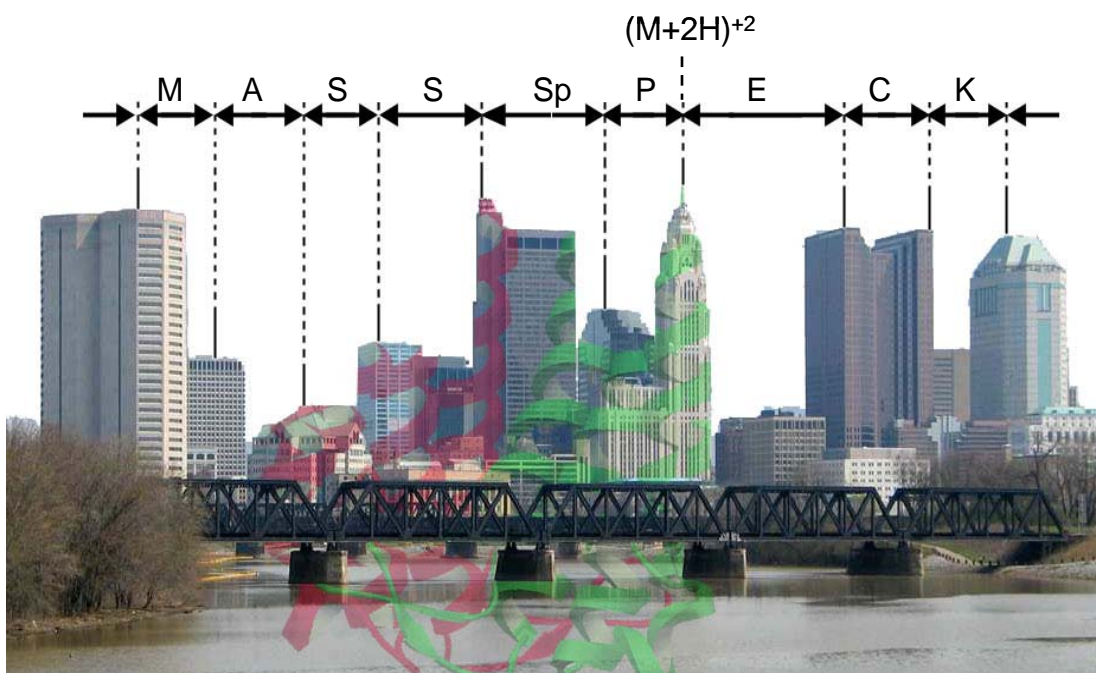# 10th Annual
Ohio Mass Spectrometry Symposium

April 15, 2013

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2013 OMSS ORAL ABSTRACTS

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Evidence of Third Residue Involvement in Diketopiperazine and Oxazolone b_2 Ion Formation in NAXIG and QAXIG Pentapeptides

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Introduction

The N-terminal fragments of peptides in MS/MS studies, or "b" ions, have been the subject of controversy for many years. The result of amide bond cleavage, these ions can be generated by two main pathways, forming either a head-to-tail cyclic peptide or a 5-membered oxazolone ring structure. The b_2 ion is particularly interesting because the head-to-tail isomer, or diketopiperazine, is typically more stable than the oxazolone but requires a high energy trans-cis isomerization of the peptide bond in order to form. To date, spectroscopic and labeling studies have demonstrated that residue identity in the first two positions of the peptide is a significant factor in determining which of these structures forms but have not explored influence of the third residue.

Methods

All peptide precursors were synthesized in house using solid-phase FMOC chemistry. A Bruker Daltonics 9.4T FT-ICR at the University of Arizona was used for hydrogen-deuterium exchange (HDX) and MS^3 experiments. Action infrared multi-photon dissociation (IRMPD) spectroscopy was performed on a Bruker Daltonics Esquire ion trap coupled to the CLIO free electron laser (FEL) in Orsay, France. Computational modeling was performed by first generating a collection of reasonable conformations for each structure and protonation site using a Monte Carlo conformational search. Unique conformations were then optimized at the B3LYP/3-21G, B3LYP/6-31G, and B3LYP/6-311++G** basis sets using the Gaussian 03 software package. Frequency calculations were performed at the 6-311++G** level and a 0.978 scaling factor applied to theoretical IR spectra.

Preliminary Data

MS^3 and HDX experiments were initially performed on the pentapeptides NALIG and QALIG to examine the influence of an amino acid with an amide side chain on diketopiperazine and oxazolone b_2 ion formation. Analogues QADIG, QAEIG, NADIG, and NAEIG were additionally investigated by these methods to evaluate the role of acidic amino acids in the third position on diketopiperazine and oxazolone b_2 ion formation in systems with an N-terminal amide residue. Differences in HDX and fragmentation patterns in the NA and QA series of b_2 ions are observed when the third residue of the pentapeptide is varied from leucine to aspartic acid to glutamic acid, suggesting that the structural composition of the NA and QA b_2 populations are dependent on the side chain of the third amino acid.

A combination of computational modeling and action IRMPD spectroscopy will be presented to demonstrate the influence of the third position amino acid on diketopiperazine and oxazolone b_2 ion formation. Moreover, a computational approach directed towards understanding how the precursor pentapeptide bridging conformations contributes to this effect will be presented.

Novel Aspect

The third residue of pentapeptides is shown to be influential in forming diketopiperazine vs. oxazolone b_2 ions.

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Identification of tRNA Modifications in *Thermus thermophilus* HB8 Wild Type and GidA Null Mutant Strains

Lihua Fu and Patrick A. Limbach

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Introduction

Transfer RNA (tRNA) is intensively modified post-transcriptionally. These modifications have many roles, such as reducing codon misreading and modulating frame-shifting events. The modified nucleoside family xm^5s^2U (5-methyl-2-thiouridine derivatives) is found in tRNA at the wobble position (position 34). tRNAs that decode codons ending with A or G in two-codon sets often contain this modification family to help prevent frame-shifting. To examine the functional aspects of this modification family in *Thermus thermophilus*, a gene disruption mutant, $\triangle$TTHA1897, from the HB8 strain has been constructed. TTHA 1897 has been annotated in the HB8 genome as GidA, which is an enzyme in the xm^5s^2U biosynthetic pathway for *E. coli*. LC-UV-MS analysis of total nucleoside digests from both wt and mutant strains were conducted.

Methods

To determine whether modifications in this family are affected by TTHA1897, LC-MS/MS analysis of total nucleoside digests from *T. thermophilus* HB8 (wt) and $\triangle$TTHA1897 was performed. HPLC was performed using a Hitachi D-7000 HPLC system with a Phenomenex Synergi 250 × 2.0 mm (4 μm particle size) hydro-RP 80A reversed-phase column. Mobile phase A was 5 mM ammonium acetate buffer, pH 5.3; Mobile phase B was 40% acetonitrile. For direct LC-MS analysis of nucleosides, the HPLC described above was connected in-line with a UV flow cell and the output from the flow cell was directed into a Thermo LTQ-XL ion trap mass spectrometer. Nucleosides were detected in positive polarity using an electrospray ionization source. Typically, 60 μg of total tRNA nucleoside digest, prepared in 20 μL of 5 mM ammonium acetate buffer, was injected per analysis.

Preliminary Data

We have identified 28 nucleosides in *T. thermopilus* total tRNA HB8 wt including mnm^5s^2U. In $\triangle$TTHA1897 strain, 27 nucleosides were identified, but not the modification mnm^5s^2U. The modified nucleoside family xm^5s^2U is found to have a different fragmentation pattern than most nucleosides. Further effort will focus on developing a method for enhanced identification of xm^5s^2U in tRNAs for the $\triangle$TTHA1897 mutant.

Novel Aspect

The first time the unique fragmentation pathway of xm5s2U as a family of nucleosides was found.

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Highly Efficient Ionization of Phosphopeptides at Low pH by Desorption Electrospray Ionization Mass Spectrometry

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Introduction

Phosphorylation is one of the most common post-translational modifications (PTMs) of proteins and it plays an important role in biological processes such as signal transduction. Phosphopeptide signal in the positive ion mode is severely suppressed when a phosphoprotein digest is ionized by traditional ionization methods such as electrospray ionization (ESI) in a commonly used "bottom-up" approach. One intrinsic cause is that the phosphate groups of phosphopeptides tend to lose protons to carry negative charges, decreasing the phosphopeptide ionization efficiency. Our strategy to tackle this problem is to use the liquid sample DESI approach via adding a strong acid into sample solution so that phosphate deprotonation can be inhibited. DESI turns out to be more tolerant to strong acid than traditional ESI.

Methods

A home-built apparatus of liquid sample DESI was used throughout the experiments. A Thermo Finnigan LCQ DECA ion trap mass spectrometer (Thermo Scientific, San Jose, CA) was mainly employed for ion detection. The sample are acidified with strong acids, then it was flowing out of the fused silica transfer capillary (i.d., 0.1 mm) underwent interactions with the charged microdroplets generated from DESI spray for ionization. The spray solvent for DESI was methanol/water (1:1 by volume) containing 1% acetic acid and was injected at a flow rate of 10 μL/min with a high voltage of 5 kV applied to the sprayed solvent. The flow rate of the sample solutions injected for DESI ionization was 2-5 μL/min. Then it will be detected by the nearby mass spectrometer.

Preliminary Data

As a proof-of- principle experiment, a mixture of O-phospho-L-tyrosine and L-tyrosine was ionized directly by ESSI (a variant form of ESI), which does show the suppression of the phosphoamino acid signal in comparison to non-phosphorylated amino acids. Interestingly, when the amino acid mixture was acidified with HCl to pH 2 and ionized by DESI with the spray solvent of MeOH/H_2O/HOAc, the signal intensity of O-phospho-L-tyrosine exceeds L-tyrosine. We thus examined phosphoprotein digests with this DESI approach. α-Casein, a phosphoprotein containing a major component α-S1-casein and a minor component α-S2-casein , was trypsin

digested and tested. Using ESSI, among 17 peptide ions identified, four phosphopeptide ions were detected, covering only one phosphorylation site of the α-S1-casein protein that has eight phosphate groups in total. When the sample was acidfied by HCl to pH 2 and analyzed by DESI, besides the 17 peptide ions seen in the ESSI-MS spectrum, two additional phosphopeptide ions were detected, covering all eight phosphorylation sites of the α-S1-casein. Three phosphopeptide ions from α-S2-casein were observed, covers 9 out of 11 possible phosphorylation sites in the DESI spectrum. In the case of β-casein tryptic digest, only +2 ion at *m/z* 1562 was observed in the ESSI-MS spectrum. However, abundant +3 ion at *m/z* 1042 was generated by DESI. The increased charge state is valuable in providing increased sequence coverage via electron-based tandem MS analysis such as electron-capture dissociation (ECD). This DESI method is also applicable to the analysis of acidic sulfated peptides. The sulfated hirudin was suppressed and no corresponding peptide ion was observed in the ESSI-MS spectrum. In contrast, the doubly charged sulphated hirudin (*m/z* 746) arose *via* DESI ionization. This result shows the possibility for detecting sulfopeptides of low stoichiometry by DESI, analogous to the detection of the α-S2-casein phosphopeptides mentioned above.

Novel Aspect

This work provides a novel approach to effectively ionizing phosphopeptides/sulfapeptides in complicated mixtures.

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Automated Data Mining using C13 Depleted tRNA

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Introduction

Mapping and sequencing of individual transfer ribonucleic acids (tRNAs) can prove to be very difficult, requiring rigorous sample preparation prior to analysis. Previously our group has presented a simplified method for the comparative analysis of RNA digests (CARD). In the CARD approach two complete sets of digestion products from total tRNA are compared using the enzymatic incorporation of heavy O18 isotopic labels. By doing this we are able rapidly screen total tRNA from mutants or even comparatively sequence total tRNA from two species with high genomic homology. One short coming of this approach has been data analysis can become quite complex due to convoluted mass spectra; Here, we demonstrate that culturing our bacteria in C^{13} depleted media interferences greatly simplifies our spectral interpretations by removing interferences cause by isotopic overlap. Further this approach is illustrated by using automated data analysis tools provided through open source software.

Methods

Chromatographic separation was conducted using a Hitachi LaChromeUltra UPLC system. IP-RP-HPLC separations were performed using a Poroshell™ 2.7-μm C18 solid-core stationary phase column from Agilent®. Mobile phase A consisted of 16.3mM TEA/400mM HFIP at pH 7.0 in water; equal amounts of mobile phase A and methanol were combined to produce mobile phase B. Flow rates from 50 to 150uL were tested with a gradient slope of 1.5% per minute. Final, optimized high throughput assay used a 150uL/min flow rate with a gradient of 9%B per minute. MS analyses were performed using a Thermo LTQ XL™. Software used for data analysis include MZmine™ and MSight™.

Assays were produced using theoretical digestion products for total tRNA from E.coli using the guanine specific enzyme RNaseT1. *E.coli* were grown in MONOEXPRESS (NATURAL ABUNDANCE-N) from Cambridge isotopes. Data analysis was performed using MZmine™ and MSight™ open sources software.

Preliminary Data

Growing bacteria in C^{13} depleted media significantly reduces spectral complexity and simplifies biological data analysis. Both MZmine ™ and MSight™ provide adequate 2D alignment features for comparing MS data and should be valuable tools for future comparative analysis.

Novel Aspect

An automated method for total tRNA sequencing using isotopic labeling.

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SILAC Ratio Calculator - An Innovative Tool that Automates Normalization and Peptide Level Quantitation of SILAC-MS/MS Data Sets

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Introduction

Histone post-translational modifications (PTM) play a crucial role in stabilizing chromosomal structure and regulating gene transcription. Here, stable isotope labeling by amino acids in cell culture (SILAC) combined with LC-MS/MS was implemented to quantify relative changes in histone PTMs. SILAC is a powerful tool for measuring relative changes in proteins. However, challenges arise when using SILAC for peptide level quantitation. To address these challenges, we developed a novel informatics approach that enables robust SILAC quantitation of peptides. The algorithm uses peptide clustering and novel global normalization to allow for robust calculation of SILAC ratios. The algorithm was used in a systematic study of histone modification crosstalk to calculate relative changes in histone H3 K79 methylation and H3 K56 acetylation.

Methods

The algorithm is provided a precursor ion mass (or mass list) and information about the SILAC label. First, the program scans the mzXML file to identify all light: heavy signal pairs for all possible charges and isotopes. Next, a global normalization factor is determined from the median and abundance weighted median in addition to the slope of light vs. heavy abundances fitted by linear regression and robust linear regression. The program then iterates over all the MS1 scans to cluster signals by mass and retention time that correspond to the peptide(s) of interest. The ratio for the peptides is calculated in the same manner as for the normalization factor.

Preliminary Data

The "SILAC Peptide Ratio Calculator" enables robust relative peptide quantitation. First, the algorithm utilizes four strategies to determine a global normalization factor: median ratio, abundance weighted median ratio, linear regression slope and robust linear regression slope. For better normalizing performance, a two-fold ratio filter is implemented that rejects all ratios greater than a two-fold change for normalization. Next, the algorithm determines the SILAC ratio for the target peptide(s). Mass accuracy tolerance and signal rejection threshold are used to aggressively filter noise, improving the accuracy of SILAC ratios. For example, we used the calculator to determine the ratio for the peptide H3 EIAQDF[79]KTDLR. As the mass accuracy improved from 0.1 to 0.01 and signal rejection threshold from 10,000 to 100,000, the number of

signal cluster decreased from 17774 to 157. Retention time clustering with a 0.5 min elution window was able to effectively cluster the true peptide signal from an interfering peptide that eluted a minute later. The SILAC ratio of the target peptide cluster was determined by the slope of a robust linear regression of the light and heavy abundances. The ratio is then normalized by the global normalization factor. The program outputs light vs heavy abundance plots and spreadsheets necessary to evaluate the quality of normalization and peptide ratio. We applied the SILAC ratio calculator to determine histone post-translational modifications changes associated with histone crosstalk. In this study, 44 site-directed yeast histone mutants were used to mimic changes in histone PTMs while we probed other modifications sites for crosstalk induced changes. The final relative ratios for the histone modifications, H3 K79me and H3 K56ace were compared pair-wise to the wild-type using an unpaired t-test. Using this approach we identified several novel crosstalk pathways in yeast, demonstrating the utility and power of this approach.

Novel Aspect

A novel informatics approach for robust quantitation of peptide post-translational modifications from SILAC LC-MS/MS data.

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Quantitative Global Phosphoprotemics of a MAPK-AKT Dual Pathway Inhibitor Anti-Cancer Drug

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Introduction

A novel anti-cancer drug TRC-382, a single agent dual AKT-MAPK pathway inhibitor, has been shown to possess anti-proliferative and anti-tumorigenic activity in both non small-cell lung cancer (NSCLC) cell culture and mouse models. Subsequent analysis identified TRC-382 as a potent activator of PP2A, a protein phosphatase and tumor suppressor protein, and a major negative regulator of AKT and ERK signaling. To better understand global pathway perturbation and downstream effectors of this molecule, phospho-proteomic profiling of TRC-treated H1650 cells was performed and compared to treatment with presumed single agent inhibitors MEK (Selumitinib,AZD 6244) and Akt inhibitor (MK2206) treated cells. This approach provides a global, unbiased evaluation of the perturbation of phosphorylation signaling pathways upon treatment of this phosphatase activator.

Methods

The H1650 lung adenocarcinoma cell line was chosen to perform triplicate global proteomic profiling across different drug treatments. Each sample was split to provide a technical replicated and these samples were digested and enriched for phosphopeptides using TiO2 column followed by a C18 cleanup. Subsequent to enrichment, label-free LC-MS/MS was performed using the Waters Nano-acquity UPLC-LTQ Velos Orbitrap XL proteomics analysis platform. Automated differential quantification of peptides was accomplished using Rosetta Elucidator. Peptide and protein identifications were integrated with these quantifications and used for statistical analysis via a standard one way ANOVA. Significantly changed proteins were imported into Ingenuity Pathway Analysis (IPA) and drug specific molecular networks were generated.

Preliminary Data

Proteomic data based on 4-hour LC-MS/MS analyses of H1650 lysates yielded a successful quantification of over 3531 peptides after enrichment that mapped to 1693 non-redundant phopsphoproteins. The protocol had good specificity with 79% detected peptides seen to be phosphorylated. In addition, the method was very reproducible with over 80% of the phosphopeptides measured having less than a 30% coefficient of variance. Pathway analysis of the detected phosphoproteins indicates the protocol can accurately compare many important signaling pathways, including: MEK, AKT, Fak-Src and RhoA.

Novel Aspect

Discovery and further mechanistic understanding of a first-in-class, novel anti-cancer drug TRC-382 using global phosphoproteomics.

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Ligating Oligonucleotide and NAD$^+$ with T4 RNA Ligase

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T4 RNA ligase can be used to join two single-stranded oligonucleotides together. As such, this ligase may be a potential tool for site-specific labeling of oligonucleotides in analytical methods that benefit from differentially labeled samples. The donor molecule in this ligation reaction must contain a 5'-phosphate group, and the acceptor molecule must contain a 3'-hydroxyl group. In this research, the model acceptor consists of a 10-mer oligonucleotide and the donor is nicotinamide adenine dinucleotide (NAD$^+$). Variables in the ligation reaction, including reaction time, ratio between acceptor and donor, T4 RNA ligase amount and dimethyl sulfoxide (DMSO) concentration, were investigated and optimized. Liquid chromatography mass spectrometry (LC-MS) is used to monitor these reactions. Data from reaction optimization and new potential donor and acceptors in the ligation reaction will be presented.

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Changes in Volatile Compounds of Human Urine as it Ages

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The urinary odors emitted from toilet facilities and from individuals suffering from either incontinence or metabolic disorders are perceived as unpleasant. While numerous studies have been conducted to investigate the volatile organic compounds (VOCs) released from urine, the odorants responsible for the urine odor are not well characterized. Furthermore, anecdotal reports suggest that the odor of aged urine is different from that of fresh urine. However, no study has investigated the VOCs released from aged urine. In this study, we analyzed and compared the VOCs released from fresh and aged urine samples, investigating the changes in the urinary VOCs as urine aged. Solid phase microextraction followed by gas chromatography-mass spectrometry was employed for extraction and analysis of VOCs from urine, and the resulting chromatograms and mass spectra were then analyzed by using Metabolite Differentiation and Discovery Lab, a novel metabolite profiling software. There was an overall decrease in concentration of many urinary VOCs due to evaporation of urine as it aged. On the contrary, some highly water-soluble compounds such as short and branched chain organic acids and trimethylamine, increased. Their increased release is most likely due to the loss of water and the subsequent release of water soluble VOCs as urine ages. We suggest that these VOCs may contribute to the odor of the aged urine.

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Exploring High Energy Barrier Pathways for Better Structural Characterization of Stable Non-Covalent Protein Complexes by Surface Induced Dissociation (SID)

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Introduction

Native mass spectrometry (MS) is a method to study the quaternary structures of labile non-covalent protein assemblies. Tandem MS (MS/MS) and ion mobility (IM) are coupled with native MS to dissociate the protein complexes, and study the conformations, respectively. Different from the widely used collision induced dissociation (CID), surface induced dissociation (SID) deposits more energy into the ions in a shorter time by colliding the ions into a surface. Previous MS/MS and IM studies in our laboratory suggest that SID is able to access high energy barrier pathways which cannot be observed in CID. In this work, SID shows its ability to dissociate stable protein complexes which are resistant to be dissociated in CID, to reveal more structural information.

Methods

IM-MS was performed on a modified Synapt G2 HDMS (Waters MS Technologies, Manchester, UK) and the custom SID device located between the CID cell and the IM cell. By adjusting the voltages of the SID device electrodes, the ions can be transmitted through for CID and IM experiments or collide with the surface. IM seperated the products based on their collision cross section (CCS).

Glycogen phosphoralase B kinase (PHB) dimer from rabbit muscle and L-glutamic dehydrogenase (GDH) hexamer from bovine liver were used as model proteins in this study. Triethylammonium acetate (TEAA) was used to reduce the charge states for comparing CID/SID fragmentation behaviors of different charge states.

Preliminary Data

Two charge states of PHB dimer and GDH hexamer were selected for CID and SID. No obvious dissociation was observed in CID and the precursors are unfolded. In contrast, SID produced various fragmentation products.

PHB dimer produced monomer in SID. In the SID spectrum of the +27 PHB dimer, the folded and unfolded monomers were observed. The percentages of folded and unfolded monomer at different SID acceleration voltages show that the folded monomer has lower onset energies than the unfolded monomer. At higher SID acceleration voltage, the folded monomer is less than the unfolded monomer, indicating the unfolded monomer is thermodynamic control product while the folded monomer is kinetic control product. Also, the precursor ion needs to be partly unfolded (CCS increase) to be dissociated. SID of the +21 PHB dimer shows mainly

folded monomer. The dissociation pathway of +21 is different from that of the +27. More folded monomer indicates that lower charged precursor better preserves its folded state in MS/MS.

In the SID spectrum of +39 of GDH hexamer, monomers, dimers, trimers and charge-stripped hexamers were observed. Similar to PHB, monomer and trimer of GDH products have folded and unfolded forms. Again, the precursor needs to partly unfolded before dissociation. The SID spectrum of +27 of GDH hexamer is obviously different from that of +39. The major product is the folded trimer while the percentages of all the other products in the total signal are lower than those in +39. Similar to PHB, the lower charge state of GDH also produces more folded products.

Because SID deposits more energy into the complex in a shorter time than CID, high energy barrier pathways can be accessed.

Novel Aspect

This study shows SID's ability to reveal substructure of stable protein complexes that do not dissociate in CID.

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Thermus Thermophilus tRNAs-Asn, Asp, His and Tyr are not Modified at the Wobble Position

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Thermus thermophilus is a gram-negative thermophilic bacteria that has served as the model organism for recent studies into ribosome structure and function. Unlike a majority of bacteria, *Thermus* does not posses the hypermodified nucleoside queuosine (Q) within its tRNAs. In other bacteria, tRNAs coding for asparagine, aspartic acid, histidine and tyrosine contain Q, modified in place of guanosine, at position 34 – the wobble position. While the exact function of Q is still not understood, it appears that this modification may enhance translational fidelity through reading frame maintenance or improved decoding. Before undertaking a detailed study into the significance of queuosine's absence in *Thermus*, we first sought to determine whether some other modified nucleoside is found at the wobble position of these four tRNAs. To answer this question, individual tRNAs from *Thermus* were purified using hybridization probes attached to biotinylated streptavidin beads. These purified tRNAs were digested with RNase A or T1 and the resulting oligonucleotides were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mass spectrometry sequencing of the anticodon region of these four tRNAs reveals each to contain the genome-encoded guanosine, with no evidence that other modifications are found in place of queuosine for *Thermus* tRNAs. In addition to presenting the data characterizing the anticodon sequences for these four tRNAs, additional sequence information from each will be presented, including placement of modified nucleosides found at other sequence locations.

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Mass Spectrometry and Tandem Mass Spectrometry Analysis of Alkyl Polyglysoside (APG) Surfactants

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Introduction

Surface active agent (Surfactant) means active at the surface. The surfactants have the ability to adsorb at surfaces or interfaces between any two immiscible phases and they are categorized by this property. Surfactants reduce the surface tension. They consist of a hydrophilic head group and a hydrophobic tail. They are divided in four classes (cationic, anionic, nonionic and zwitterionic) depending on the charge in the hydrophilic head when it dissolves in aqueous solution. They are primarily utilized in personal-care products, and they are also added to other industrial and pharmaceutical products. Alkyl polyglysosides (APGs) are a kind of nonionic surfactant which does not produce ions in aqueous solution. APGs contain a sugar head and a hydrophobic hydrocarbon tail. They have low toxicity, fast biodegradability, and they are prepared from renewable resources. They are typically prepared by reacting fatty alcohols with starch or glucose during the Fischer synthesis. These products are very complex because they contain a large number of different chemical compounds. Different analytical techniques can used to study PAGs including LC-MS and GC-MS. APGs are generally macromolecular mixtures. In this study, APG surfactant blends have been characterized by matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometry.

Methods

The three samples analyzed are plantacare 818, plantaren 1200 and plantaren 2000 supplied by BASF. All samples are poly (1, 4-glycosides) substituted at the reducing end with C8-16 alkyl chains. MS and MS/MS investigations of these samples were carried out on a Bruker HCT ion trap MS equipped with an ESI source and a Bruker UltraflexIII MALDI-TOF/TOF mass spectrometer equipped with a Nd:YAG laser. The samples were dissolved in MeOH at a final concentration of 0.01 mg/ml (ESI) and 20 mg/ml (MALDI). For MALDI analysis, the sandwich method was used with dithranol as a matrix. The cationizing agent used was NaTFA (MALDI) and LiTFA (ESI).

Preliminary Data

With MALDI, all samples form $[M+Na]^+$ ions. Oligomers with glucose (162 Da) and hydrocarbon (28 Da) repeat units and the same end groups (2H, O) are present in all mass spectra. Plantacare 818 is found to contain 1 to 8 glucose (G1-G8) units and C8-C16 alkyl chains. Plantaren 1200 gives a narrower distribution, viz. G1-G7 and C12-C14. On other hand,

Plantaren 2000 shows a G1-G6 and C8-C14 distribution. Upon MALDI-MS/MS, sodiated APG oligomers undergo losses that identify both the saccharide unit as well as the alkyl chain (R), which are eliminated as dehydrated moiety and ROH alcohol, respectively.

With ESI, all samples form $[M+Na]^+$ and $[M+Li]^+$ ions. The ESI spectra are more complex than the MALDI spectra because APGs form dimers in MeOH solution. MS/MS shows that the dimers break to monomers at low collision energy, consistent with noncovalent (hydrogen) bonding between the two monomeric APG units. The ESI-MS and MS/MS spectra further reveal that both homodimers (identical alkyl groups) and heterodimers are present in the MeOH solution. Such aggregation agrees well with the micelle-forming properties of APGs.

Overall, the multidimensional MS approaches discussed offer a simple, fast, and detailed analysis of the macromolecular structures present in APG blends.

Novel Aspect

MALDI and ESI provide rapid and definitive information about the hydrophilic units and alkyl chain distributions in Alkyl polyglysosides surfactants.

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Sequencing the Transfer RNAs from *Thermus thermophilus* Using Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

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Post-transcriptional modifications of ribonucleic acids play an important role in structural and functional regulations. Sequencing of transfer RNAs, the most highly modified species, sheds light on the details of modification-function relationships and how tRNA serves in the process of protein translation. However, the application of typical sequencing methods is limited by the frequent occurrence of modifications in tRNAs, such that only five organisms have their total tRNAs sequences (including post-transcriptional modifications) uncovered to date. Here we report a method that holds the potential to provide a complete modification map of total cellular tRNAs from thermophilic bacterium *Thermus thermophilus* by the use of liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). As enzymatic digests of total cellular tRNAs are of similar properties, many efforts have been taken to improve the detection and fragmentation of RNase digestion products from tRNAs. Improved quality of MS/MS spectrum is achieved for identification and sequence location of modified nucleosides, as well as increased number of detected ions. Conserved modifications among the bacterial thermophiles help make reasonable predictions on the location of modifications, followed by further confirmation by the in-house software and Excel-Macro. By combining site-specific enzymatic digestion (using Ribonuclease T1) with data-dependent MS/MS, some post-transcriptional modifications in *T. thermophilus* have been mapped onto specific tRNA sequences.

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Modification of the Prolyl Ring of Val-Pro-Ala and the Impact of this Modification on b₂ Ion Structure

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Introduction

The fragmentation of peptides via collision induced dissociation in mass spectrometry results in the formation of b and y ions. These fragments result from the cleavage of the peptide bond and are used in protein identification in the field of proteomics. Literature reports for solution experiments show that substituents placed on proline can alter the shape of the ring and therefore change the cis/trans isomerization, which might affect the structure of gas phase fragments containing these homologues. Here we present b2 ion studies on one tripeptide (VPA) and show how the addition of a fluorine or a hydroxyl group on the 3rd position of the proline ring can affect the b2 ion formation.

Methods

Peptides were all synthesized using FMOC solid-phase synthesis and analyzed via tandem mass spectrometry on a Thermo Velos Pro, ion mobility-mass spectrometry (IM-MS) on a Waters Synapt G2S, hydrogen-deuterium exchange on a Bruker Apex Qe 9.4 T FT-ICR MS, and action infrared-multiphoton dissociation (IRMPD) spectroscopy on a Bruker Esquire ion trap. Density functional theory (DFT) was used to determine computationally the stability of different structures. Calculated structures were used both to justify the likelihood of specific structures existing in the gas phase as well as to directly compare experimental mobility drift times and IRMPD spectra to theoretical ones.

Preliminary Data

IRMPD results of ValHyp (Hyp=hydroxyproline), ValFlp (Flp=trans-fluoroproline), and Valflp (flp=cis-fluoroproline) all show the presence of a strong oxazolone band in the CO region at 1900 cm^{-1}. The presence of peaks in the diketopiperazine region between 1700 and 1800 cm^{-1} varies depending on which substituent is placed on the prolyl ring. Recently published data from our group showed a pair of medium sized diketopiperazine bands at 1760 and 1701 cm^{-1} for ValPro and we observed similarly intense bands for Valflp at 1752 and 1689 cm^{-1}. ValHyp and ValFlp fail to show any significant diketopiperazine bands, but if zoomed in x10 a small band can be observed at 1756 cm^{-1} for ValHyp. This stretch could correspond to the amide I CO but the lower band representing the protonated amide CO stretch is not present.

In the order of diketo band intensity based on IRMPD data alone, the ranking of ValPro and its substituent homologues goes from Valflp with the most intense bands, ValPro, ValHyp and finally ValFlp, which has no bands in this region close to the calculated bands. In solution

studies performed by Ron Raines, Hyp and to a greater extent Flp have been shown to retain the trans conformation for the N-terminally adjacent peptide bond. In the same work, Pro was shown to be relatively more suitable for influencing the isomerization to cis while peptide bonds N-terminal to flp had the greatest ratio of cis/trans
character. Because formation of diketopiperazine requires a cis bond, our data shows that the in solution trans/cis isomerization trend carries over to gas phase fragmentation.

To supplement this ion spectroscopy data, MS^3 fragment analysis, ion mobility with MOBCAL calculated structure comparisons, and H-D exchange data will be presented. To date, this supporting information is consistent with the chemical information found by action IRMPD.

Novel Aspect

Second residue prolyl ring substitution that favors cis favors formation of the b2 diketopiperazine.

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Quantitative Proteome Turnover in *C. elegans*

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Introduction

      Caenorhabditis elegans (*C. elegans*) is a useful model organism for studying the turnover of individual proteins throughout its life cycle as the worm has short life cycle (around 3 weeks). Isotope Labeling with Amino acids in Cell culture (SILAC) is a useful approach for *in-vivo* incorporation of a labeled amino acid(s) into proteins for mass spectrometry (MS)-based quantitative proteomics. We, in this study, have utilized SILAC proteomic analysis to monitor the proteome turnover in the *C. elegans* during its life span.

Methods

      Using the M9 minimal media, ^{12}C- or ^{13}C-lysine was incorporated into the proteins of lysine auxotroph bacteria *E. coli* AT713. *C. elegans* was then fed with the ^{13}C-lysine labeled *E. coli* for two generations and at the L-4 larvae stage, they were transferred to the plates containing ^{12}C-lysine labeled *E. coli*. The worm samples were harvested every other day up to day 16, digested by Lys-C, and analyzed by LC-MS/MS. The protein identification and the quantification were performed by the "ProteomicsTools". The ^{12}C-peptide/(^{12}C-peptide + ^{13}C-peptide) ratios for individual peptides from the same protein were used to calculate the ^{12}C /(^{12}C + ^{13}C) ratio of the protein at each time point , and the ratios were plotted as a function of time.

Preliminary Data

      Life span assay of *C. elegans* grown on ^{13}C-Lys and ^{12}C-Lys determined that the heavy isotope has no effect on the worm's life span. Using the SILAC pulse chase approach, the labeled and newly synthesized unlabeled proteins were quantitatively monitored during the life span of the worms using mass spectrometry. As expected the ratios of the ^{12}C /(^{12}C + ^{13}C) peptide decreased as the worm ages, for all the proteins. We have quantified more than 500 proteins for most of the time points. We observed the general trend of fast protein turnover rates from day 1 - 5 and also from day 11 – 16 for most of the proteins. Lipid binding proteins, proteases and DNA transcription regulators were found to have fast turnover rates until day-5. The turnover rates of the structural proteins such as actin/myosin and calcium binding proteins were slow. While the turnover rates of ribosomal proteins involved in the protein synthesis were much faster in the later stages of the worm's life. Comprehensive data analysis is underway and the results will be discussed. This study gives us a glimpse as to what kinds of proteins are synthesized at which stages of an organism's life. Such information may be useful to better understand the physiological changes during aging.

Novel Aspect

Use of SILAC pulse chase approach with *C. elegans* to profile the proteome turnover.

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Inosine at the Wobble Position of tRNA[Arg] in *Thermus thermophiles*

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Thermus thermophilus is a gram-negative thermophilic bacteria that has served as the model organism for recent studies into ribosome structure and function. Unlike a majority of bacteria, the gene coding for TilS and TadA is fused in one operon. *Since* no one has reported the functionality of this fused gene product, whether this TilS and TadA are functional in *Thermus* is unknown. In other bacteria, tRNA carrying Arginine contains Inosine at position 34 – the wobble position, modified in place of adenosine by TadA. To determine the functionality of this gene product, we first need to determine the nucleoside at the position 34 of tRNA[Arg]. The individual tRNA was extracted from the total tRNA and digested with RNase T1. The digestion products were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The nucleoside at position 34 was determined to be I by denovo sequencing. In addition, the existence of I in *Thermus* was further verified by UV-LC-MS/MS analysis of nucleosides. Other than that, we also found the existence of methyl-inosine in *Thermus*.

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A Label-free Shotgun Proteomic Characterization of Multiple Myeloma Derived Extracellular Vesicles

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Introduction

Multiple myeloma is the second most common hematological malignancy. Extracellular vesicles have become a prominent research focus due to the growing evidence that these molecules can functionally manipulate the surrounding microenvironment through transfer of bioactive proteins, mRNA and miRNAs. This study uses label-free shotgun proteomics to examine differences between multiple myeloma vesicle and cellular lysates from different patient derived cell lines. Analysis was performed using a novel spectral count quantitation tool that combines three separate tag-count based hypothesis tests. The data show that cell line of origin and protein packaging are key factors effecting the protein composition of vesicles. These data in future research projects to prioritize protein targets for further functional assays as well as markers for myeloma progression.

Methods

Extracellular vesicles were isolated from 48 h serum starved multiple myeloma cell culture media by differential centrifugation. In triplicate, vesicle isolations and cell pellets were subjected to overnight (>16 h) tryptic digestion and peptides were separated by Dionex HPLC and characterized by ThermoFisher Orbitrap-XL mass spectrometer. LC-MS/MS data was searched against the UniProt human reference proteome database using the MassMatrix search engine for protein identifications. Protein matches were retained based on an FDR of 5% or 2 total decoy matches and with a minimum of 2 unique peptides per protein identification. Significance testing of relative protein abundance from spectral count data was performed by the MultiSpec tag-count software with cross-algorithm q-value equivalence testing at an FDR level of 5%.

Preliminary Data

The functional consequences of extracellular vesicles released from both normal and cancer cells has become a significant research topic. This is due to the ability of these molecules to transfer bioactive proteins, mRNA and miRNAs to distant target cells. However, to date no proteomic or label-free spectral count comparisons between extracellular vesicles and their

cellular origin have been completed. Using six distinct multiple myeloma cell lines, MM.1S, MM.1R, U266, L363, OPM2, and RPMI 8226, each associated with different disease states, we sought to identify mechanistic pathways associated with the pathogenesis of multiple myeloma.

The comparison of protein identifications between vesicles and cellular lysates show a large ID overlap. Similarly, identification comparisons between vesicles of different cell lines show even greater overlap (>335 IDs). These results suggest the unique protein identifications in the vesicles are not the cause of functional differences. Hierarchal clustering of the spectral count data showed clustering by both sample type (vesicle or cellular lysate) and by vesicle cell of origin suggesting differences in protein abundances between samples. MultiSpec significance analysis of the label-free spectral counts determined that a large number of proteins had variable expression across sample types. For example, label-free comparison of the MM.1S and U266 vesicles yields 125 proteins with significant expression differences. Bioinformatic analysis of gene ontological annotations shows many biological process and molecular functions represented by the differentially expressed proteins between sample types. Collectively, these results suggest the functional differences observed from extracellular vesicles were not necessarily a result of specific vesicle protein packaging but a result of protein abundance within the vesicles.

The results from these experiments are significant for two reasons. First, we are the first to apply a label-free spectral count approach to analysis of extracellular vesicles. Second, our data establishes a starting point for mechanistic understanding of myeloma pathogenesis.

Novel Aspect

Our research applied label-free spectral count quantitation to the analysis of multiple myeloma extracellular vesicles.

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Yeast Lipidomics via LC-MS Profiling Using the Q Exactive Orbitrap Mass Spectrometer

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Mitochondrial lipids from wild-type (WT) yeast (*S. cerevisiae*) and a knockout (KO) strain that does not produce coenzyme Q (CoQ) were profiled by liquid chromatography mass spectrometry (LC-MS) using a benchtop quadrupole – Orbitrap mass spectrometer[1]. Mitochondria were isolated from WT and KO yeast, and mitochondrial lipids were extracted in triplicate with cold isopropanol. The LC-MS data was obtained at 70,000 mass resolving power in positive ion mode using a previously published LC method[2,3]. Lipid species were identified by analysis of the MS/MS data (35,000 resolution) using Lipid Search software (MKI, Tokyo, JP). The fully automated analysis identified over 300 lipids from over 600 different ions in four data files. Statistical analysis of differences in the abundance of individual lipids from WT and KO yeast revealed significant changes in multiple lipid species, including coenzyme Q as well as a variety of phospholipids, sphingolipids, and glycerolipids from this complex mixture.

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Comprehensive, Accurate, and Precise Quantification of Acylcarnitines and Acyl-CoAs in Tissues using On-Line Ion-Exchange Trapping and UHPLC-MS/MS

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Introduction

(U)HPLC-MS/MS analysis of acylcarnitines and acyl-CoAs is usually not comprehensive due to the different polarities of the short-chain, medium-chain, and long-chain acyl species. We have developed rigorously quantitative UHPLC-MS/MS methods using on-line ion-exchange trapping followed by UHPLC and applied it to tissue analysis of acylcarnitines and acyl-CoAs. All categories (short-, medium- and long-chain, dicarboxylic, and hydroxy-) are quantified in a single 13.5 min chromatogram for acylcarnnitines and a 15 min chromatogram for acyl-CoAs.

Methods

Tissues (25 mg) were homogenized in 1 mL of acetonitrile / isopropanol / potassium phosphate buffer, centrifuged, and 10 µL of the supernatant applied to an Oasis MAX SPE plate for the isolation of acylcarnitines. Following derivatization with pentafluorophenacyl trifluoromethanesulfonate, samples were injected into a UHPLC equipped with a loading pump, analytical pump, and a valve for on-line SCX trapping of all acylcarnitine species. Acylcarnitines were then flushed onto a UHPLC reversed-phase column, separated using an acetonitrile gradient, and detected using a QQQ mass spectrometer. For acyl-CoAs, a 500 µL aliquot of the same tissue homogenate was applied to an SPE column containing 100 mg of 2-(2-pyridyl)ethyl-functionalized silica gel. On-line WAX trapping was used to trap all the acyl-CoAs.

Preliminary Data

Quantitative isolation of acylcarnitines from tissues was followed by complete derivatization for maximum sensitivity and chromatographic performance. On-line SCX trapping followed by UHPLC reversed-phase chromatography separated isomeric acylcarnitine pentafluorophenacyl esters, from acyl-chain C2 - C18. Detection was by positive ion MRM. Quantification was performed using class appropriate internal standards and multiple-point calibration curves, i.e. 13 points over a 200-fold range. Quantitative isolation of acyl-CoAs from tissues, followed by on-line WAX trapping and UHPLC reversed-phase chromatography, separated isomeric acyl-CoAs (from dicarboxylic to long-chain acyl-CoAs). Detection was by positive ion MRM and quantification was performed using internal standards and multiple-point calibration curves. Qualitative analysis of acylcarnitines and acyl-CoAs was also successfully performed, by removing the UHPLC column and collecting a precursor ion scan for acylcarnitines (positive ion 400-1000 m/z on the precursor 293 m/z) or a neutral loss scan

(positive ion 700-1600 m/z on the neutral loss of 507) for acyl-CoAs. Tissue samples from animal studies were analyzed.

Novel Aspect

All categories (short-, medium- and long-chain, dicarboxylic, and hydroxy-) of acylcarnitines and acyl-CoAs were quantified, each in a single chromatogram.