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Thursday, March 14, Session I

L1

HYDROGEN EXCHANGE MASS SPECTROMETRY TO PROBE THE CONFORMATION OF PROTEINS BOTH IN SOLUTION AND IN MEMBRANES

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A continually growing area of mass spectrometry is the analysis of protein conformation and dynamics. One classic approach is to label protein molecules in solution under physiological conditions as the incorporation of the labeling agent is a function of the folded conformation. Hydrogen exchange (HX) methods label the backbone amide hydrogens of proteins with deuterium and the location and magnitude of the labeling can then be determined with mass spectrometry (MS). HX MS studies are particularly well suited for analysis of proteins that will not crystallize, proteins not amenable to NMR, or proteins available in only small quantities. This presentation will explore current methodology and applications of HX MS, both for multi-protein systems in solution and for the analysis of membrane proteins using liposomes or phospholipid nanodiscs.

L2

IN-SOLUTION STRUCTURE OF CELLOBIOSE DEHYDROGENASE PROBED BY HYDROGEN / DEUTERIUM EXCHANGE MASS SPECTROMETRY

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Cellobiose dehydrogenase (CDH) is an enzyme involved in the early processes of lignocellulose catabolic degradation. Being the only currently known extracellular flavocytochrome, CDH is unique from the point of view of its molecular architecture. It is a monomeric glycoprotein consisting of two domains connected by a flexible linker. The combination of a haem with a FAD molecule within a single protein gives CDH the ability to exchange electrons with a variety of interaction partners, thus making it an interesting enzyme for research in the fields of biocatalysis and biosensors.

To probe the in-solution structural organization of this multidomain enzyme as well as its proposed conformational changes under different pH conditions, we employed amide hydrogen/deuterium exchange in combination with mass spectrometry (HDX-MS).

Prior to HDX-MS experiments we thoroughly characterized primary sequence of CDH using mass spectrometry methods. We examined the disulfide bond organization of CDH, found all six potential N-glycosylation sites in CDH molecule to be decorated with high-mannose type glycans and moreover, we uncovered an extensive O-glycosylation of the interdomain linker region. Next, we optimized digestion conditions and performed HDX-MS experiment examining the conformations at pH 5.4 (enzymatic pH optimum) and pH 7.4 (only residual activity remaining). The data indicate a conformational change near the proposed domain interface, which could be explained by "opening" of the two-domain assembly at higher than optimal pH. These results are in agreement with sedimentation velocity data from analytical ultracentrifugation, which demonstrate a global conformational change in the CDH molecule at pH 5.4 and pH 7.4.

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THE USE OF MASS SPECTROMETRY AND MOLECULAR MODELING TO DESIGN STRUCTURAL MODEL OF MOUSE NKR-P1 PROTEINS

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Introduction

Determination of protein conformation has traditionally been realized by X-ray crystallography and NMR spectroscopy. Although these techniques provide high resolution atomic data, they have some limitations. Both NMR and X-ray require large amounts of pure analyte and are time-consuming techniques. Mass spectrometry combined with chemical cross-linking offers alternative approach to identify the protein fold. This method is fast and uses small amounts of material. Our aim was to gain insight into low-resolution structure of NKR-P1C receptors. NKR-P1C is an activating immune receptor expressed on the surface of mouse natural killer cells. Using distance constraints derived from chemical cross-linking and disulfide arrangement in combination with computational methods, protein conformation was designed. The validation of structural model was addressed using ion-mobility mass spectrometry.

Methods

In order to design structural model of NKR-P1C, protein was cross-linked using homobifunctional cross-linking reagents disuccinimidyl suberate (DSS) and disuccinimidyl glutarate (DSG). After cross-linking reaction, SDS-PAGE of cross-linking reaction mixture was performed. Also, disulfide bound arrangement was determined after non-reducing SDS-PAGE. The band of cross-linked or non-reduced protein was excised and subjected to in gel digestion by Asp-N and trypsin. The peptide mixtures from the enzymatic digest were analyzed by LC/ESI-FT-ICR MS. Cross-links and disulfide-linked products were identified using Links software. These distance constraints were used for molecular modeling. Homology modeling followed by a short steepest descent minimization was performed using the MODELLER 9v7 package. To verify the fold of NKR-P1C, native mass spectrometry with ion mobility measurements were performed.

Preliminary Data

Restraint-based computational modeling was used to generate a model that represents the experimentally determined constraints with a minimum of violations. Molecular dynamics was used to refine the model and to describe the most populated protein conformers in solution. In addition to the positional constraints obtained from the disulfide mapping and from cross-linking experiments the model needs to preserve the overall C-type lectin-like fold in these simulations, as the protein core is strongly conserved, and the template and modeled structure share a sequence identity of 88%. However, as crystal structures are rigid contrary to protein dissolved in solution we allowed the side chains in the core to be more flexible and adapt to the given experimentally determined constraints. Specific attention was paid to the extended loop region proposed to be involved in protein-ligand interactions and ligand specificity. The only crystal structure published to date for the entire NKR-P1 family, mouse NKR-P1A, shows this extended loop pointing away from the protein core, in a conformation in which the loop would be fully exposed to the solvent. Such a conformation could be clearly excluded from the cross-links of the protein in solution. This was further supported by IM-MS measurements corresponding to the compact form of the molecule based on the experimentally derived collisional cross section. Therefore, in the most populated conformation in solution, NKR-P1C most likely adopts the conformation similar to the solution structure of NKR-P1A. Our model enables us to describe this conformation on an atomic scale.

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ANALYSIS OF HETEROGENEOUS HINGE-REGION *O*-GLYCOSYLATION OF HUMAN IGA1 USING MALDI-TOF/TOF MASS SPECTROMETRY

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Changes in the glycosylation patterns of various glycoproteins are associated with several diseases. Hence determining disease-associated glycosylation patterns and heterogeneity provides a better understanding of disease mechanisms. This work focuses on the *O*-glycosylation of immunoglobulin A1 (IgA1), where aberrant glycosylation plays a key role in the pathogenesis of IgA nephropathy (IgAN). IgA1 hinge region carries 3-6 *O*-glycans consisting of *N*-acetylgalactosamine (GalNAc) with galactose (Gal); both glycans may be sialylated. In IgAN patients, some *O*-glycans on a fraction of IgA1 molecules are Gal-deficient. Here we describe a sample preparation protocol with optimized cysteine alkylation of a Gal-deficient polymeric IgA1 myeloma protein prior to in-gel digestion and analysis of hinge-region glycopeptides by MALDI-TOF/TOF mass spectrometry (MS) as a novel strategy. IgA1 hinge-region glycopeptides were fractionated by reversed-phase liquid chromatography using a microgradient device and identified by MALDI-TOF/TOF tandem MS (MS/MS). The acquired MS/MS spectra were interpreted manually and by means of our own software, which allowed assigning up to six *O*-glycosylation sites and suggested possible isomeric *O*-glycoforms. The most abundant Gal-deficient *O*-glycoforms were $GalNAc_4Gal_3$ and $GalNAc_5Gal_4$ with one Gal-deficient site and $GalNAc_5Gal_3$ and $GalNAc_4Gal_2$ with two Gal-deficient sites. The most frequent Gal-deficient sites were at Ser230 and/or Thr236.

Thursday, March 14, Session II

L5

EVOLUTION OF GENETIC DIVERSITY OF REPETITIVE EXTRAGENIC PALINDROMIC ELEMENTS (REPS): A COMPARATIVE STUDY

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Repetitive extragenic palindromic elements (REPs) constitute a group of bacterial genomic repeats known for their high abundance and several functions of importance for host cells´ physiology. We analyzed the phylogenetic distribution of particular classes of REP elements in genomic sequences of sixty-three bacterial strains belonging to the *Pseudomonas fluorescens* species complex and ten strains of *Stenotrophomonas* sp., in order to assess intraspecific REP diversity and to gain insight into long-term REP evolution.

Based on proximity to RAYT (REP-associated tyrosine transposase) genes, twenty-two and thirteen unique REP classes were determined in fluorescent pseudomonads and stenotrophomonads, respectively. REPs were generally occurring in hundreds or even over a thousand of perfect copies of particular REP class per genome. REP sequences showed highly heterogenous distribution. The abundances of REP classes roughly followed host strains´ phylogeny, differing markedly among phylogenetic clades. High abundances of particular REP classes appeared to depend on the presence of cognate RAYT gene, and deviations from this state could be attributed to recent or ancient mutations of *rayt*-flanking REPs, or RAYT loss. RAYTs of both studied bacterial groups are monophyletic, and their cognate REPs show species-specific characteristics, suggesting shared evolutionary history of REPs, RAYTs and their hosts.

Our results show that REP elements constitute intriguingly dynamic components of genomes of fluorescent pseudomonads and stenotrophomonads, and indicate that REP diversification and proliferation are