiment. Our results on ubiquitin suggest that such EX1/EX2 exchange behavior does not adversely affect SUPREX analyses, at least in the case of ubiquitin. Our results also indicate that the cooperativity of α -lactalbumin’s unfolding reaction as determined by SUPREX was essentially identical to that determined by circular dichroism (CD) spectroscopy and that the m -values generated in each case (see Figure 1) were relatively small for a two-state folding protein of α -lactalbumin’s size. Such a small m -value is consistent with the formation of partially folded equilibrium intermediate state(s).

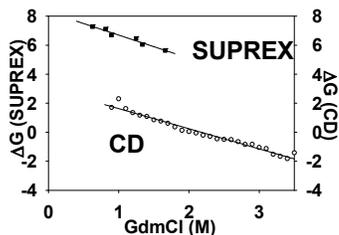


Figure 1. Results on α -lactalbumin . SUPREX(closed squares) vs. CD (open circles).

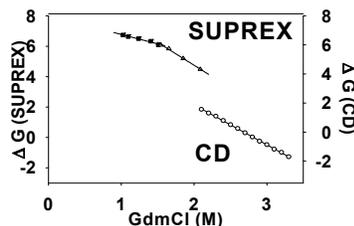


Figure 2. Results on cytochrome *c* . SUPREX(closed squares and open triangles) vs. CD (open circles).

Interestingly, our SUPREX results on cytochrome *c*, IFABP, and myoglobin revealed different unfolding behavior at different denaturant concentrations. At higher denaturant concentrations (i.e. denaturant concentrations similar to those accessible in the CD experiment), the SUPREX data yielded the same m -values as those generated from CD denaturation curves (see, for example, cytochrome *c* results in Figure 2). However, different unfolding behaviors were observed for these proteins at the lower denaturant concentrations that are uniquely accessed by SUPREX (see, for example, cytochrome *c* results in Figure 2). These results suggest that SUPREX is a useful probe of the high energy equilibrium unfolding intermediates in protein folding reactions that can often go undetected in more conventional spectroscopy-based studies of the chemical denaturant-induced equilibrium unfolding reactions of proteins.

Poster 9

Hydrogen Exchange and FT-ICR Mass Spectrometry Reveal Structural Differences Between Ovine Prion Protein Monomer and Oligomers

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Mass spectrometry has become a powerful tool for the analysis of biomolecules in the post-genomic era. Most notably, it can provide information about the three-dimensional structure of proteins and protein complexes in solution by employing amide hydrogen exchange, enzymatic digestion and analysis of the resulting peptides by mass spectrometry [1]. Differences in solution phase surface accessibility of different regions of the protein are reflected by differences in deuterium incorporation in the peptides corresponding to those regions.

The prion protein (PrP) is a 22 kDa protein whose exact cellular function is still unknown. However, PrP is believed to play a crucial role in mammalian transmissible spongiform encephalopathies (TSE) that are fatal neurodegenerative disorders including BSE, scrapie and Creutzfeldt-Jacob disease [2]. The key event in the development of these diseases is thought to be the conversion of the host prion protein (PrP^C) into a pathogenic conformer (PrP^{Sc}).

Recently, the spontaneous formation of soluble PrP oligomers during heat induced partial unfolding of the prion protein has been reported [3]. Currently, no three-dimensional structure for these oligomers is available. As small, soluble oligomers are thought to play an essential role in diseases associated with protein misfolding [4] we thus aimed at obtaining information about their structure by hydrogen exchange and FT-ICR mass spectrometry. Due to the high resolution as afforded by FT-ICR mass spectrometry, a chromatographic separation of the peptides prior to MS analysis was not necessary, allowing for direct observation of hydrogen exchange of all peptides in one single spectrum.

The hydrogen exchange pattern observed for the monomeric PrP is in good agreement with the crystal structure, which validates our approach. The hydrogen exchange pattern of PrP oligomers, on the other hand, differs significantly from that of the monomeric form. Our findings are in very good agreement with FT-IR studies [3] and cloning experiments. The hydrogen exchange experiments on monomeric and oligomeric PrP define for the first time the regions that undergo structural transitions during the oligomerization process of the protein.

[1] L. A. Busenlehner, R. N. Armstrong, *Archives Biochem. Biophys.* **433** (2005) 34-46.

[2] S. B. Prusiner, *Science* **216** (1991) 136-144.

[3] H. Rezaei, F. Eghiaian, J. Perez, B. Doublet, Y. Choiset, T. Haertle, J. Grosclaude, *J. Mol. Biol.* **347** (2005) 665-679.

[4] M. Stefani, C. M. Dobson, *J. Mol. Med.* **81** (2003) 678-699.

Poster 10

Hydrogen/Deuterium (H/D) Exchange is a Versatile Tool to Determine Regions of Destabilization in ALS-Causing SOD1 Mutant Proteins

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Mutations in the metallo-enzyme Cu,Zn superoxide dismutase (SOD1) cause amyotrophic lateral sclerosis (ALS) and ongoing research points to SOD1 aggregation as the underlying cause. While SOD1 aggregation is diminished by the high stability of the holo-protein ($T_m \sim 90^\circ\text{C}$), the protein may exist in vivo in a demetallated, disulfide-reduced state for a considerable time. Considering that unstable or partially unfolded proteins have higher propensities to aggregate than their stable counterparts, the disulfide-reduced, apo state may be the pathogenic form of SOD1.

In our studies we use ESI-MS to measure the extent and rate at which the backbone amide hydrogens undergo H/D exchange in deuterated buffer. We have conducted studies to examine the global H/D exchange behaviors of disulfide-intact and disulfide-broken hWT SOD1 and several ALS-causing SOD mutants. We have found that reducing the disulfide bonds in different SOD1s leads to increased rates (and extents) of deuterium incorporation into the proteins' backbones. In some ALS mutants, reduction of the disulfide bond leads to a protein that has H/D exchange behavior consistent with an unstructured polypeptide.

We have used also time-resolved, site-specific H/D exchange to determine the regions in which the A4V ALS mutant protein is destabilized relative to human wild-type SOD1 and to characterize the regions in which the exchange properties of hWT SOD1 change upon reduction of its disulfide bond. Many regions of the A4V mutant have the same H/D exchange kinetics of the corresponding regions in human wild-type SOD1, but we have found some regions in which A4V exhibits increased rates of H/D exchange. Interestingly, the H/D exchange behavior of disulfide-broken hWT SOD is identical to that of disulfide-intact A4V in many regions. In addition, we have found that the electrostatic loops of both A4V and (disulfide-intact and disulfide-broken) hWT exhibit H/D exchange behavior consistent with these regions being unstructured. In contrast, we have observed that the greek-key loops in these proteins are structured and exhibit protection from complete exchange.

Poster 11

Proton-Deuteron Amide Exchange Studies by Mass Spectrometry for Protein Resonance Assignment in Nuclear Magnetic Resonance

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Assigning peaks from each amino acid in 2D ^1H - ^{15}N HSQC spectrum is often a first step for the structural characterization of biomolecules by nuclear magnetic resonance (NMR). It is especially important when those molecules fail to form crystals suitable for diffraction studies. Here we explore a novel approach for protein resonance assignment that relies on the ability of NMR and Mass Spectrometry (MS) to monitor the rates of exchange of an amide proton for a water deuteron. MS can provide information on the peptide sequence containing a position that exchanges, while NMR can provide resolved cross peaks that yield structure and drug binding information. By correlating amide exchange rates from the two sources, we hope to achieve the assignment of NMR peaks from proteins that are difficult to pursue by the usual triple resonance NMR techniques.

The feasibility of our strategy has been previously tested on a small protein system, human ubiquitin (1D3Z, 8.5kDa).¹ The NMR measurements employ Hadamard transform (HT) methods to extend the time scale of exchange rate measurement and improve measurement precision.² High mass resolution and high mass accuracy allow peptic peptide identification and H/D amide exchange rate quantification by MS.

The current obstacle to assignment is the need for localization of deuterium incorporation to a specific amino acid of a peptide fragment. Here we demonstrate the possibility that the same digested peptides analyzed by MS methods can be collected from HPLC columns and deuterium incorporation assessed site-by-site from variation in resolved amide resonance intensities of peptides in NMR spectra. Our target is ST6Gal1, a 38 kDa sialyltransferase, a protein with considerable biological significance. Assignment in this case is aided by ^{15}N -labeling of specific amino acids to reduce numbers of peaks that must be monitored and correlated with MS data.

As a second approach to site-specific measurement of hydrogen-deuterium exchange, electron capture dissociation-Fourier transform ion cyclotron resonance (ECD-FTICR) MS is explored as a means of improving resolution. This technique provides ultra high mass resolution and more extensive fragmentation. The absence of a pepsin digestion step is anticipated to further minimize the severe back-exchange issue. The proton/deuteron scrambling issue anticipated for ECD is under exploration by comparing H/D exchange MS data to NMR data.

Reference

1. Feng, Lianmei; Orlando, Ron; Prestegard, J.H. Mass Spectrometry assisted assignment of NMR resonances in ^{15}N labeled proteins. *Journal of the American Chemical Society* 126(44):14377-14379, 2004
2. Bougault, Catherine; Feng, Lianmei; Glushka, John; Kupce, Eriks; Prestegard, J. H. Quantitation of rapid proton-deuteron amide exchange using hadamard spectroscopy. *Journal of Biomolecular NMR* 28(4):385-390, 2004.

Poster 12

The Determination of High-Affinity Protein/Inhibitor Binding Constants by ESI/HXMS

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Novel Aspect:

The use of a SUPREX based method to determine protein/compound binding constants using automated liquid handling and ESI.

Introduction:

The determination of protein/ligand binding constants is a critical phase of pharmaceutical drug discovery. Often, the molecular assay used to measure binding affinities reaches a "wall" due to the low concentrations of the reagents necessary for very potent ligands.

Recently, SUPREX has been shown to be a method which can measure protein/ligand binding constants. SUPREX offers the advantage of having no theoretical upper limit to the binding affinity as well as requiring only a small amount of protein and ligand. The use of ESI offers advantages over MALDI including greater mass accuracy and amenability to automation.

Methods:

Protein was diluted into a series of deuterated exchange buffers containing increasing denaturant concentrations. After a period of time, exchange was quenched and the sample immediately analyzed by HPLC/ESI. All sample handling was performed with a Gilson 215 liquid handler.

Results:

The instrument set-up presented here utilizes automated liquid handling to perform dilutions, incubations, quenches, sample injections, HPLC, and ESI analysis. This automation yields a moderate throughput method, with each stability curve taking approximately 1.5 hours to generate.

The method has been shown to generate accurate K_{dS} for a protein with a number of small molecule inhibitors having a wide range of potencies. In these experiments, m -values were generated with and without ligand by varying the exchange time.

Poster 13

Phosphoinositide Binding Regulates α -Actinin CH2 Domain Structure: Analysis by Hydrogen/Deuterium Exchange Mass Spectrometry

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α -Actinin is an actin bundling protein that regulates cell adhesion by directly linking actin filaments to integrin adhesion receptors. The phosphoinositides, phosphatidylinositol (4,5)-P₂ and phosphatidylinositol (3,4,5)-P₃, bind to the calponin homology 2 domain of α -actinin regulating interaction with actin filaments and integrin receptors. Both phosphoinositides inhibit α -actinin bundling activity. However, only phosphatidylinositol (3,4,5)-P₃ can disrupt α -actinin bundled actin filaments and interaction with integrins. The goal of this study was to determine the mechanism by which phosphoinositide binding regulates α -actinin function using hydrogen-deuterium exchange mass spectrometry to monitor the solvent accessibility of the calponin homology 2 domain. Global hydrogen-deuterium exchange data suggested that phosphatidylinositol (3,4,5)-P₃ binding increases calponin homology 2 domain dynamics, whereas phosphatidylinositol (4,5)-P₂ appeared to decrease the structural dynamics of the domain. Local hydrogen-deuterium exchange, based on analysis of peptic fragments from the calponin homology 2 domain, showed that phosphatidylinositol (3,4,5)-P₃ binding significantly increased solvent accessibility of the α -E helix whereas phosphatidylinositol (4,5)-P₂ binding decreased solvent accessibility of the neighboring α -F helix. Although structural modeling shows that the α -E and α -F helices are in close proximity to the phosphoinositide binding site, the results suggest that phosphoinositides do not directly regulate the actin-binding sequence within the CH2 domain. We propose that the α -E and α -F helices are involved in intramolecular interactions between the N- and C-terminal domains of the full-length α -actinin anti-parallel homodimer and that phosphoinositides regulate bundling activity and interaction with integrin receptors by influencing this interaction and altering the orientation of the N-terminal actin-binding domain.

Poster 14

Exploring Folding Intermediates of Indole-3-glycerol Phosphate Synthase (sIGPS), a $(\beta/\alpha)_8$ Barrel Protein, by Hydrogen Exchange Mass Spectrometry

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A previous circular dichroism study of the equilibrium and kinetic folding mechanisms of indole-3-glycerol phosphate synthase (sIGPS), a member of the $(\beta/\alpha)_8$ barrel family, demonstrated the presence of stable and transient intermediates with substantial secondary structure. To obtain detailed structural information on these intermediates, the equilibrium and kinetic folding reactions of sIGPS were monitored by hydrogen/deuterium exchange-mass spectrometry. The intact protein and pepsin-digested fragments at various urea concentrations or refolding intervals were studied by electrospray and MALDI-TOF, respectively, after $^2\text{H}_2\text{O}$ labeling and quenching in acid. The results indicate that the regions from $(\beta/\alpha)_{2-5}\beta_6$ were protected in the equilibrium intermediate, while N-terminal $(\beta/\alpha)_1$ and C-terminal $\alpha_6(\beta/\alpha)_{7-8}$ regions were not protected against exchange. The manual mixing refolding study revealed a kinetic intermediate possessing similar protection patterns, but with decreased protection against exchange in the boundary regions. When compared with the structure of equivalent intermediates in the alpha subunit of Trp synthase, another $(\beta/\alpha)_8$ barrel protein, this study suggests that the N-terminal region plays a crucial role in directing the folding of the family of $(\beta/\alpha)_8$ barrel proteins.

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Microsecond Oxidation by Laser Flash Photolysis Differentiates between the Apo and Holo Forms of Myoglobin

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We recently detailed a method using a 248-nm KrF laser to form hydroxyl radicals from hydrogen peroxide [1]. Using a scavenger, the radical is consumed within one microsecond, a timescale so rapid that the oxidation induced unpacking of secondary structure cannot occur [2]. When applied to apomyoglobin, we observed that Phe 138 located at the back of the porphyrin binding pocket is not oxidized, indicating that the pocket is closed. Furthermore, His 81, Leu 86, Pro 88, Leu 89 and His 93 (Table 1) are oxidized demonstrating that the F-helix is exposed to solvent as suspected by NMR data [3]. As the F-helix is essential for binding of the porphyrin to myoglobin, we demonstrate that there are significant differences in the oxidized residues on the F-helix. His 93, which is critical for ligating the iron atom of the porphyrin ring, is not oxidized in this experiment. Secondly, Leu 86 and Leu 89 are mostly buried when the F-helix is ordered, and these residues are not oxidized. Pro 88 is exposed in the holomyoglobin crystal structure, so it is not surprising that it is oxidized in both the apo and holo forms. Interestingly, Lys 87 is oxidizing only in the holo form – most likely because the more reactive Leu 86 and Leu 89 residues are now buried, resulting in oxidations on the less reactive, but still exposed, Lys 87. Finally, we demonstrate that Leu 137, which is oxidized in the apo form when the pocket closes, is not oxidized in the holo form, where the protein is more structured. Taken together, the data indicates that the laser flash photolysis method is capable of differentiating between different conformational states present in the apo versus the holo form of myoglobin. We are extending this work to other protein-ligand systems including protein-peptide and protein-protein systems.

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Gross laboratory members: Drs. Rohrs, Vidavsky & Walters;
GAM Laser Inc, Orlando, FL.
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References:

- Hambly, D. M.; Gross, M. L. Laser Flash Photolysis of Hydrogen Peroxide to Oxidize Protein Solvent-Accessible Residues on the Microsecond Timescale. *J Am Soc Mass Spectrom* **2005**.
- Gilmanshin, R.; Williams, S.; Callender, R. H.; Woodruff, W. H.; Dyer, R. B. Fast events in protein folding: relaxation dynamics of secondary and tertiary structure in native apomyoglobin. *Proc Natl Acad Sci U S A* **1997**, *94*, 3709-13.
- Eliezer, D.; Wright, P. E. Is apomyoglobin a molten globule? Structural characterization by NMR. *J Mol Biol* **1996**, *263*, 531-8.

Table 1: Solvent exposure of the holo form and observed oxidations for the F-helix apo and holo forms of myoglobin.

		F-helix		Solvent Accessibility (Å ²)
		Apo	Holo	Holo
GLY	80			10
HIS	81	Y	Y	64
HIS	82	N	N	9
GLU	83			54
ALA	84			44
GLU	85			33
LEU	86	Y	N	10
LYS	87	N	Y	58
PRO	88	Y	Y	46
LEU	89	Y	N	22
ALA	90			0
GLN	91			32
SER	92			22
HIS	93	Y	N	0
ALA	94			5
THR	95			36
LYS	96			62

The Use of Hydrogen-Deuterium Exchange to Investigate the Role of Structural Conformation and Protein Folding of the Amyloidogenic Protein beta-2-Microglobulin

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The pathological deposition and fibril formation of the normally soluble protein beta-2-microglobulin is central to the onset of the disease dialysis-related amyloidosis. The population of non-native conformations of the protein is thought to be key to initiating the fibrillation process.

We have used ESI-MS hydrogen-deuterium exchange (HDX) to investigate the structural dynamics of beta-2-microglobulin, in addition to selected protein variants which exhibit altered stability, under physiologically relevant pH conditions. Our investigations show beta-2-microglobulin to exhibit a dynamic nature even in its folded state at pH 7, and also indicate a core region encompassing approximately 30 residues which has significantly higher resistance to HDX than the remainder of the protein. The core region undergoes HDX by an EX1 mechanism (Figure 1(a)), thus suggesting that whilst beta-2-microglobulin unfolds globally on a slow timescale ($t_{1/2} \sim 24\text{h}$) there is evidence for a globally unfolded species ($\sim 5\%$) in equilibrium with the native state. The global unfolding rates for the variants are faster than that of the wild-type protein ($t_{1/2} \sim 1\text{-}2\text{h}$) (Figures 1(b) and (c)), whilst the protected core region is significantly smaller.

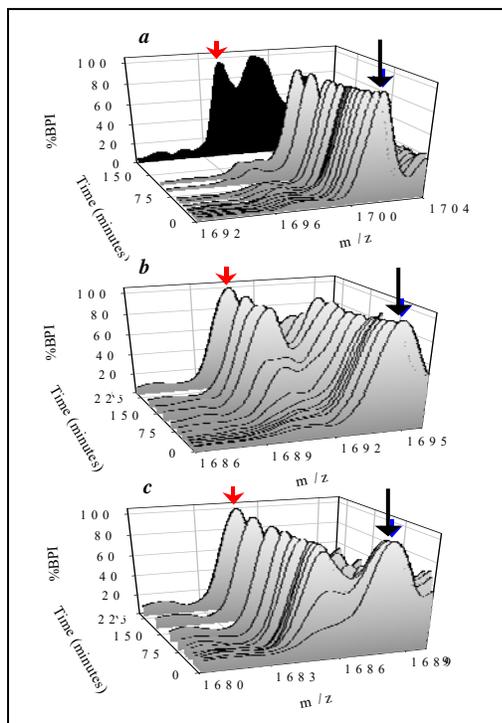


Fig. 1: ESI-MS 7+ ions of deuterated:

- (a) beta-2-microglobulin,**
 - (b) variant I7A,**
 - (c) variant F30A,**
- undergoing HDX over time.**

Long arrows denote the base peak at $t=0$ that exchanges by EX2 kinetics.

Short arrows denote the peak that emerges via EX1 kinetics i.e. fully exchanged protein.

Quantitative Mapping of Native Protein Conformational Landscapes by HXMS

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In evolving proteins, Nature balances the need to design a specific function into a particular fold with the requirement to ensure the ability of the polypeptide chain to reach and maintain that fold. Many proteins appear to have evolved native states to which they can spontaneously fold, but which are vulnerable to misfolding and aggregation under certain conditions, leading to the toxic deposition of amyloid fibers as plaques in neurodegenerative and other amyloid diseases such as Parkinson's, and Dialysis Related Amyloidosis. These examples underscore the fact that the native state is actually an ensemble of conformations, including rare partially and fully unfolded species, which can contribute to the protein's role in both biology and disease. Therefore, understanding that role requires the ability to determine the conformations populated from the native state, together with the energetics and kinetic barriers between them, or "map the conformational landscape", under the physiological and pathological conditions of interest.

Following the rate of hydrogen-deuterium exchange (HX) from the native state by ESI mass spectrometry is an ideal approach to map native landscapes. The high sensitivity of HX allows detection of even rarely sampled non-native conformations, and the observed rate of exchange can be related to either the kinetic barrier or the thermodynamics of the underlying conformational equilibrium, under the appropriate regimes. In contrast to NMR, which detects the ensemble-averaged rate of exchange for each amide independently, MS monitors all labile sites simultaneously, enabling the direct detection of cooperatively populated intermediates. However, the contribution of all sites to the observed distribution also complicates interpretation of the HX behavior, especially when not at the two extremes of the HX regimes. In addition, unambiguously extracting the features of the ESI mass spectrum relevant only to the deuterium distribution for an exchanging protein is often difficult, due to instrument, ionization and sample artifacts that cause multi-peak distributions even for native samples not undergoing HX.

Here we describe developments to address both challenges, and their application to HXMS of the amyloidogenic protein beta-2-microglobulin, and the chaperonin substrate actin. We apply analytical solution and numerical simulations approaches to model simple HXMS behavior over the full range of regimes, and probe the relationship between the experimental observables and the rate constants of the conformational equilibrium underlying exchange. In addition, we use a deconvolution approach to extract unobscured deuterium distributions from complex HXMS experimental data, to allow more quantitative comparison to the modeled behavior. These advances in quantitating and interpreting HXMS behavior will make native conformational mapping by HXMS a more powerful tool to understand the critical interplay between conformation and function for biologically important and disease related proteins.

Poster 18

Electron Capture Dissociation Proceeds with a Low Degree of Intramolecular Migration of Amide Hydrogens

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Considerable controversy exists in the literature as to the occurrence of intramolecular migration of amide hydrogens upon gas phase fragmentation of protonated peptides and proteins. This phenomenon has important implications for the application of gas phase fragmentation as an experimental tool to obtain site-specific information about the incorporation of deuterium into peptides and proteins in solution. Using a unique set of peptides having a polarized deuterium incorporation (i.e. with their carboxyl-terminal half labeled with deuterium) we have showed unambiguously, that hydrogen (¹H/²H) scrambling is such a dominating factor during low-energy and high-energy collisional activation of protonated peptides, that the original deuterium pattern of these peptides is completely erased (Jorgensen, et al., J. Am. Chem. Soc. 2005, 127, 2785-2793; Jorgensen, et al., Mol. & Cell. Proteomics 2005, 4, 1910-1919). Taking further advantage of this unique test system we have now investigated the occurrence of scrambling upon electron capture dissociation (ECD). Our results show that the degree of scrambling upon ECD is significantly reduced. We observe, however, a remarkable increase in the degree of scrambling with increasing c-ion size. These findings are discussed in the context of the nonergodic fragmentation model.

Poster 19

Characterization of Inter-subunit Interactions in Bacteriophage P22 Portal Complexes using Mass Spectrometry based Hydrogen/deuterium Exchange

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The dsDNA bacteriophage P22 has a ring shaped dodecameric complex of the 84 kDa portal protein which forms the central channel of the DNA packaging motor.

CryoEM reconstruction of the C-terminally truncated P22 portal (1-602) complexes at ~8Å has revealed a ring-like morphology shared by portal complexes of phi-29, SPP1, T3 and herpes simplex virus. Although a cryoEM reconstruction at this resolution can provide global structural information, it lacks sufficient resolution to directly identify the amino acids involved in subunit-subunit contacts.

To identify the amino acids involved in subunit-subunit contacts in the P22 portal ring complexes, we performed comparative hydrogen/deuterium exchange analysis of monomeric and dodecameric portal protein using FT-ICR mass spectrometry (LTQ-FT). The high resolution and sensitivity of FT-ICR provided high sequence coverage (~95% of 732 residues) without extensive chromatographic separation. H/D exchange experiments provided evidence of inter-subunit interactions at regions which were predicted to be conserved helical modules based on threading using the crystal structure of the phi-29 portal complexes.

An Aptly Positioned Azide Group Enables Facile MS Detection of Cross-Linked Peptides

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Chemical cross-linking is used to identify nearest neighbours in protein complexes, while identifying cross-linked amino acids residues is a powerful method to validate models of the 3-D structure of proteins. However, an efficient identification of cross-linked sites remains a challenging task. We present a new type of cross-linker containing an aptly positioned azide group in the spacer (see **Fig.1**). Treatment of prepared cross-linked peptides with phosphine or dithiol leads to cleavage in a fraction of the cross-linked species and reduction of the azide without cleavage in the rest (reaction's basics described by JW Back *et al.**). The unprecedented simultaneous occurring of cleavage and reduction without cleavage in cross-linked peptides (see **Fig.2**), along with the ease to chromatographically separate products from unmodified peptides, provide a firm basis for a powerful analytical strategy, combining diagonal chromatography and mass spectrometry, for the rapid identification of cross-linked sites in protein complexes, even in complex mixtures.

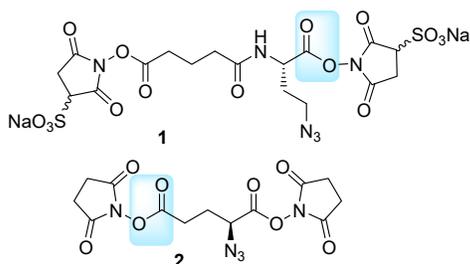


Figure 1. Structures of two cross-linkers containing an aptly positioned azide in the spacer. **1**, BACOP; **2**, BAG; The activated ester bonds in these cross-linkers are nucleophilically attacked by amines in proteins resulting in formation of amide bonds. Highlighted ester bonds are converted into scissile amide bond upon cross-linking, patent pending.

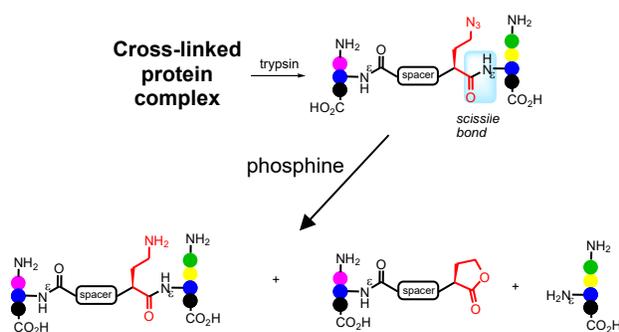


Figure 2. Phosphine-induced reactions in cross-linked peptides. In a complex mixture only the target cross-linked peptides selectively react with phosphine to give both cleavage of the cross-linker and reduction of the azide. Reversed phase liquid chromatography of the fractions before and after phosphine treatment (diagonal chromatography) reveals the cross-linked peptides and their cleavage products in shifted fractions. Mass spectrometry of the combined shifted fractions enables facile identification of cross-

*) Back, J.W., David, O., Kramer, G., Masson, G., Kasper, P.T., de Koning, L.J., de Jong, L., van Maarseveen, J.H., and de Koster, C.G. (2005) Mild and Chemoselective Peptide Bond Cleavage of Peptides and Proteins at Azido-homoalanine, *Angew Chem* **117**, 8160–8164; *Angew Chem Int Ed Engl*, **48**, 7946–7950

Poster 21

Site-Specific Amide Hydrogen Exchange in Melittin Probed by Electron Capture Dissociation Fourier Transform Ion Cyclotron Resonance Mass Spectrometry

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Abstracts

Backbone amide hydrogen/deuterium (H/D) exchange in combination with proteolytic digestion and electrospray ionization mass spectrometry (ESI-MS) has been widely used to probe protein conformation, interaction, and folding. However, most MS/MS strategies involve heating, deuterium scrambling prior to or during dissociation and thereby loss of site-specific information. Electron capture dissociation (ECD) has been suggested to occur faster than energy randomization, *i.e.* be a non-ergodic process. That hypothesis suggests ECD can fragment deuterated peptides without causing hydrogen scrambling and thereby provide amino acid residue-specific amide hydrogen exchange rates (as a result of minimal scrambling). Such a feature would improve the resolution of approaches involving solution-phase amide hydrogen exchange combined with mass spectrometry for protein structural characterization. We explored this hypothesis using melittin, a small bee venom protein, as our model system. Exchange rates of amide hydrogen in methanol were calculated from consecutive *c*-type ion pairs and compared with previous NMR data: the amide hydrogens of leucine 13 and alanine 15, located at the unstructured kink surrounding proline 14 in the melittin structure adopted in methanol, appear as fast exchangers and the amide hydrogens of leucine 16 and lysine 23, buried within the helical regions of melittin, appear as slow exchangers. However, calculations based on *c*-type ions for other amide hydrogens do not correlate well with NMR data, and evidence for deuterium scrambling in ECD was obtained from $z\bullet$ -type ions. The latter observation may be a result of the radical character of z ions.

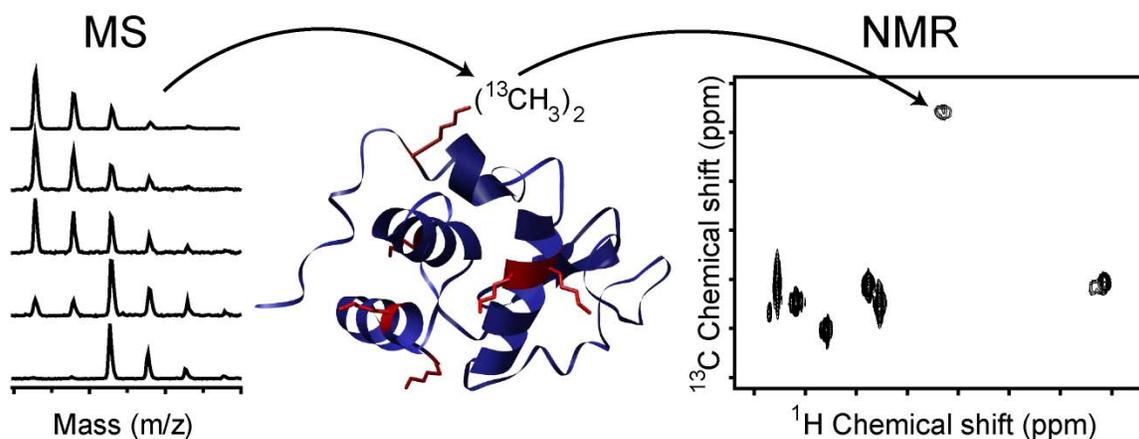
Mass Spectrometry Assisted Assignment of NMR Resonances in Reductively ^{13}C -Methylated Proteins

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Reductive ^{13}C -methylation of proteins has been used as an isotope labeling strategy to study protein structure, function, and dynamics by nuclear magnetic resonance (NMR) spectroscopy. Two ^{13}C -methyl groups are added to each primary amine, the ϵ -amine of lysine and the N-terminal amine, of a protein under mild reaction conditions to produce ^{13}C -dimethylamines. The NMR signals from the ^{13}C methyls introduced in this way can be sensitive probes of protein-protein and protein-ligand interactions. Distance restraint data for the determination of protein structure can also be acquired by measuring paramagnetic perturbations to the ^{13}C methyl NMR signals. However, in order to make full use of this ^{13}C labeling strategy, the resulting ^{13}C -dimethylamine peaks in the NMR spectrum must be assigned to the N-terminal amino acid and lysines of the protein.

The assignment approach presented here relies on the site-specific reactivity of the lysines and N-terminal amine of a protein and the measurement of isotope incorporation by MS and NMR. A protein sample is prepared with a sub-stoichiometric amount of ^{13}C -formaldehyde followed by excess natural abundance formaldehyde. Under these conditions, a protein sample is produced with varying $^{13}\text{C}/^{12}\text{C}$ ratios at each dimethylamine. The percentage of ^{13}C incorporation at each dimethylamine depends on the rate of reductive methylation at each site, which is affected by the amine's pKa and steric accessibility. Mass spectrometry (MS) is used to quantitate the isotope incorporation levels assigned to specific peptide sequences containing the dimethylamines. These same levels are measured with NMR from peak intensities in ^1H - ^{13}C HSQC or HMQC spectra and correlated with the MS data to make assignments. Hen egg white lysozyme and concanavalin A were used as model proteins to demonstrate this assignment strategy. Preliminary results for the application of the method to alpha-2,6-sialyltransferase will also be discussed.



Macnaughtan, M. A.; Kane, A. M.; and Prestegard, J. H. *J. Am. Chem. Soc.* **2005**, *127*, 17626-17627.

Poster 23

Amide Hydrogen Exchange Reveals Conformational Changes in Hsp70 Chaperones Important for Allosteric Regulation

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Hsp70 chaperones assist protein-folding processes by a nucleotide-driven cycle of substrate binding and release. Although structural information is available for the isolated nucleotide-binding (NBD) and substrate-binding domains (SBD) in the high affinity conformation, the low affinity conformations and the conformational changes associated with mutual allosteric regulation remained largely enigmatic. Using amide hydrogen exchange in combination with mass spectrometry we analyzed the *Escherichia coli* Hsp70 homologue DnaK as full-length protein and its individual domains in the nucleotide-free and ATP-bound conformation. We found a surprising degree of flexibility in both domains. The comparison of the full-length protein with the isolated domains demonstrates a mutual stabilization of both domains. Interestingly, the linker region that connects NBD and SBD is solvent exposed in the absence of nucleotide and completely buried in the presence of ATP suggesting a pivotal role in inter domain communication.

Poster 24

Cross-Linking of a Small Molecule to the Hcv Ns5b Polymerase

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Hepatitis C virus (HCV) is a flavivirus that is the etiological agent of non-A non-B hepatitis, which chronically infects over two percent of the world population. Current therapies consist of a combination of pegylated-interferon and ribavirin. A sustained response is not achieved for a large proportion of patients so specific anti-HCV chemotherapies are in demand.

To meet the clinical need, attention has focused on the essential activities of the linear polyprotein encoded by HCV viral RNA genome. The HCV NS5B RNA-dependent RNA polymerase emerged as a prime target in the search for specific HCV anti-virals. As a consequence a series of benzimidazole 5-carboxamide compounds, which function as allosteric inhibitors, had been discovered through a high-throughput screening enzyme assay. Later the compounds were shown to inhibit the cellular RNA replication of a HCV sub-genomic replicon. Further studies distinguished this class of compounds from other non-nucleoside NS5B inhibitors that bind to distinct allosteric sites through the isolation and characterization of resistant replicon mutants localized in a particular structural region.

Over the past decade of intense scrutiny, the three dimensional structure of the NS5B polymerase has been determined as the apo-enzyme form and with some inhibitory molecules. It has an organization comparable to other nucleic acid polymerases with the familiar features of fingers, palm and thumb domains that are organized in a "right-hand" motif. A distinctive feature of the HCV polymerase active site cavity is the protrusion of a unique beta-hairpin from the thumb subdomain that both structural and biochemical studies indicate plays a role in the initiation of de novo RNA synthesis. The HCV polymerase also features two loops that bridge the fingers and thumb subdomain and result in an encircled active site. This feature is now known to be shared by other RNA-dependent RNA polymerases from rhinovirus, bacteriophage phi6, rabbit hemorrhagic disease virus, bovine viral diarrhea virus, Norwalk virus and poliovirus. Interestingly, the interface between the HCV polymerase N-terminal λ 1 loop and the thumb subdomain is the location of a GTP binding site although its precise biological role is unsolved.

To investigate the binding of benzimidazole 5-carboxamide compounds and to elucidate the binding region(s) of the NS5B polymerase, cross-linking experiments with a photo-reactive analogue followed by capillary chromatography and high resolution mass spectrometry analysis of the enzyme was also undertaken. Through these experiments and other biochemical information, a novel binding site was localized for these inhibitors at the junction of the thumb-domain and the N-terminal finger loop consistent with detection of attachment to a tryptic peptide identified as NMVYSTTSR.

Poster 25

Free Radical Driven Isotopic Scrambling in the Electron Capture Dissociation of Linear Peptides

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Deuterium scrambling of labeled peptides is well known during Collisionally Activated Dissociation (CAD), but since Electron Capture Dissociation (ECD) is supposedly non-ergodic, it is expected that deuterium scrambling will not impact tandem mass spectrometry experiments if ECD is used as the dissociation method. The presented data demonstrate that, while the initial cleavage may indeed be non-ergodic, secondary cleavages cannot, and many of the products from these long-lived radical intermediates exhibit lifetimes sufficient for certain deuterium migration events to occur. In these experiments, 16-mer peptides were synthesized with four glycine residues with each glycine having either d0- (undeuterated) or d2- (deuterated) form. Comparison of the ECD spectra of these two forms shows that deuterium atoms that are positioned on the alpha carbon of glycine can migrate during ECD – provided that the peptide involved has the capacity for forming hydrogen bonds to maintain a tertiary structure in the gas phase after electron capture. Glycine alpha-carbon positions were chosen because they are known to efficiently stabilize a radical, so the initially formed radical can easily migrate to this position via hydrogen (or in this case deuterium) abstraction. This data is unlikely to impact deuterated peptides that are formed using H/D exchange in the solution phase because the deuterium labels generated in H/D exchange are usually placed on heteroatoms such as –OH or –NH, and such positions are unable to efficiently stabilize a radical. However, this data also shows that ECD does involve secondary reactions beyond the initial cleavage and it will be important to test ECD under H/D exchange conditions with standards before rushing into using it for mapping surface exposure of proteins.

**Determination of the Oligomeric State of Phage Portal Protein Complexes by
Electrospray Mass Spectrometry**

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The portal protein complexes of the dsDNA phages are located at one of the twelve otherwise identical vertices and represent the conduit through which the viral genome translocates during both DNA packaging and later during infection. As such, they represent a crucial component of the ATP-dependent packaging motor. *In situ*, portal complexes exhibit two striking symmetry mismatches. The first is their localization to only one of the twelve otherwise identical icosahedral vertices, and the second is the fact that they are dodecameric protein complexes positioned at a pentavalent vertex (12 on 5 symmetry).

While portal complexes are apparently invariantly dodecameric *in vivo*, they often deviate from dodecameric symmetry when assembled *in vitro* suggesting that all the information required for correct assembly is not contained within the portal protein alone.

We have used native electrospray mass spectrometry to characterize the *in vitro* assembly process. We have accurately determined the oligomeric states of the portal complexes of phages P22, Phi29 and SPP1, complexes which range in mass from 430 kDa to 1 MDa. The highest order oligomers were found to be dodecamers for both P22 and Phi29 and tridecamer (13-mer) for SPP1. For both P22 and SPP1 portal complexes a progression of smaller oligomers ranging upward from monomers were detected in the mass spectra, although the undecamers (P22) and tridecamer (SPP1), were by far most abundant. These results are in agreement with size exclusion chromatography data suggesting that mass spectra represent a snapshot of the equilibrium distribution of oligomeric species. Intriguingly, the most abundant species of P22 portal protein in the mass spectrum was an undecamer. The mass spectrometric data suggest that the undecamer is not a product of gas phase dissociation of the dodecamer but rather that it pre-exists in solution. However rotationally averaged cryo-electron microscopy images of the P22 portal complexes failed to identify an undecameric ring suggesting that this complex might be an incompletely closed yet stable dodecameric ring.

These studies demonstrate that mass spectrometry can accurately identify and characterize the different subpopulations of macromolecular complexes present in complex biological assembly pathways.

Identification and Structure Determination of Tyrosine Nitration in Human Eosinophils Using FTICR Mass Spectrometry in Combination with Immunoanalytical and Isotope- Labelling Procedures

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Nitration of Tyrosine residues in proteins has been shown to be associated to important physiological functions, as well as pathophysiological effects such as in Alzheimer's disease, atherosclerosis, and broncho-alveolar diseases. The identification of Tyrosine nitrations is hampered, *inter alia*, by low stabilities and levels of modifications, and possible structural changes introduced by the nitration. High resolution FTICR mass spectrometry (FTICR-MS), in combination with immuno-analytical procedures have been developed as powerful tools for unequivocal and sensitive identifications of Tyr-nitrations, (i) upon peroxyxynitrite treatment at the active site Tyr-430 residue of bovine prostacyclin synthase, and (ii) specific endogenous nitration at Tyr-488 of human eosinophil-peroxidase (EPO) [1,2]. Detailed structural studies were performed with Tyr-nitrated model peptides, prepared by chemical synthesis and by *in vitro* peroxyxynitrite nitration, using FTICR-MS in combination with NMR, structure modelling, and isotope-labelling. In contrast to ESI-MS providing unequivocal identifications, 3-nitro-tyrosyl-peptides undergo rapid photochemical fragmentation at UV-MALDI conditions which renders standard MALDI-MS critical for identification of biological protein nitrations. In contrast, stable molecular ions of nitrated peptides were obtained using infrared(IR)-MALDI-FTICR-MS with a 2.97 μm NdYAG-laser [3]. Recent applications reveal specific Tyr-nitration substrates in blood eosinophils mediated by EPO containing the single, surface-exposed nitration at Tyr-488, such as major basic protein and eosinophil-cationic protein. These results suggest that Tyrosine nitration may provide increased concentrations of cationic toxins in eosinophil granules that may lead to improved toxin binding to biological surfaces, thereby exerting an important function for host defense.

- [1] Schmidt, P., N. Youhnovski, A. Daiber, A. Balan, M. Przybylski, V. Ullrich (2003) *J. Biol. Chem.* **278**, 12813-12819.
- [2] Ulrich, M., N. Youhnovski, A. Petre, D. Worlitzsch, M. Schirle, M. Schumm, J. Lee, J. Checkel, M. Dinauer, P. Schmid, F. Ratjen, M. Przybylski, G. Döring (2005) *Nature*, submitted for publication.
- [3] Petre A., N. Youhnovski, J. Lukkari, R. Weber, M. Przybylski (2005) *Eur. J. Mass Spectrom.* **11**, 513-518.

Poster 28

The Allosteric Activation of Coagulation Factor VIIa Visualized by Hydrogen Exchange

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Coagulation factor VII (FVIIa) is a trypsin-like protease which plays a pivotal role in the initiation of blood coagulation. Unlike trypsin, FVIIa is cofactor-dependent and requires binding of tissue factor (TF) in order to fully express its enzymatic activity and become biologically active. Here we use amide hydrogen exchange (HX) monitored by mass spectrometry to reveal the molecular details of FVIIa activation by comparing the HX kinetics of FVII in several activation states, including zymogen FVII, FVIIa and FVIIa variants with enhanced intrinsic activity, in the presence or absence of TF. FVIIa is seen to undergo significant structural stabilization upon binding of TF as indicated by decreased HX rates localized to the second β -barrel of the protease domain of FVIIa and to several interconnecting loops around the active site. Several areas remote from the active site also display decreased HX rates revealing a concerted interplay between functional sites in FVIIa during activation. Furthermore, the allosteric activation response is not confined to the protease domain, as decreased HX rates are seen in a region of the light chain containing the disulfide bond linking the protease domain to the light chain and in the first epidermal growth factor-like domain, located approximately 50Å from the active site. The results presented provide novel insights into the cofactor-dependent activation of this important protease and an intriguing example of dynamic interplay between functional sites, revealing the potential for allosteric regulation of this common family of trypsin-like serine proteases.

Determining the Mechanism of Bacterial Fibre Assembly using Non-Covalent Electrospray Ionisation- Mass Spectrometry

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The highly pathogenic *Salmonella enterica* subspecies I produces extra-cellular Saf fibres, composed of β -sheet subunits^{1,2}. The Saf operon codes for four proteins: SafA, the fibre subunit; SafB, the chaperone; SafC, the usher; and SafD, a fibre capping adhesin¹. Fibre assembly is known to occur by a β -strand exchange mechanism, in which completion of a seven-strand immunoglobulin-like fold in a subunit protein is achieved by the donation of an N-terminal extension 'Nte' strand from an adjacent subunit^{3,4}. This donor strand exchange mechanism causes dissociation of the chaperone-subunit complex and formation of the subunit-subunit complex, at the site of the usher in the bacterial outer membrane⁵. The details of this mechanism in systems such as Saf are largely unknown, but it is hypothesised to proceed *via* a ternary intermediate in a concerted manner whereby the Nte strand 'zippers' into the chaperone-subunit complex, displacing the chaperone.

Here, the reaction between a chaperone-subunit complex and the Nte peptide of an incoming subunit is analysed. Mass spectrometry with nano-electrospray ionisation has been used to follow this reaction with time, observing the decrease in concentration of the SafA-SafB non-covalent complex (Mw = 37,123Da), and the appearance and increase in concentration of the SafA-Nte peptide non-covalent complex (Mw = 15,193 Da). The presence of a SafA-SafB-Nte peptide ternary complex (Mw = 39,191 Da) has been detected, confirming directly and for the first time that the reaction proceeds by a concerted mechanism involving a stable ternary intermediate (Figure 1). Peptide variants of the Nte strand have also been used to probe the roles of individual residues. In this subunit exchange mechanism, the reaction rates change significantly depending on the sequence of the peptide, and this can be attributed to a 'zipping' mechanism, whereby hydrophobic residues on the N-terminal extension sequentially replace residues of the chaperone, causing dissociation of the chaperone-subunit complex.

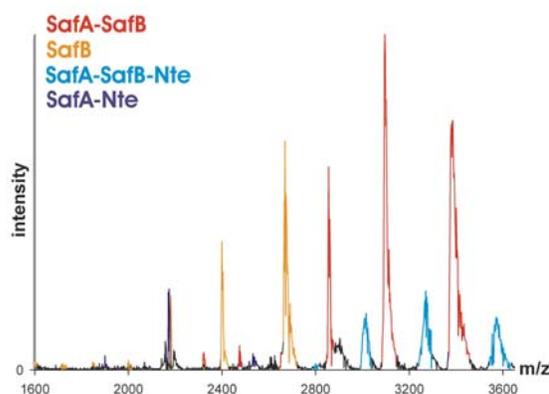


Figure 1. Mass spectrum of the reaction between SafA-SafB (red peaks) and wild-type Nte peptide (not shown) 4 minutes after reaction initiation. This is the first evidence of the existence of a ternary intermediate (light blue peaks) during donor strand exchange. The products of the reaction, free SafB and SafA-Nte, are shown by the ochre and dark blue peaks, respectively.

1. Townsend S.M *et al. Infect. Immun.*, 2001, **69**, 2894-2901
2. Thanassi D.G. *et al. Curr. Opin. Microbiol.*, 1998, **1**, 223-231
3. Sauer F.G. *et al. Cell*, 2002, **111**, 543-551
4. Sauer F.G. *et al. Science*, 1999, **295**, 1058-1061
5. Sauer F.G. *et al. Biochim. Biophys. Acta*, 2004, **1694**, 259-267

The Collaboratory for MS3D

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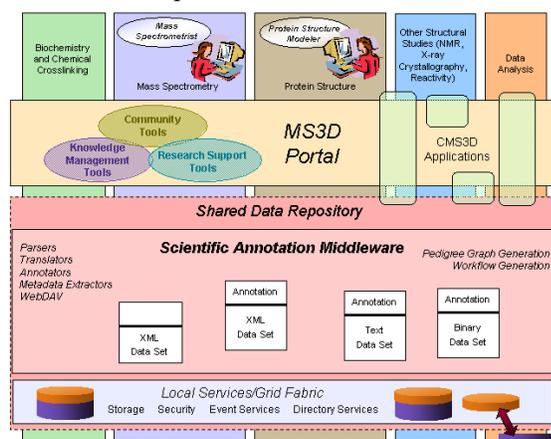
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The Collaboratory for MS3D (C-MS3D) is a multi-institution project that is building web-based data sharing and tool development technologies to support collaborative development of innovative new approaches to biomedical research. C-MS3D is validating its approach by focusing on facilitating the work of an emerging MS3D collaborative community as they develop new tools, analysis approaches and data schema for the application of the MS3D approach to the investigation of macro-molecular structures that are not directly amenable to high-resolution methods alone. MS3D is a mass spectrometry based approach to measuring distances in macromolecules and macromolecular complexes using chemical cross-linking, proteolysis and mass spectrometry [1-2]. The objective of C-MS3D is to enable the MS3D collaborative community in the following ways:

- To create a data-centric collaboration infrastructure for MS3D projects.
- To build an extensible portal for sharing MS3D data and tools.
- To enable information interoperability by creating new schemas/tools for data sharing.
- To enable the creation and storage of new MS3D metadata in an interoperable format.
- To automate the capture of data provenance and workflow in a comprehensive knowledge management system.
- To spur collaborations among scientists in the MS3D community.

The C-MS3D builds on an open source portal and knowledge management infrastructure, Knowledge Environment for Collaborative Science (KnECS) [3], to which new data management and analysis capabilities to support MS3D research are adaptively added. The C-MS3D has adopted mzXML [4], a common standard for MS data. Translators from vendor-specific MS data into mzXML are being developed to enable the sharing of raw MS data. Researchers



will be able to use existing open source tools as well as develop new ones for viewing and manipulating mzXML formatted MS data. The KnECS metadata browser allows researchers to add, view, browse, and search this metadata.

Using a development library for creating asynchronous web services in KnECS, we are integrating a tool for assigning MS peak lists to pairs of chemically cross-linked peptides (Links), and a tool for assigning MS/MS fragmentation spectra of cross-linked peptides, nucleic acids, and proteins (MS2Links) into the C-MS3D portal. These tools are the first components of a MS3D collaborative problem solving-environment that will improve data analysis workflow. Other tools including software for designing optimal sets of cross-linking experiments and performing molecular modeling tasks will be integrated into C-MS3D in a similar fashion.

We encourage interested scientists to join this MS3D pilot effort by going to <http://ms3d.org>. Ultimately, our objective is to make this infrastructure available as an open source 'collaboration tool kit' that will enable similar emerging collaborations across the biomedical community.

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References

1. Young, M.M., *et al.*, High-Throughput Structure Determination: Rapid Identification of Protein Folds Using Mass Spectrometry and Intramolecular Cross-linking. *Proc Natl Acad Sci U S A*, 2000. 97(11): p. 5802-6.
2. Schilling, B., *et al.*, MS2Assign, automated assignment and nomenclature of tandem mass spectra of chemically crosslinked peptides. *J Am Soc Mass Spectrom*, 2003. 14(8): p. 834-50.
3. The Collaboratory for Multi-scale Chemical Science (CMCS) website. Available at <http://cmcs.org/>, 2006.
4. Pedrioli PGA, *et al.*, "A Common Open Representation of Mass Spectrometry Data and its Application in a Proteomics Research Environment." *Nature Biotechnology* 22(11):1459-1466 (2004).

Poster 31

Investigating Hyaluronan-Protein Interactions using Hydrogen/Deuterium Amide Exchange Mass Spectrometry

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Hyaluronan (HA) is an unbranched linear polysaccharide comprised entirely of a repeating disaccharide of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by alternative β 1-3 and β 1-4 linkages, which plays significant roles in embryonic development, ovulation and inflammation mainly through its interactions with HA-binding proteins [1]. Therefore, tools for investigating protein-HA interactions are essential to gain further insight into the biological function. The majority of HA-binding proteins interact with the polysaccharide via common structural domains of ~100 amino acids, termed Link modules. A single independently folded Link module can support high affinity HA binding, as found in the protein product of tumor necrosis factor stimulated gene-6 (TSG-6), which functions during inflammation. The tertiary structure of the Link module from human TSG-6 referred to as Link_TSG6 in both its free and HA₈ (octasaccharide) bound conformation was determined previously by NMR spectroscopy [2]. In this study, hydrogen/deuterium (H/D) amide exchange mass spectrometry is used to map the contact surface of Link_TSG6 in the absence or presence of HA₈.

Link_TSG6 with or without a seven fold molar excess of HA₈, was incubated in a buffered solution containing 70% deuterium oxide. H/D exchange was quenched by dilution with ice cold acetic acid to pH ~2.5 and the samples analyzed immediately by electrospray ionization-Fourier transform ion cyclotron resonance (ESI-FTICR) mass spectrometry, or digested prior to dilution by adding an equal volume of porcine pepsin (1 mg/ml in ice cold acetic acid, pH 2.5) for 4 min on ice. Since pepsin cleavage is non-specific, yet reproducible, it cannot be predicted in advance. Therefore, prior to H/D exchange experiments, Link_TSG6 peptic fragments were sequenced using collision induced dissociation (CID) in the ion-trap. In some instances peptic fragments with or without deuterium were also analyzed using electron capture dissociation (ECD).

Results for undigested Link_TSG6 indicate that a maximum amount of protection is observed 5-10 min post quenching; approximately 7-10 amide hydrogens are prevented from undergoing exchange in the presence of HA₈. This corresponds well with NMR data, which shows that 11 amino acids comprise the Link_TSG6 binding groove (Lys¹¹, Tyr¹², Val⁵⁷, Tyr⁵⁹, Pro⁶⁰, Ile⁶¹, Phe⁷⁰, Ile⁷⁶, Tyr⁷⁸, Arg⁸¹, and Trp⁸⁸; [2]). Deuterium incorporation in a peptic fragment corresponding to amino acids 1-19 (monoisotopic mass = 2198.2) was also analyzed with or without HA₈. Results show that approximately four amide hydrogens undergo exchange in the peptide in the absence of HA₈ compared to the control peptide derived from a Link_TSG6 sample incubated in water. However, in the presence of HA₈, only two amide hydrogens undergo exchange compared to the control. This indicates that the oligosaccharide might be protecting two amide hydrogens, possibly those associated with Lys¹¹ and Tyr¹².

In conclusion HA octasaccharides appear to effectively protect certain Link_TSG6 amide hydrogens from deuterium exchange as analyzed ESI-FTICR mass spectrometry. This technique will hopefully lead to the mapping of the entire Link_TSG6 binding groove and the contact surfaces of other HA binding proteins.

References:

- [1] Blundell, C.D., Seyfried, N.T. and Day, A.J. (2004) Structural and functional diversity of hyaluronan-binding proteins. *Chemistry and Biology of Hyaluronan*, (H. G. Garg and C.A Hales Ed), Elsevier Press, Amsterdam, The Netherlands, 184-204.
- [2] Blundell, C.D., Mahoney, D.J., Almond, A., DeAngelis, P.L., Kahmann, J.D., Teriete, P., Pickford, A.R., Campbell, I.D. and Day, A.J. (2003) The link module from ovulation- and inflammation-associated protein TSG-6 changes conformation on hyaluronan binding. *J Biol Chem*, **278**, 49261-49270.

The Role of Oxidative Protein Damage and Localized Conformational Change in Sporulation—A Case for Oxidative Stress Signaling

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The initiation of sporulation in *Bacillus subtilis* is tightly regulated by a multicomponent phosphorelay pathway. A critical component of the sporulation phosphorelay pathway is Spo0F, a single-domain response regulator protein. Under oxidative stress conditions, sporulation of *B. subtilis* has been shown to be inhibited through an unknown mechanism. In order to examine the role of Spo0F in oxidative stress response, a novel technique was developed, in which the kinetics of oxidation of many side chains in the protein were measured simultaneously by nano-electrospray coupled to a hybrid quadrupole-time of flight mass analyzer. Analysis of the oxidation kinetics of the sporulation regulator Spo0F revealed that, at low levels of protein oxidation, Spo0F exhibits a drastic shift in the kinetics of oxidation of regions near the essential phosphorylation site, while the kinetics of oxidation of other regions remain unaffected. As the kinetics of oxidation are primarily governed by the chemical nature of the side chain and the solvent accessibility of the side chain, these data indicate a localized conformational change of Spo0F triggered by oxidative damage. Analysis of the multi-site oxidation kinetics reveals that, upon oxidation of Met81, the loops surrounding the phosphorylation site unfold. Oxidative signaling via Spo0F may play an important role in inhibiting sporulation in response to oxidative stress in *Bacillus*. Sequence analysis of members of the single-domain response protein family reveals that Met81 is conserved in a subset of family members, suggesting a potential subfamily of oxidative stress sensors within the domain superfamily.

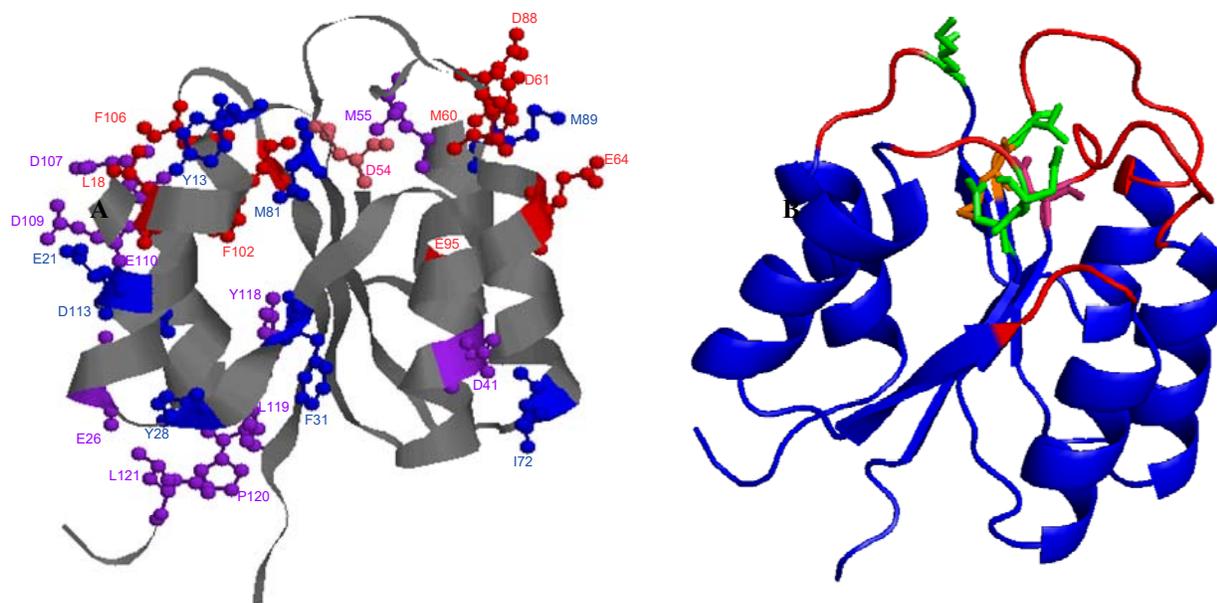


Figure 1: A ribbon illustration of the average NMR solution structure of Spo0F. The phosphorylation site Asp54 is shown in pink, with the sites essential for phosphorylation and activity shown in green (Asp10, Asp11, Thr82 and Lys104). **A.** The change in oxidation kinetics for each identified or implicated residue mapped onto the structure of Spo0F. Residues that showed ≥ 5 -fold increase in oxidation kinetics after the conformational change are shown in red. Residues that showed between a 2- and 3-fold increase in oxidation kinetics after the conformational change are shown in purple. Residues that showed less than a 2-fold increase in oxidation kinetics after the conformational change are shown in blue. **B.** A model for the oxidative damage-induced conformational change of Spo0F. The regions shown in red are thought to become disordered after the conformational change, while the regions in blue are thought to mainly remain in the native conformation. Oxidation of Met81 (orange) triggers the conformational change.

Structural Insights of Human Centrin 2 Revealed by H/D Exchange, Pepsin Digestion and Correlation Studies

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Introduction

Human centrin 2 (20 kDa), which contains 4 EF hand motifs, is a member of the Ca²⁺-binding protein family. Although there is currently no crystal or NMR structure of the protein, the N- and C-terminal domains have been determined separately by NMR. Human centrin 2 (HC2) is known to form aggregates in the presence of saturating calcium levels; however, a construct, in which the first 25 amino acids at the N terminus are truncated (Δ 25-HC2), does not. The Δ 25-HC2 contains all four EF hand motifs and behaves similarly to the full length human centrin 2. The goal of this work is to use H/D exchange, combined with pepsin digestion, to reveal areas of protection within the protein in the presence and absence of Ca²⁺. Correlation of the product-ion spectra of the peptic peptides reveal areas of the protein that become more, or less, protected upon ligand binding. We expect that these studies will give insight to the conformational changes that occur in the native state and provide additional information into the calcium binding properties of the protein.

Experiment

The Δ 25-human centrin 2 was from the Mayo Clinic. H/D exchange was carried out at various times in 10 mM ammonium acetate (pH = 7.4) and 25 °C containing 98% D₂O. Immobilized pepsin on agarose (Pierce) was added to the quenched solution (pH = 2.5, 0 °C) for 2 minutes. The digested solution was desalted on a C₁₈ guard column, and the peptides separated on a LC Packings C₁₈ cartridge (1.0 mm x 10 mm). A Waters CapLC was used for the gradient (40 μ L/min) and the eluent was analyzed by ESI/MS/MS using a Thermo LTQ/FTMS operating in the positive-ion mode.

Results

The pepsin digestion resulted in a set of peptides that gives 100% sequence coverage, with overlapping residues, of human centrin 2. From previous H/D exchange kinetics experiments, we know that there is little or no change in the uptake between the apo and holo forms of the protein. A new program developed in our laboratory allows us to compare product-ion spectra of modified vs. unmodified peptides to identify sites of modification. These correlation data show that some peptic fragments take up more deuterium in the presence of calcium, indicating that the region of the protein represented by the peptide becomes less protected in the holo state. These data also show that some peptic fragments become more protected in the presence of calcium, thus decreasing the overall deuterium uptake. While there are still questions regarding deuterium scrambling in collision induced dissociation, we do find consistent and complementary identification of sites of protection using both y and b ion series. Product-ion spectra of peptides from the EF hand regions of human centrin 2 reveal protection near the loop region. The most protected areas of the protein are in EF hand regions 1 and 3.

Structural Flexibility of Membrane-Fusogenic Peptides Examined by D/H-Exchange Reactions

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SNARE proteins drive different types of intracellular membrane fusion. Recent evidence suggests that their transmembrane segments (TMSs) play a direct role in lipid mixing. Using a FRET-liposome fusion assay, we have previously shown that reconstituted synthetic peptides representing SNARE TMSs are capable of driving membrane fusion in vitro. Interestingly, the fusogenic activity of these peptides is inversely correlated to the stability of their α -helical conformation. This suggested that structural flexibility of SNARE TMSs is important for fusion.

Here, we examined the structural flexibility of these peptides via D/H-exchange reactions and mass spectrometry at conditions where they exhibit high α -helix contents.

First, the peptides were fully deuterated. Subsequently, they were diluted into protonated solvent.

Irrespective of the type of peptide, > 50% of the deuterons exchanged extremely rapidly suggesting that they are unprotected. The exchange kinetics of the protected deuterons revealed three groups of deuterons in each peptide with different exchange rate constants. It is likely that these different rate constants reflect partial unfolding reactions of the α -helices.

Interestingly, those peptides that exhibit higher membrane fusogenicity in the fusion assays display a higher number of rapidly exchanging deuterons and higher exchange rate constants of their “slow” deuterons. This indicates, that determination of D/H-exchange reactions by electrospray mass spectrometry is suited to determine functionally relevant structural flexibility of membrane fusogenic peptides.

References:

1. Hofmann, M. W., Weise, K., Ollesch, J., Agrawal, A., Stalz, H., Stelzer, W., Hulsbergen, F., deGroot, H., Gerwert, K., Reed, J., and Langosch, D. (2004) De novo design of conformationally flexible transmembrane peptides driving membrane fusion, *Proc Natl Acad Sci U S A* 101, 14776-14781.
2. Langosch, D., Crane, J. M., Brosig, B., Hellwig, A., Tamm, L. K., and Reed, J. (2001) Peptide mimics of SNARE transmembrane segments drive membrane fusion depending on their conformational plasticity, *J. Mol. Biol.* 311, 709-721.

DXMS: Software for Automated Analysis of H-D Exchange Mass Spectral Data

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Abstract

We describe software for automated analysis and reduction of amide H-D exchange liquid chromatography / mass spectrometry datasets. For each peptide in a list of peptides generated from the parent protein by proteolytic digest, the software determines the retention profile of the peptide, sums the spectra under the LC peak, fits an isotopic distribution to the peptide's molecular ion, then computes the mass-weighted centroid. The results can be exported for use in protein confirmation, exchange kinetics, ligand binding sites, or other studies.

Experimental Protocol

The software assumes an experimental protocol in which an unlabeled (*i.e.* non-deuterated) protein is first digested under the same conditions as will be used for labeled protein. The peptides thus generated are analyzed in an LC/MS (or LC/MS/MS) experiment to determine the retention behavior and identity of each. Subsequently, LC/MS data of labeled peptides are obtained, and the retention profiles and identities determined from the unlabeled data are used to locate and determine the degree of deuteration of each labeled peptide.

Details of the Software

The software is written for the Microsoft Windows XP operating system, using the C++ programming language and the ActiveX and the Microsoft Foundation Classes tools. The software uses a model of "peptide pools" and an "experiment set". Each peptide pool is comprised of a set of identified peptides. These identities may be determined by LC/MS/MS analysis and identification using SEQUEST or by accurate mass LC/MS. The results of the identification are imported and used to create a peptide pool. An experiment set consists of at least one non-deuterated LC/MS dataset and one or more deuterium-exchanged LC/MS datasets. Each peptide pool is paired with the experiment set to create an "analysis set". There may be more than one peptide pool (one containing peptides identified with high confidence, and others of lower confidence, for example), with the result of one analysis set per peptide pool. Each analysis set contains the group of analysis results for the peptide pool against each of the LC/MS datasets in the experiment set.

A two-step procedure is used to locate peptides in labeled LC/MS datasets. First, a rough estimate is made of the degree of deuteration, and from this an isotopic distribution is computed. This distribution is matched to the LC/MS spectra in the elution range to isolate the peptide. Next, an accurate isotopic distribution is fit to the actual molecular ion isotopic distribution. This isotopic distribution is then used to compute the centroid for the isotopic cluster and thus the extent of deuterium labeling of the peptide.

Results

The software provides high quality centroid values for correctly-identified peptides that are cleanly separated in time and m/z from other peptides with similar retention and m/z . For peptides that are weak or have noisy spectra or that have interferences from other peptides in the same time and m/z range, manual editing of either the retention range used for spectral co-addition or the m/z range used for centroid calculation may be necessary.

Poster 36

Amide $^1\text{H}/^2\text{H}$ Exchange Reveals Changes in I κ B α Conformational Flexibility Upon Binding to NF- κ B

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More than 150 target genes, involved in a wide variety of cellular functions, are regulated by the nuclear factor kappa B (NF- κ B) family of eukaryotic transcription factors. Given the central role that NF- κ B plays in regulating cellular development and proliferation, it is not surprising that misregulation of NF- κ B transcriptional activity is observed in many disease states. NF- κ B activity is regulated in the cell by its inhibitor, I κ B α , which binds to NF- κ B and prevents its nuclear localization. Following degradation of I κ B α , free NF- κ B translocates to the nucleus and activates transcription of its target genes, including I κ B α . Newly synthesized I κ B α freely enters the nucleus, binds to NF- κ B, and exports it to the cytosol. Thus, I κ B α inactivates NF- κ B transcriptional activity through a negative feedback mechanism. The crystal structures of NF- κ B bound to DNA and NF- κ B bound to I κ B α revealed that I κ B α binding causes a conformational change in NF- κ B that prevents it from associating with DNA by burying the DNA-contacting surfaces. However, the molecular mechanism by which I κ B α regulates NF- κ B transcriptional activity remains unknown. I κ B α 's conformational flexibility seems to be important for its regulation of NF- κ B transcriptional activity. Characterization of how the dynamic properties of I κ B α change upon binding to NF- κ B will be important for understanding this regulatory mechanism, and it will provide insight into the biological roles for intrinsically disordered proteins. I κ B α is composed of six ankyrin repeats (ARs), a ~33 amino acid structural motif thought to mediate protein-protein interactions. While I κ B α bound to NF- κ B was well ordered in the crystal structure of the I κ B α •NF- κ B complex, I κ B α is highly flexible in solution. I κ B α amide $^1\text{H}/^2\text{H}$ exchange experiments followed by MALDI mass spectrometry show that ARs 1, 5, and 6 readily exchange most of their amide protons, but ARs 2–4 are more protected from solvent. This suggests that ARs 1, 5, and 6 are highly flexible, whereas ARs 2–4 form a more compact region of the protein. Amide $^1\text{H}/^2\text{H}$ exchange experiments comparing I κ B α and NF- κ B bound I κ B α show that ARs 5–6 exchange far fewer amide protons when I κ B α is bound to NF- κ B than in the free protein. While some of this protection from solvent is undoubtedly due to the interface, the number of amide protons that are protected exceeds that which is predicted based on the contacts made in the crystal structure. This suggests that I κ B α undergoes conformational changes and/or changes in conformational flexibility upon binding to NF- κ B. Remarkably, it is the interactions of AR 6 and NF- κ B that are primarily responsible for the preclusion of DNA binding in the I κ B α •NF- κ B complex. These data provide strong evidence that the highly flexible conformation of I κ B α is crucial for its regulation of NF- κ B transcriptional activity.

**Plans for Improved DXMS Software for Automated Analysis of H-D Exchange
Lc-Mass Spectral Data**

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Abstract

Our present generation DXMS data reduction software is the result of a two- year, \$500,000 software design effort which began in 2001 (see accompanying poster). It markedly speeds DXMS data reduction when compared to the "manual" approaches pioneered by David Smith, but is imperfect, and requires extensive human operator intervention and editing of its output to detect and correct errors. The requirement for editing has been of little impediment to the successful DXMS study of the numerous individual proteins we have performed over the past three years. In these studies, collaborating graduate students with great interest in a particular protein are happy to make the investment in software training and subsequent editing, as a few months of such work typically results in a first rate DXMS- focused publication. We train collaborators in the use of the software while they visit our DXMS lab at UCSD, and then provide such trained operators with ready access to the software through web-based servers.

Our recent demonstration of the utility of DXMS- guided crystallographic construct re-design to high-throughput structural genomics efforts has highlighted the throughput limitations of our current-generation software. Improvements to the software that significantly reduce the need for operator input would render DXMS analysis feasible to a larger number of investigators and proteins.

We have in hand the funding to produce the next generation DXMS analysis software. We aim to decrease the need for human operator support by a factor of 10 or more, resulting in the ability of the software, operating on a single workstation, to fully process the DXMS data for 50-75 proteins per week, with the assistance of a single human operator.

Many of the present DXMS software shortcomings that require operator editing are well-understood by us, and have relatively straightforward solutions that can be accomplished by algorithmic improvement:

- A. *Difficulties due to retention time variation and computational compensations for such variation.*
- B. *Occasional ambiguity of peptide identification.*
- C. *Correlation chromatogram generation problems.*
- D. *Determination of the precise deuterium content of correlation chromatogram- identified peptides, most particularly when contaminating isotopic envelopes overlap peptides of interest.*

We also plan to implement machine learning approaches to perform much of the editing function that remains after algorithmic improvement.

Poster 38

Structural Changes of Microtubules Induced by Taxol

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Microtubules are the target of anti-tumor drugs such as Taxol. GTP bound to the β subunit of the α/β tubulin heterodimer is hydrolyzed upon assembly of tubulin to microtubules. Taxol induces the assembly of stable microtubules. Although photoaffinity labeling and electron crystallography have localized the binding pocket for Taxol on β -tubulin, they do not provide insight into the mechanism by which Taxol stabilizes microtubules. Hydrogen/Deuterium exchange (HDX) in combination with Liquid Chromatograph-Electrospray ionization mass spectrometry (LC-ESI MS) permits studies of structure and dynamics of biomolecules in solution. A time course of deuterium incorporation was carried out with purified chicken erythrocyte tubulin heterodimers, or microtubules assembled in the presence of GTP or GTP plus Taxol. The global exchange kinetics of tubulin dimers and microtubules was monitored by LC-ESI MS and the deuterium incorporation into tubulin polypeptide chains was calculated. After 100 minutes of exchange, 207 out of 430 (48%) total exchangeable amide protons of the amide hydrogens in the tubulin α -chain were replaced by deuterium. The corresponding number for the β -chain was 221 or 52% (425 total exchangeable amide protons). After Taxol-induced polymerization, a marked reduction in deuterium incorporation is apparent with only 161 (37%) and 157 (37%) amide protons in the tubulin α -chain and β -chain, respectively, replaced by deuterium. This protection reflects decreased solvent accessibility or a more rigid conformation in both polypeptide chains. GTP-dependent polymerization reduced the flexibility of microtubules to a lesser extent. The reduced flexibility is preferentially on the β -chain, as suggested by the significantly reduced deuterium incorporation (205 amide protons) on the β -chain, while only slightly reduced deuterium level was observed on the α -chain (205 amide protons). Local HDX on microtubules was analyzed on peptic peptides by LC-MS. As expected, peptides from the Taxol binding pocket were significantly protected against HDX. A comparison of the deuterium incorporation in the presence or absence of GTP- and Taxol allowed us to determine not only the regions involved in Taxol binding, but also the longitudinal and lateral dimer-dimer interactions. The data demonstrate that HDX coupled to mass spectrometry can be used to study microtubule-drug interactions.

Poster 39

Hydrogen-Deuterium Exchange Mapping of the Arp2/3 Complex using LC-ESI-MS

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Cell motility depends on the assembly of a branched network of actin filaments at the leading edge. Actin polymerization provides the driving force that propels the plasma membrane and results in directional cell movement. A central component in this process is the Arp2/3 complex, a 220kD seven subunit assembly, which requires ATP and protein activators to nucleate new actin filaments. However, it is not clear where the protein activators bind to the protein. Amide Hydrogen/Deuterium exchange coupled with mass spectrometry allows us to examine the structural and dynamic properties of the mammalian Arp2/3 complex. We assessed differences in global exchange rates, measuring changes in deuterium incorporation in the apo-complex, the complex in the presence of ATP, and the complex in the presence of both ATP and the activating peptide segment from the Wiskott-Aldrich syndrome protein (WASp). Using a fast gradient, we were able to separate each of the seven subunits and obtain exchange data. In the presence of ATP, we note a significant decrease in the rate of deuterium incorporation in some but not all subunits. Slightly more protection was observed when WASp is added. This preliminary data suggests the subunits that interact with the ATP and WASp. A pepsin digestion of the complex, coupled with LC/MS, will allow a more detailed analysis of the regions that are responsive to nucleotide and activator binding.