A1

Probing the Molten Globule Structure of Aspartate Aminotransferases on a Ion Cyclotron-Fourier Transform Mass Spectrometer in Combination with Hydrogen Exchange

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Two forms of the enzyme aspartate aminotransferase coexist in the eukaryotic cell, one cytosolic (cAAT) and another mitochondrial (mAAT). The molecular chaperone hsc70 discriminates between these two highly similar isoenzymes, and binds exclusively to some early folding intermediates of the mitochondrial form. The nature of this discrimination is not fully understood, but one possibility is that hsc70 recognizes some folding intermediates that are absent, or poorly populated, during the refolding of cAAT. Here we examine the structure of these early folding intermediates for each isoenzyme form by a combination of deuteration exchange and mass spectrometry. Unfolding of the proteins at pH 2.0 in D2O results in the labeling of about 90% of all amide linkages for both proteins. During the first few seconds after refolding of these proteins in protiated buffer, mAAT exchanges back all of the deuterium that was acquired during labeling of the unfolded state. However, cAAT retains a protected core of 20% of all amide linkages. These protected regions have been mapped by mass spectrometry on a Fourier Transform Ion Cyclotron Mass Spectrometer (LTQ FT, ThermoFinnigan), and have been compared to the corresponding regions of the mitochondrial enzyme. Our results are consistent with the rapid collapse of the cytosolic isoenzyme, resulting with the formation of a highly structured hydrophobic core that contains protected amide linkages. These structural changes are global and not confined to a particular region of the protein. Thus, the ability of hsc70 to discriminate between mAAT and cAAT can be explained by sequestering sequence elements on cAAT into this hydrophobic core, therefore making them unavailable for binding to the chaperone.

A2

Monitoring Chemical and Oxidative Modifications in Human Estrogen Receptor α Isoform

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Introduction: Accumulated scientific evidence has demonstrated the importance of an activated estrogen receptor (ER) mechanism that not only drives both the initiation and promotion of most human breast cancers but also serves as the molecular target for lifesaving endocrine therapy. Our previous published studies indicate that different chemical oxidants cause alternative types of ER structural change, which in a few clinical tumors appear to be reversible, but in the majority, ER changes are apparently irreversible. Quinone oxidants are known to generate both reactive oxygen species and arylated proteins. Comparing the action of menadione and other thiol-reacting agents on model peptides and the 67 kDa recombinant ER alpha isoform, we are optimizing the detection and analysis of modified ER by mass spectrometry.

Preliminary Results: Double enzyme digestion of recombinant ER, using endoprotease Lys-C and Asp-N, gave rise to diagnostic zinc finger peptides derived from the ER-DNA binding domain (DBD), each containing two cysteine residues. By comparing peptide mass fingerprints generated from the chemically treated recombinant protein samples with those from controls, cysteine residues within the DBD that exhibit heightened sensitivity to modification by vit.k3, BrB, and IAA are characterized. Analysis of MALDI-TOF spectra revealed that arylation by vit.k3 gave rise to distinctive quinone/hydroquinone thioether adduct ions. Unlike alkylation reactions performed with IAA, treatment with BrB or vit.k3 resulted in a fraction of the zinc finger peptides remaining unmodified, suggesting that steric factors may have a negative impact on reactions between the latter reagents and cysteine thiols. Additionally, the mono and doubly alkylated peptide adduct ions of the selected reagents were identified by MALDI-TOF/MS. Tandem-MS analysis of peptide adducts of each reagent produced conclusive b and y fragment ions that confirmed the exact location of modified residues. Tandem-MS analysis performed on mono alkylated peptide adducts of the selected reagents also established that Cys thiols within the ER-DBD are not chemically equivalent. The presence of basic lysine or arginine residues in the neighborhood of Cys thiols appears to influence the thiol dissociation constants (pKa’s) and consequently their chemical reactivity. By spiking breast cancer cell lysates (MCF-7 cells) with recombinant ER, followed by immunoprecipitation with antibodies from different sources, we have optimized an affinity purification protocol for ER enrichment and successfully analyzed endogenous ER derived solely from MCF-7 cells by MS. Treatment of growing MCF-7 cells with chemical oxidants/quinones should allow us to catalogue chemical modifications of amino acids in ER alpha. This will help to elucidate some of the mechanisms underlying chemical modulation of ER alpha and breast tumorigenesis.
A.3
The Elucidation of the Disulfide Framework of Conotoxin GIIIB Using Electron Capture Dissociation Mass Spectrometry

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The conotoxins are a series of peptide families that have been isolated from the venom of the Conus genus of tropical cone snails. The venom contains a variety of peptides many of which are rich in cysteine residues. Generally, the cysteines are involved in disulfide bridges that contribute to the overall 3D structure of the peptides and thus influence their biological activity. Standard mass spectrometric fragmentation analysis (i.e. CID) requires that the disulfide bonds, be reduced and alkylated prior to analysis to ensure adequate fragmentation for determination of the primary sequence. As a result, the disulfide framework information is lost. Other methods are available for the determination of the framework but these are often tedious and time consuming. Electron Capture Dissociation (ECD) mass spectrometry is a non-ergodic process that has been shown to cleave disulfide bonds thus making reduction and alkylation redundant. ECD mass spectrometry was used to elucidate the disulfide framework of conotoxin GIIIB, a peptide that contains 3 disulfide bridges, utilizing fragmentations unique to this type of mass spectrometry. The fragmentation allowed the correct framework to be elucidated from the 15 possible arrangements.

We would like to acknowledge the financial support of the NIH (Grant: NIH NCRR RR001614).

A.4
Capturing the Dynamics of GRP94 Homodimer by Using Chemical Cross-linking and Mass Spectrometry

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Glucose-regulated protein of 94 kDa (GRP94), the endoplasmic reticulum (ER) paralog of heat shock protein 90 (Hsp90), is known to perform an essential function(s) in the folding and assembly of numerous membrane and secretory proteins. GRP94 is highly homologous to its cytosolic counterpart, Hsp90, which facilitates protein folding via ATP-binding and hydrolysis-linked interactions with client protein substrates. In current views, ATP binding functions to promote transient intermonomeric dimerization of the Hsp90 N-terminal ATP binding domains; it is this conformation that is thought to support client protein activation. In this study, we analyzed GRP94 quaternary structure using chemical cross-linking in conjunction with tandem mass spectrometry and report the identities of six cross-linked peptides, representing two distinct interaction surfaces between the monomeric subunits of the GRP94 dimer. Two of the cross-links localize near the C-terminal dimerization domain, consistent with previous biophysical and biochemical studies. The remaining four cross-linked pairs identify a novel, ATP-independent intermonomeric contact between the N-terminal and middle domains of the subunit polypeptide chains. These data provide direct experimental evidence for a compact conformation with two monomers intertwined in native, full-length GRP94. The broader implications of these structural insights will be discussed.

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A.5
Characterization of IgG1 Molecule by Peptide Map

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Peptide map was developed for an IgG1 molecule following a standardized method to shorten development timeline. In brief, the antibody is reduced and alkylated under denaturing conditions. After buffer exchange to TRIS solution, it is deglycosylated with PNGase F, then digested with Lys-C. A Kromasil C18 reverse phase column is used for peptide separation.

The method was developed to characterize the IgG1 molecule within 2 weeks, with more than 97% sequence coverage. Combined with data from a second enzymatic digestion, 100% sequence coverage is achieved for the molecule.

This peptide map can successfully detect oxidation, deamidation, degradation, N-terminal cyclization and C-terminal Lysine variability. It can be used as an identity assay with preset acceptance criteria, and/or for other characterization purposes.

The method is robust, shows lot to lot, system to system, and analyst to analyst reproducibility.
A New Technique (COMSPAR1) Was Used to Identify 3-Isopropylmalate as the Major Endogenous Substrate of the Saccharomyces cerevisiae trans-Aconitate Methyltransferase

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Trans-aconitate, a spontaneous degradation product of the citric acid cycle intermediate cis-aconitate, appears to be detoxified in bacterial and fungal cells by a methylation pathway. While characterizing the homolog of the trans-aconitate methyltransferase in the yeast Saccharomyces cerevisiae (Tmt1), we found that this enzyme catalyzes an additional reaction forming an unknown type of methyl ester. This endogenous product was partially purified using anion-exchange HPLC, extracted with acidic ethyl acetate, derivatized with TMS, and subjected to GC/MS analysis. Comparing the mass spectra of samples prepared from wild type and tmt1 knockout cells, we found a 334 m/z ion using CI that was present in the wild type but not the mutant. This product was identified as one of the two possible 3-isopropylmalate methyl esters by chromatographic co-elution and El fragmentation patterns. Using NMR analysis, we were able to show that the C-1 carboxylate, and not the C-4 carboxylate was methyl esterified.

3-Isopropylmalate is a late intermediate in the leucine biosynthesis pathway in yeast in a reaction sequence that parallels the chemistry of the conversion of citrate to isocitrate via cis-aconitate in the citric acid cycle. Four stereoisomers exist for 3-isopropylmalate and we then asked whether the Tmt1 methyltransferase may be responsible for modifying one or more of the three “unnatural” isomers, or even for the trans-isomer of the isopropylmalate intermediate (isopropylfumarate). All of these compounds can form spontaneously and interfere with the biosynthetic pathway in a similar fashion that trans-aconitate interferes with the citric acid cycle. However, we detected no activity of the Tmt1 methyltransferase with isopropylfumarate and the stereoisomer of 3-isopropylmalate involved in the biosynthetic reaction appears to be the best substrate. Thus, these results suggest that the methylation of 3-isopropylmalate is not a detoxification reaction but may represent a new metabolic branch. 3-Isopropylmalate methyl ester is not present in yeast cells with a double mutant in the leu4/leu9 genes that encode the enzymes that synthesize the precursor 2-isopropylmalate, suggesting that the substrate for the methylation reaction is formed in the normal leucine biosynthetic pathway. Current research is focused on observing a phenotype between wildtype and tmt1- cells.

This approach in identifying methylated small molecules in wild type and mutant yeast will be used to identify other novel methyltransferases in yeast, particularly in conjunction with labelling with S-adenosyl[methyl-D3]methionine. Data analysis is facilitated using COMSPAR1 software (a free downloadable software package) that allows for rapid comparisons between 2 MS data sets in mirrored displays.
A.8

**Analysis of Cell Polarity Protein Regulation Using High Performance LC MALDI**

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An o-MALDITM Q-TOF mass spectrometer with high efficiency IDA data acquisition was combined with a unique LC-MALDI deposition device for the analysis of protein expression in cell polarity regulation.

The ability of cells to organize their internal structure such that particular regions of the cell become specialized in function is essential to life. Such cellular polarity allows a single cell to develop surfaces which are functionally and biochemically distinct, e.g., gut epithelial cells. The disruption of cell polarity has been associated with the onset of metastasis behavior in tumor development. RICH1 is a Rho GTase activating protein that, in addition to the RhoGap domain, contains a C-terminal proline-, serine-, and threonine-rich domain. A number of phosphorylation sites were suspected in this C terminus region, associated with various binding domains.

An investigation of the sequence and post-translational modifications of RICH1 was explored using the technique of LC oMALDITM Q-TOF. A unique LC MALDI deposition device was employed utilizing pneumatically driven pressure amplifiers and fast mixing dynamics to generate rapid high efficiency separations. The device is capable of electrostatically depositing LC eluant and matrix solution onto MALDI targets at frequencies of up to 1 kHz. An oMALDITM Q-TOF mass spectrometer exhibiting high efficiency IDA data acquisition and a fast 1.5-kHz Nd:YAG laser was utilized in this analysis of protein expression in cell polarity. In addition to exceptional sequence coverage, a number of biologically relevant and previously unknown sites of phosphorylation were readily identified.

A.9

**Subcellular Organelles: We Can Take the Cell Apart, but Can We Put It Back Together?**

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In pursuit of increased understanding of the multiple mechanisms that contribute in parallel to acquired drug resistance, we are measuring changes in protein abundances and structures in drug resistant human breast cancer cells. These investigations were designed from the beginning to be carried out at the organelle level, and thus far, unbiased proteomic studies of the cytosol, nucleus, mitochondria and plasma membrane confirm that a multiplicity of proteins and pathways are altered in drug resistant cancer cells. Working at the organelle level allows us to discuss altered proteins in the context of organelle biochemistry and function. In addition, some pan-cellular themes appear, notably changes in protein abundances that may stabilize apoptosis. Improved proteomic methods resulting from this program include enzyme-catalyzed O-18 labeling, pellicle isolation of the plasma membrane, and peptide isoelectrofocusing.

A.10

**Multidimensional Separation of Membrane Protein Digests Using Immobilized pH Gradients Coupled to LC-MS for Enhanced Sequence Coverage and Confidence**

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Introduction: Shotgun proteomics strategies whereby complex protein lysates undergo proteolytic digestion prior to sample fractionation offer significant advantages over traditional gel-based techniques, especially for hydrophobic proteins. Using such bottom-up approaches, protein identification is derived solely from MS/MS information; consequently a post-acquisition bottleneck is produced at the data analysis stage. Additional physical information, such as isoelectric point (pI) enhances the confidence of in-silico identification. Separation of peptides by immobilized pH gradients (IPG) is an effective method of sample fractionation and provides valuable pI data for sequence identification. We and others have found that combining pI fractionation with LC-MS in bottom-up analysis of complex peptide mixtures validates peptide sequence, and enhances sequence coverage of hydrophobic proteins.

Methods: Cytochrome P450 and nAChR were digested with trypsin (Promega) or endoproteinase AspN (Roche) overnight in the presence of surfactants. 5 ug of the digested samples were loaded directly onto 3–10 NL IPG strips (Invitrogen) in addition to a 1 ug aliquot of myoglobin. After electrophoretic focusing, the strips were cut into 8 equal pieces and peptides were extracted using 5% TFA and 50%ACN/2.5% TFA. The extracted peptides were dried using a speed vac (Savant) and were reconstituted in 20% ACN/2.5% TFA. The samples were then analyzed with both 4700 MALDI-TOF/TOF (Applied Biosystems) and Q-TOF LC-ESI/MS (Waters). The data were analyzed by GPS Explorer (Applied Biosystems), Mascot Distiller (Matrix Science) sequence database searching and GP-MAW 6.0 (ChemSW).

Preliminary Results: High recovery yields of digested peptides from IPG strips were obtained and thus we were able to analyze the same samples in parallel using the Q-TOF LC-ESI/MS and 4700 MALDI-TOF/TOF (via LC separation using automated spotting). Electrophoretic migration of peptides exhibited good reproducibility between multiple separations which allowed for assignment of peptides into pI GEBins. These bins represent a pI range that is 1/8th of the nonlinear 3 to 10 pH range of the strip. Sequence assignments were made based on 1) the exact mass of the peptide, and 2) by comparing agreement between the predicted pI and the known pI of a myoglobin proteolytic fragment co-migrating within the same pI bin. We confirmed our sequence identifications by MS/MS analysis to determine if sequence assignments by our method were successful. This study demonstrates that exact mass and assigning peptides to a narrow pI range is sufficient to successfully identify peptide sequences. Further, we demonstrate that this method is ideal to enhance sequence coverage of hydrophobic proteins, especially for the purpose of detecting membrane-spanning protein segments.
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Microbial communities play key roles in the Earth’s biogeochemical cycles, but for the most part are very poorly understood. For example, microorganisms from a number of lineages thrive in acid mine drainage (AMD), one of the most extreme environments on Earth. Natural communities, but for the most part are very poorly understood. For example, microbial communities play key roles in the Earth’s biogeochemical cycles. We validated 400 hypothetical proteins from the AMD sample, with identifications corresponding to quadrupole mass spectrometry provided confident measurement of over 2000 proteins from the AMD sample, with identifications corresponding to all five dominant species in the biofilm. Proteins involved in protein refolding and radical defense were abundant, indicating that damage to biomolecules is a key challenge for survival. We validated 400 hypothetical proteins, a small subset of which are encoded within blocks of genes apparently acquired by lateral transfer. We detected entire operons of abundant, novel, lineage-specific proteins that may be important to the survival in the extremely acidic, metal rich environment. One-half of the predicted proteins from the most abundant microbe Leptospirillum group II were detected; however, a substantial fraction of these proteins were hypothetical/unknown. The extracellular fraction was dominated by a novel protein shown to be a cytochrome central to iron oxidation and AMD formation. DNA sequencing of the cytochrome directly from the biofilm used in the proteome analysis allowed for the prediction of two single nucleotide polymorphisms resulting in changes of two amino acids in the protein. Re-analysis of the proteomics data based on the new amino acids and a theoretical N-terminal cleavage site allowed for 100% sequence coverage of this protein from the proteome of the extracellular fraction. In total, this proteogenomic approach enables identification of the major investments of cellular resources and inference of the physiological challenges faced by a self-sustaining, chemoautotrophic community. In addition to the extreme acidity and metal toxicity, reactive oxygen species are a significant challenge in this environment. Moreover, biofilm polymer production and nitrogen fixation appear to be partitioned among community members.

Research support provided by the U.S. Department of Energy, Office of Biological and Environmental Research Microbial Genome Program.
Identification and characterization of a post-translation modification is often significant in the elucidation of biological function. It has been common practice for many years to detect post-translationally modified peptides by detecting specific product ions during a mass spectrometric analysis. A time-of-flight based analyser (TOF) can measure these product ions at very high mass accuracy. The sampling efficiency for the ion of greatest mass is typically around 25%, and less for the lower m/z ions. Through the synchronization of a travelling wave collision cell with an oa-Tof mass analyzer, the instrument duty cycle can be significantly enhanced, over a narrow m/z range, for specific ions of interest. This results in increased sensitivity for specific product ions, without compromising mass accuracy.

Utilizing a collision cell made of stacked RF ion guides, in combination with a travelling DC wave, ions can be transported and ejected from the collision cell as packets. The ion packet release from the gas cell can then be timed with the pusher pulse, therefore, increasing the duty cycle on a particular m/z of interest. We will show the application of this enhanced duty cycle (EDC) mode to current product ion based detection methods; increasing sensitivity using the technique, for sample infusions and in conjunction with capillary HPLC. Detection of a variety of species in mixtures of varying complexity will demonstrate the selectivity, increased sensitivity and accurate mass measurement capabilities of this method. Data presented will show the analysis of tryptic digests from known glycoproteins and phosphoproteins.

The use of EDC has previously been shown to produce around an extra order of sensitivity when detecting product ions. This has been shown on m/z 204, HexNAc oxonium ion, for fetuin glycopeptides and on the m/z 216 phosphotyrosine product ion from a synthetic phosphopeptide. Product ion based experiments have been shown to detect small amounts of glycopeptide in complex mixtures, with glycopeptides detected and selected for MS/MS from a tryptic digest of whole serum. Enhancement of current methodologies with this new EDC technology result in a new highly sensitive method for detection and mass measurement of post translationally modified peptides.

Detailed analysis of protein glycosylation is usually performed after chemical or enzymatic separation of glycans from the protein backbone. This procedure is time-consuming, often requires high initial amounts of sample, and therefore is not complementary with current high-throughput proteomic techniques. Here we explore a more direct approach, where glycoprotein of interest is digested in solution with a protease and its glycosylation is assessed using LC-MS/MS of the resulting glycopeptides. Immunoglobulin G (IgG) is suitable model for such studies, since its glycosylation is well described and, due to its heterogeneity, the proteolytic digest results in complex peptide mixture.

IgG was purified from human serum, and digested in solution with trypsin. Resulting digest was pre-fractionated on a microbore HPLC column, and analysis of collected fractions was performed using a nano-HPLC, coupled to ESI Q-TOF mass spectrometer (Micromass, Manchester, UK). LC-MS was performed with moderately increased collision gas pressure and acceleration potential to induce gas-phase deglycosylation. This has resulted in appearance of carbohydrate oxonium ions, which were used as reporter ions to identify eluting glycopeptides in the total ion chromatogram.

Thirteen glycoforms of the IgG glycopeptide EEQFNSTFR were detected, eluting in two chromatographically separated peaks. Masses of all observed glycoforms corresponded to glycan structures described for the IgG molecule. All glycoforms contained a trimannose core and a chitobiose unit with the core fucose attached. Nano-electrospray has revealed monosialylated glycoforms in spite of their low abundance, as well as bisecting GlcNAc-containing glycoforms.

The use of induced gas phase deglycosylation in MS measurements has an inherent problem of assessment which of the observed glycoforms are produced in the mass spectrometer as gas-phase deglycosylation products of the larger co-eluting glycoforms. This issue was successfully addressed by ion current extraction for each glycoform and analysis of its exact elution time in the extracted ion chromatogram.

Our results demonstrate that a considerable amount of structural detail can be inferred directly from the MS spectra of glycopeptides acquired under MS conditions that favour partial gas-phase deglycosylation. This approach can therefore be useful for rapid and large-scale analysis of protein glycosylation at the glycopeptide level.
A.15

Proteomics on a Thermo Finnigan LTQ-FTICR Mass Spectrometer

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The introduction of linear ion traps, with their increased trapping efficiency and ion storage capacities, has dramatically increased the utility of these instruments in proteomics laboratories. The linear ion traps are capable of faster scan rates than 3D traps and can now provide high quality MS3 spectra within nanoLCMS time frames. With these improvements in mind, we have developed a series of methods for the analysis of in-gel proteolytic digests using both MS2 and MS3 scans. We analyze the effect of adding MS3 scans on data base searches of the results, with emphasis on its effect on protein identification false positives and false negatives. We also investigate the effect of reduced chromatographic separation time on data base search results with the aim to determine the minimum run time consistent with accurate data base searches. This will lead to the ability to analyze more samples per day than is currently achievable. Finally, we will compare the use of MS3 scans with or without FTICR accurate mass measurements on the accuracy and effectiveness of data base searches.

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A.16

A Quantitative Study on the Membrane-associated Proteins of Salmonella Enterica: the Effects of Osmotic Stress

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Salmonella enterica serovar Typhi is the causal agent of typhoid fever in humans, affecting a wide sector of the worlds population and causing an estimated 600,000 deaths per year. A further three million fatalities per year are caused due to acute gastroenteritis and diarrhoea. We have looked at the change in protein expression profiles of the cell membrane when Salmonella is exposed to a change in environmental osmolarity. Cell membrane fractions were obtained from the wild type and ompR-ve mutant Salmonella after exposure to NaCl and also from a control grown under normal conditions. Tryptic digests of cell membrane extracts were analysed by a simultaneous qualitative and quantitative LC-MS method.

In these experiments, 1/100g aliquots of sample from each condition (control and osmotically stressed) were run in quadruplicate and separated on a 2-hr reverse phase gradient and analyzed via LC-MS. Protein identifications and quantitative information were extracted by the use of powerful specialised algorithms. This provides an isotope tag free quantification method coupled to simultaneous protein identification.

Eluting peptides were matched across analyses by their retention time and within the tolerance of the measured mass accuracy to form Exact Mass Retention-time Pairs (EMRTs). Normalization of datasets was performed using an exogenously spiked internal standard; subsequently intensities of all peptides were compared to ascertain the relative change in intensity of each protein between the two conditions. In the EMRT route peptides are plotted, (condition versus condition), and ions exhibiting a similar regulation, or fold change, can be grouped associated with their high-energy, fragment ion data, and searched against a database to determine their identity.

Results generated, thus far, have returned around 1,000 protein identifications (including protein homologues). The outer membrane porins ompA and ompH are clearly up regulated in both experiments, but the extent of expression increase in the ompR-ve mutant is seen to be about half of that in the wild type. This is in line with the evidence that the ompR gene regulates other porins. In addition a number of proteins unique to the salt stressed condition have been identified, including the Tol system outer membrane stability protein. We will present data summarizing the effect of osmotic stress on the membrane proteins of Salmonella.
A.17

**LC-Laser Induced Fluorescence Detection Combined with ESI Mass Spectrometry for Quantitative Proteomics**

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Quantitative methods are required to address multiple problems in proteomics research. Stable isotope labeling methods are currently widely utilized because they provide relative quantification based on the ratio of peaks in mass spectra. Laser induced fluorescence detection (LIFD) is expected to have equivalent sensitivity to mass spectrometry and could provide an alternative method to generate high accuracy quantitative data. In this study, we report a system for inline fluorescence detection consistent with peak identification by mass spectrometry.

Microscale or nanoscale reversed phase HPLC is desirable for LC separations with ESI-MS because of the physical compatibility of the eluate with ESI and the improved mass sensitivity due to the effective analyte concentration. A flow cell preceding ESI-MS must be compatible with low flow rates without adding significant dead volume. In this study, the flow cell was constructed by removing approximately 2 mm of the polyimide coating from a length of cubic fused silica capillary (100 μm × 100 μm), producing a detection volume of approximately 6 nL. An independent flow cell box was placed near the ESI interface with optical fibers as the connections for the laser source for excitation and a photomultiplier for emission.

For evaluation, a tryptic digest of apo-myoglobin was used as a standard sample. Naphthalene dialdehyde (NDA) derivatization produces efficient N-terminal fluorescent labeling at room temperature. The reaction proceeds for 30 seconds after the automated addition of NDA into an injector vial at pH 6.0 in 200 mM phosphate buffer containing 50 mM KCN solution, and samples are then analyzed by HPLC-LIFD-ESI-MS. An excitation wavelength of 410 nm solid-state laser was used. A band-pass filter was placed between the photomultiplier and the 600 μm diameter optical fiber for the NDA emission maximum of 490 nm.

Fluorescence chromatograms for aliquots of fluorescently labeled apo-myoglobin peptides were recorded for concentrations from 100–500 fmol with excellent S/N and reproducibility. ESI-TOF mass spectra of the fluorescently labeled peptides are consistent with the formation of single NDA derivatives of each peptide. The results of the LIFD detector evaluation including reproducibility and dynamic range will be described in this presentation.

A.18

**Microfluidic Nano LC/MS/MS Increases Sequence Coverage and Number of Proteins Identified from Complex Samples**

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The MudPIT approach to proteomic profiling of complex samples identifies only a subset of the proteins present in the sample. Factors affecting this limitation include peak capacity, separation efficiency, the MS/MS scan acquisition rate, and the sample path from injection through detection. This work examines the impact of an integrated microfluidic separation and ionization platform coupled to a high-performance ion trap mass spectrometer on the level of protein identification achieved from complex samples. Results are compared with conventional 2D LC-nanoESI-MS/MS. Studies using HSA digests showed that the microfluidic chip-based system resulted in chromatographic peaks that were at least two times narrower than with the conventional system. The improved chromatographic performance is due to elimination of peak dispersion caused by fittings and connections in the conventional system. Results from offline 2D LC/MS/MS of immunodepleted human serum were consistent with the HSA study. The microfluidic system resulted in greater sequence coverage for all proteins identified compared to the conventional nanoflow system. On average a 20% increase in sequence coverage was observed for the top scoring proteins identified using the microfluidic system. Additionally, the observed peak intensities were at least five times higher on average for the microfluidic system than for the conventional system. Studies from E. Coli lysates using an orthogonal separation step at the protein level (i.e. 1D SDS gels and reverse phase chromatography) followed by LC/MS/MS analysis are currently underway and preliminary results illustrate the advantage of the microfluidic device over conventional nanoESI in terms of sequence coverage and number of proteins identified.
Acquisition of Tandem Mass Spectral Data in the Profile Mode Dramatically Improves Data Quality in Multidimensional Protein Identification with TurboSEQUEST Search

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In MudPIT experiments in which tandem mass spectra are recorded in the profile mode, the accuracy of protein identification performed with TurboSEQUEST™ is dramatically improved when compared to searches using MS/MS data recorded in the centroid mode. When matched measurements are made under exactly identical experimental and analyte conditions (the only difference being profile vs. centroid mode of MS/MS data), at least four striking differences are observed: (a) protein database searches with profile MS/MS input data identifies at least two times the number of proteins that meet acceptance criteria than those of centroid input data. (b) All indices of positive identification of the short-listed peptides and proteins are two times (or more) higher than those of centroid input data. (c) With profile MS/MS data, the ranking of the candidate peptides and proteins reflect a more accurate rejection of false positives. (d) The percentage peptide coverage in an identified protein is substantially higher than those whose input data are recorded in the centroid mode.

In this poster, an integrated Multidimensional Protein Identification Technology (MudPIT) that employs a Michrom BioResources Paradigm MS4 Multi-Dimensional Separations Module, an Endurance Autosampler, a Michrom NanoTrap Platform, and an LCQ Deca Xp Plus ion trap mass spectrometer, is presented. In this method, the five most abundant ions in the mass spectrum are selected and fragmented to produce tandem mass spectra. In this data-dependent scan with Xcalibur 1.4 SR1, six scan events are created. The program does not allow data acquisition in the profile mode on the first scan event - data must be acquired in the centroid mode. However, the program does allow profile data recording with the remaining five (and subsequent) scan events.

The new method is demonstrated here with 12-cycle MudPIT runs on each of: (a) whole cell lysates of human SW-13 cell line (adenocarcinoma of the human adrenal gland), (b) horse heart myoglobin, and (C) Bovine Serum Albumin (BSA). All TurboSEQUEST™ search results are filtered by targeted validation of putative biomarkers. The cumulative work on this project needed for biomarker discovery. The combined use of iTRAQ reagent, Pro Group, twenty-one P450 proteins were unambiguously identified and quantified. The observed expression changes correlate well with previous targeted MRM quantification work on these proteins. In addition to assessing the changes in the P450 proteins, the PANTHER Gene ontology tool can be used to further understand the changes in the drug treated liver samples. PANTHER can be used to search for protein expression patterns based on protein molecular functions and biological processes as a result of phenobarbital treatment. The combined use of iTRAQ reagent, Pro Group Viewer and the mass spectrometer's ion trap MS/MS capability enabled the robust identification and relative quantitation of proteins needed for biomarker discovery. The cumulative work on this project demonstrates a prototypical workflow using stable-isotope chemistries and a single MS platform for non-targeted biomarker discovery followed by targeted validation of putative biomarkers.
A.21

**Use of Cross-linking and Tandem Affinity Purification (TAP) Methodology to Identify New Interaction Partners of the Kinase Mst2**

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Characterization of the complex net of interactions the proteins establish in the living cell is vital to the fully understanding of the various functions they perform, as well as their regulatory mechanisms. *In vitro* interaction studies fail sometimes to reproduce the native environment inside the cell or to simulate the dynamic conditions in the subcellular compartments where proteins are located in response to stimuli. Mst2 kinase has been reported to be activated by apoptotic signals as well as other stress conditions. We have used a proteomic strategy to identify proteins that interact with Mst2 in living cells. Transfection of human breast carcinoma cell line MDA-MB-435 with a retroviral vector containing an N-terminal TAP (Tandem Affinity Purification) tagged Mst2 allowed us to build a robust expression system from which we could purify in 2 sequential affinity chromatography steps complexes containing the tagged Mst2. In order to detect weak or transient interactors which could be lost during the purification we have developed a crosslinking approach, in which cell cultures are fixed with formaldehyde prior to lysis and then subjected to purification of the complexes. At the present, we identified several Mst2 interacting proteins using tandem affinity purification combined with mass spectrometry, which is today the method of choice to characterize protein-protein interactions. Some of these partners are known to interact with Mst2, like the protein WW45 or the highly related kinase Mst1, while other findings link Mst2 with different signaling pathways. The approach described will help to more thoroughly understand the protein-protein interactions and their regulatory mechanisms. The UCSF Mass Spectrometry Facility (A.L. Burlingame, Director) supported by NIH NCRR RR01614.

A.22

**Continued Explorations in Low Level Data Analysis of Peptide Time-of-flight Data**

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We continue to extend our investigations of raw data obtained from commercial TOF instruments. In this regard, we have developed a script that generates summary data for Mascot searches from QStar LCMS data. The script offers improvement over the instrument vendor’s software in both its ability to report accurate peak centroids as well as offering robust determination of isotope series in fragment spectra. We will explain the process that lead to the choice of an optimal filter for this type of raw data as well as the extension of a previously reported model (1) for deisotoping of multiply charged ions.

In addition, we have begun attempts to offer a more satisfactory method for combining single shot axial MALDI spectra over a single plate spot. This has relied on a close examination of sources of variation in the experiment. We will present results from a variety of approaches used to align replicate spectra, as well as a method that attempts to explicitly model the stochastic and expected sources of variation in time-of-flight for individual peptides.

A.23

FT-ICR Mass Spectrometry for N- and O-glycoproteomics: Accurate Mass Determination, Fragmentation, and Automatization


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For implementation of high throughput glycoproteomics high demands on analytical techniques related to mass determination accuracy, sensitivity of detection, directed fragmentation and speed of data acquisition are required. Identification of glycoconjugate structure calls for efficient strategies similar to those for identification of proteins, but additional structural parameters relevant for their biological interaction specificity are the site of the glycosidic bond attachment, patterns of branching, and stereochemistry at anomeric centers.

High speed mapping and sequencing of complex glycomixtures by MS became suitable for glycoproteomics introducing the on-line capillary electrophoresis and automated chip-based sample admission [1, 2]. High mass resolution and accuracy can be achieved on Fourier Transform Ion Cyclotron Resonance (FT-ICR) MS at 9.4 T along with the possibility of electron capture dissociation for determination of glycosylation patterns [3, 4]. Sample admission by chips coupled to FT-ICR gave a significant rise in sensitivity and speed of analysis [5–8]. Applications to hereditary diseases and tumor biology will be presented.


A.24

Identification of Post-translational Modifications via Blind MS/MS Search

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Post-translational modifications (PTMs) are of great biological importance. Most database search algorithms perform a restrictive search that can only take into account a few types of PTMs and ignore all others. We describe an unrestricted PTM search algorithm that searches for all types of PTMs at once in a blind mode, i.e. without even knowing which PTMs exist in a sample. The blind PTM identification opens a possibility to study the extent and frequency of different types of PTMs, still an open problem in proteomics. Using our new algorithm, we were able to construct a two-dimensional PTM frequency matrix that reflects the number of MS/MS spectra in a sample for each putative PTM type and each amino acid.

Application of this approach to a large IKKb dataset resulted in a largest set of PTMs reported for a single MS/MS sample so far. We demonstrate an excellent correlation between high values in PTM frequency matrix and known PTMs, thus validating our approach. We further argue that the PTM frequency matrix reveals some still unknown modifications that warrant further experimental validation. Finally, we provide multiple supporting evidence that our findings reflects real PTMs rather than artifacts of our approach.
**A.25**
Quantification of Membrane or Membrane-bound Proteins between Normal and Malignant Cells Isolated from the Same Patient with Primary Breast Cancer

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**Introduction:** More than 50% of all major drug targets are membrane proteins and their role in cell-cell interaction and signal transduction is a vital concern. Stable isotopic labeling with amino acids in cell culture (SILAC) labels all cellular proteins. Hence, normal and labeled tumor cells, for example, may be mixed and their proteolytic peptides, which are chemically identical but isotopically distinct, co-migrate in any separation method, eliminating quantification error due to unequal sampling. The mass difference between light and heavy peptides is distinguishable in the MS spectrum. The relative intensity of isotopic peptide pairs may be used to quantify differential protein expression. We describe the SILAC approach in the quantification of differential membrane protein expression between normal and malignant breast cells.

**Methods:** We labeled normal epithelial and malignant breast cells, isolated from the same patient with either light or heavy Lysine and Arginine during six doubling times. Cells proliferated in light media or those double-labeled with heavy Lys and Arg were then combined in a 1:1 ratio. Cells were lysed in hypotonic buffer and a simple cell fractionation method was used to obtain crude membranes. Membrane proteins were dissolved in SDS sample buffer and partially resolved by 1D SDS-PAGE. Protein bands were divided into 40 sections, digested with trypsin and the recovered peptides were analyzed by nESI LC-MS/MS (Q/TOF API-US, Waters). Protein identifications were performed with the Mascot search algorithm and quantification was achieved by manual analysis of corresponding peptide pairs.

**Preliminary Results:** Due to low solubility, membrane proteins are not easy to separate on 2D gels or using other liquid chromatography methods. One dimensional SDS-PAGE separation of crude membrane proteins, followed by analysis with nESI LC-MS/MS, allows us to identify up to 100 proteins in a single band. In this way, we have quantified over 1600 proteins. Among them, there are over 1000 membrane or membrane-bound proteins, 250 unknown or hypothetical proteins, and about 350 ribosomal, heat-shocked or histone proteins. A number of proteins show increased or decreased expression levels in malignant breast cells, whereas the majority of proteins remained unchanged when compared to the corresponding non-malignant samples. These results indicate that SILAC is a powerful technique for global identification and quantification of protein expression, and holds great promise in biomarker and drug target discovery efforts.

**A.26**
Proteomic Studies on Two Closely Related Species of Octocorallians with Special Reference to the Molecular Characteristics from the Organic Matrices of Endoskeletal Sclerites

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The two species of alcyonarian corals, *Lobophytum crassum* and *Sinularia polydactyla* are closely related to each other. It is reported that the calcium bound proteins in the skeletons of both contain a protein-polysaccharide complex playing a key role in the regulation of bio-calcification. However, information concerning the matrix proteins of endoskeletal sclerite is still unknown. For this reason, we have studied the proteinaceous organic matrices of sclerites for both species, to analyze the sequences and the functional properties of the proteins present. The SDS-PAGE analysis of the preparations showed four bands of proteins with the apparent molecular weights of 102, 67, 48 and 37 kDa for *L. crassum* and seven bands with 109, 83, 70, 63, 41, 30, and 22 kDa for *S. polydactyla*. A major band of about 67 kDa protein in *L. crassum* and two bands of proteins of about 70 and 63 kDa in *S. polydactyla* yielded N-terminal amino acid sequences. Periodic acid-Schiff staining indicated that the 67-kDa protein in *L. crassum*, and 83 and 63-kDa proteins in *S. polydactyla* were glycosylated. An assay for carbonic anhydrase, which is thought to play an important role in the process of calcification, revealed specific activity. Newly derived protein sequences were subjected to standard sequence analysis involving identification of similarities to other proteins in databases. Here we analyze the protein sequence statistics to evaluate distinctive attributes of residue content and arrangement in primary structure. The significantly different protein expressions and compositional analysis of sequences between two species were demonstrated.
S. subarctica SA1 (formerly identified as P. paucimobilis) capable to grow on sulfanilic acid as sole carbon, nitrogen and sulfur source was isolated [1]. As unique feature this strain contains the full set of enzymes necessary for metabolizing this compound. Sulfanilic acid could only be converted by intact cells, but not by disrupted cells. To identify the components of biodegradation, proteins specifically induced by the substrates in the membrane fractions were separated and sequenced de novo by MS. On the basis of protein sequence data genomic locuses harboring the corresponding genes were isolated. Analysis of one locus disclosed genes encoding aminotransferase type and ring hydroxylation dioxygenase enzymes, which likely participated in the conversion of sulfanilic acid to sulfocatechol. Other genes encoding proteins possibly involved in the metabolism of aromatics were also found, their characterization is in progress.

Sulfocatechol supposed to be the first intermedier in the bioconversion of sulfanilic acid is likely further oxidized by a ring clearing dioxygenase. A sulfocatechol dioxygenase of S. subarctica was partially purified by FPLC and sequenced by mass spectrometry. Based on the protein sequence data, a genomic region was isolated, which contained genes encoding sulfocatechol dioxygenase, sulfomuconate cycloisomerase, sulfolactone hydrolase, an oxidoreductase and a hypotetic permease. It was proved, that the enzymes encoded by these genes were able to metabolize sulfocatechol to β-ketoadipate.

Analysis of the cytoplasmic and membrane fractions revealed two enzymes, the aminotransferase and the oxidase to occur in both fractions. This suggest a loosely membrane associated complex responsible for conversion of sulfanilic acid tightly linked to the uptake of the compound.

Reference:

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Evaluation of Isotopic Labeling of Lysine Residues of Peptides for Quantitative Proteomics and De Novo Sequencing

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Quantitative analysis of differentially expressed proteins may be achieved by means of stable isotope labelling of protein reactive groups. In the case of proteomic strategies relying on the use of two-dimensional liquid chromatographic separation of peptides generated by enzymatic cleavage of the expressed proteins, peptides must be submitted to chemical derivatization prior to their separation.

We have evaluated a labelling reagent specifically directed towards the amino group of lysine residues. For the purpose of quantitative measurements of differentially expressed proteins, this reagent has been synthesized in a light and a heavy version (incorporation of four deuterium atoms). This reagent modifies specifically lysine side chains, and does not react with the N-terminal groups of peptides.

MALDI-MS and -MS/MS, as well as ESI-MS/MS have been used for the evaluation of the reagent, by analysing protein tryptic digests. Quantitative results obtained from the measurement of light/heavy molecular ions of peptides were the same in MALDI and ESI ionisation modes. Furthermore, for MALDI-MS analyses, a strong reduction of selective desorption effects was noticed. Indeed, whereas tryptic maps obtained with this ionisation mode are normally dominated by arginine terminated peptides, this spectral suppression effect is considerably lowered after modification of lysine groups with this reagent. Thus, as a result of derivatization, better protein sequence coverage was obtained from MALDI-MS analysis of tryptic peptides.

Another aspect of chemical derivatization of peptides concerns the quality of MS/MS spectra for protein identification, and de novo sequencing when required. With this reagent, a clear orientation of fragmentation in favour of y ions series was observed in MALDI-MS/MS, resulting in an easy sequence reading from a singly charged ion precursor. In the case of ESI-MS/MS, doubly charged ions were obtained, and b ion series were found together with y series. Nevertheless, the deuterium label introduced at the C-terminal residue greatly facilitates the sequencing process by a clear identification of y ions series.

Owing to these very promising features, this reagent is used for the analysis of differentially expressed protein in the context of hepatocellular carcinoma.

Improving Sensitivity by Combining Results from Multiple MS/MS Search Methodologies with the Scaffold Computer Algorithm

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Database-searching programs generally identify only a fraction of the spectra acquired in a standard LC/MS/MS study of digested proteins. Subtle variations in database-searching algorithms of MS/MS spectra have been known to provide different identification results. To leverage this variation, we developed Scaffold to probabilistically combine the results of multiple search engines, including SEQUEST, Mascot, and X!Tandem. We normally gain 20 to 100% more MS/MS spectrum identifications with each additional search engine, primarily due to increased confidence in low scoring matches. In this experiment, SEQUEST, Mascot, and X!Tandem were used in combination to increase the number of highly confident spectrum identifications (>95%) between 1.5- and 4.5-fold in human lens tissue experiments. These increases allow us to confidently identify a substantially larger number of proteins in LC/MS/MS studies over single database searches alone.
Developing High Throughput Methods for the Study of Modified Proteins at the Intact Protein Level

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Introduction: Peptide analysis dominates mass spectrometry (MS)-based proteomics; bottom-up proteomics has frequently been used to search for post-translational modifications (PTMs), which have a central role in protein regulation, but incomplete sequence coverage is a significant obstacle. Top-down proteomics is better suited to the discovery of PTMs, but slow gas-phase dissociation techniques can lead to severe under-sampling when used with on-line separations. An intermediate route has been developed that combines high-accuracy intact protein Fourier-transform ion cyclotron resonance (FTICR)-MS with protein identifications obtained by bottom-up proteomics measurements. Lists of possible masses for identified proteins both with and without common modifications are drawn up and compared with measured intact protein masses. When considering large numbers of proteins and modified proteins, however, even with excellent mass measurement accuracy, hypothetical masses overlap; this results in the requirement for fractionation to reduce sample complexity.

Methods: Soluble proteins obtained by disruption of Shewanella oneidensis MR-1 cells were fractionated by a number of methods: size exclusion chromatography (SEC); preparative-scale isoelectric focusing (IEF); ion exchange chromatography (IEC); hydrophobic interaction chromatography (HIC); and the combination of preparative-scale IEF with IEC. Collected fractions (and un-fractionated lysate) were then divided: one part was digested using trypsin and subjected to bottom-up analysis while another part was analyzed by on-line reversed-phase liquid chromatography (RPLC)-FTICR-MS. Peptide data were searched using SEQUEST to generate provisional protein constituent lists for each fraction, which were then used to interrogate intact protein data.

Results: Initial experiments demonstrated that collecting SEC fractions roughly doubled the number of identified S. oneidensis un-modified and modified proteins when compared with analysis of un-fractionated lysate. A majority of identified proteins were modified, with methionine loss, oxidation and methylation being detected. Although fractionation was clearly shown to be beneficial, significant sample losses and a bias towards high-abundance proteins were observed. Preparative-scale IEF was investigated as a replacement for SEC; using one-dimensional gel electrophoresis, optimal performance was found with a separation medium that included urea, thiourea, dithiothreitol, octyl-β-D-glucopyranoside, and carrier ampholytes at 0.5% by mass. IEC was also investigated, but to maintain protein stability both a cation and an anion exchanger were required; whether preparative-scale IEF was suitable for providing samples optimized for loading to contrasting ion exchangers was investigated–initial data indicated improved proteome coverage.
**A.33**

### Multidimensional Liquid Chromatography Employing Monolithic PS-DVB Capillary Columns for Bottom-up and Top-down Proteomic Analysis of Human Platelets

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Platelets, circulating in the body, play a critical role in haemostasis and contribute to essential processes in wound repair. The biology of platelets has been studied for several decades using 2D gel electrophoresis, more recently in combination with tandem mass spectrometry (MS/MS). This “classical” proteomics technique applied to platelet samples has generated over 2000 protein features of which a much smaller number of proteins has been identified so far. While 2D gel electrophoresis still provides an unsurpassed resolution for protein separations, several drawbacks of this technique have stimulated the development of alternative techniques for large-scale proteomics research.

Two-dimensional liquid chromatography (2D LC) is a valuable, complementary separation technique to 2D gel electrophoresis. The key issue in multidimensional LC methods for protein identification is whether the separations are performed at the peptide or protein level, i.e., bottom-up or top-down proteomics, respectively. Both approaches have pros and cons but share the time consuming RP chromatography step that needs to be repeated for a large numbers of liquid fractions.

In this study we have employed monolithic capillary columns to increase the speed of analysis of multidimensional LC separations. A bottom-up as well as a top-down proteomic analysis approach is applied to human platelet samples. LC/MS/MS analysis of peptides is performed on micro column switching set-up employing 200 μm ID monolithic columns. Separation of intact proteins is performed on 500 μm ID monolithic columns followed by micro-preparative fractionation and proteolytic digestion. The two approaches are compared for system reproducibility, sensitivity, the number of identified proteins and usability.

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**A.34**

### Label-free Protein Quantification Using LC-MS: Reproducibility and Statistical Analysis with Complex Proteomes

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Measurement of changes in the relative quantities of proteins in a model system under different experimental conditions is a frequent task undertaken by many investigators. As a complement to isotopic labeling, the label-free approach, based on direct comparison of mass spectral peak areas between LC-MS runs, is attractive for its simplicity and cost effectiveness for multiple samples. In the present study, a computer program capable of processing groups of multiples samples for statistical analysis was developed and applied to analyze complex proteomic samples using the label-free approach. The application was first utilized to evaluate the reproducibility and linearity of the label-free approach for complex proteomes. Various amounts of proteins from different proteomes (plasma or kidney samples, with or without the major abundant proteins removed) were subjected to repeated LC-MS analyses using ion trap or FTMS mass spectrometry. Nearly ideal Pearson’s correlation coefficients (for ion’s peak areas or retention time) and average peak area ratios were obtained between replicated runs, indicating a high reproducibility of the method. About 90% of the measured peptide ions deviated less than 20% from the average in duplicated runs, and more than half of the ions deviated less than 10% from the average. In addition, there was a good linear correlation between the multiplicity ratios of the amounts of proteins used and the observed averaged ratios of peaks areas calculated from detected peptides. Moreover, the removal of abundant proteins from the samples improved significantly the quantification reproducibility and linearity. To evaluate the method in practical applications, the computer program was used to quantify the changes in proteomes of control and experimentally treated cultured cells. By incorporating algorithms for outlier-resistant mean estimation as well as adjusting statistical probabilities in multiplicity of testing, reproducible results with a relatively low rate of false positives could be attained. Overall, this study demonstrates reliable global quantification of differentially expressed proteins in complex proteomes by combining the label-free approach with proper computational means.

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A.35

Strategies for Generating Sequence Information from High Mass Peptides/Low Mass Proteins Using Novel Tandem TOF Technology and iTRAQ™ Reagent Chemistry

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Analysis of high mass peptides/low mass proteins has been a reoccurring problem in proteomics arena. This is particularly the case for biomarker profiling experiments that often require the identification of peptides in excess of 10 kDa. Recent improvements in TOF/TOF technology have led to improved detection and fragmentation efficiency of high mass peptides and small proteins. However large gaps in the sequence coverage still create problems with de novo sequencing software tools. One way to circumvent the problem is to generate sequence tags to identify the proteins but this is limited to proteins in databases. Another approach is to induce better fragmentation by labeling the peptides/proteins with commercially available reagents such as iTRAQ™ reagent chemistry. iTRAQ™ reagent chemistry has already proved to be extremely useful in generating improved sequence information data from tryptic and non-tryptic peptides up to 4 kDa. The strategy would impact the field of proteomics especially in the diagnostic and biomarker discovery applications.

Several examples of high mass peptides/low mass proteins have been labeled with iTRAQ™ reagent chemistry to evaluate the potential of this workflow. The advantages of iTRAQ™ reagent chemistry are two fold. It not only facilitates better fragmentation but also enables quantitation of the peptides in the samples. Preliminary analysis of unlabeled proteins using novel tandem TOF technology has shown improvements in fragmentation that may well be enhanced by the use of iTRAQ™ reagent chemistry. A wide range of standard and real life high mass peptide/low mass proteins samples will be analyzed to ascertain the suitability of the iTRAQ™ reagent labeling workflow. These include amyloid peptides (AB40 & AB42) that have been implicated as causative agents for Alzheimer’s disease (AD).

An analysis strategy based on high mass peptides/low mass proteins would enable development of better diagnostic tools for diseases such as Alzheimer’s.

A.36

Characterization of Complex Oligosaccharides Using a Hybrid Ion Trap and TOF Mass Spectrometer Coupling with MALDI

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Glycosylation is a common post-translational modification to cell surface and extracellular matrix proteins as well as to lipids. Unlike proteins and nucleic acids that are linear polymers of amino acids and nucleotides respectively, with linkages at only one position, carbohydrates can adopt complex branched structures with individual monomeric units linked at one of several sites. A detailed analysis of complex carbohydrate structures has been explored with mass spectrometric techniques, and still presents a challenge for the analyst. This presentation will describe a systematic approach for carbohydrate and glycoconjugate analysis with MSn techniques. Oligosaccharides, cleaved from glycoproteins (by hydrazinolysis or enzymatically), were characterized using a hybrid MALDI Ion Trap/TOF mass spectrometer. DHB was used as the preferred matrix for ionizing oligosaccharides. High mannose, biantennary and triantennary oligosaccharides were analyzed using MS, MS2, MS3 and MS4 modes. Intact oligosaccharides were analyzed in MS mode using a cooling gas to prevent fragmentation. Individual precursor ions were isolated in the trap, subjected to fragmentation with Argon, to provide MS2 data. Product ions were selected for further fragmentation, which was achieved by increasing the energy for collisionally induced dissociation. In MS2 mode analysis of biantennary and triantennary structures also fragmented losing disaccharide units, such as the galactose- N-acetylglucosamine units that define each antennary branch, or core fucosylated-N-acetylglucosamine units. MS3 of selected MS2 products ions could be used to differentiate fragments generated from either the reducing or non-reducing ends. Cross-ring cleavages were also observed during fragmentation in MS2, and the relevant product ions could be used to differentiate branched structures by further fragmentation in MS3 mode. The MALDI ion trap/TOF mass spectrometer, therefore, provided a unique opportunity to dissect complex branched oligosaccharides using MSn and acquire as much fragmentation data as possible for structural and sequence interpretation.
Microtubule Dynamics and Drug Binding Studied by Hydrogen-Deuterium Exchange and Mass Spectrometry

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Taxol is an anticancer drug effective in the treatment of human malignancies. This anticancer agent binds to and stabilizes microtubules. Microtubules are polymers formed laterally by the self-association of α, β-tubulin heterodimers. They undergo rapid transitions from growing to shrinking resulting from the association and dissociation of tubulin heterodimers. This dynamic instability is reduced by either GTP or Taxol binding. GTP binds to both α- and β-tubulin, while taxol preferentially binds to β-tubulin in microtubules. Although photoaffinity labeling [1] and electron crystallography [2] have localized the binding sites for Taxol to a small region of the β-tubulin, they do not provide sufficient insight into the mechanism by which Taxol stabilizes microtubules.

Hydrogen/Deuterium exchange (HDX)-Electrospray mass spectrometry (ESI MS) permits studies of structure and dynamics of biomolecules in solution. High resolution x-ray diffraction and NMR studies of tubulin structure and dynamics are not possible. A time course of deuterium incorporation was carried out with purified chicken erythrocyte tubulin heterodimers, and GTP- or Taxol-stabilized microtubules. After quenching at pH 2.5, 0 °C, the protein was analyzed by LC-ESI-MS using an LTQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA).

At 37 °C global exchange kinetics of tubulin dimers, GTP- and Taxol-stabilized microtubules were monitored and the deuterium incorporation into tubulin polypeptide chains was calculated by taking into account the deuterium loss during the HPLC step. α- and β- tubulin are proteins with molecular mass of 50 kDa containing 430 and 425 exchangeable amide protons, respectively, after correction for the number of proline residues. After 100 minutes of exchange, 203 amide protons in the α-chain and 209 amide protons in the β-chain of tubulin dimer were replaced by deuterium respectively. In Taxol-stabilized microtubules, only 177 and 156 amide protons were incorporated in the α- and β-chain chain. Thus 26 and 54 amide protons in α- and β-chain tubulins, respectively, were protected from exchange, suggesting a decreased solvent accessibility in both polypeptide chains due to Taxol-binding. GTP-binding reduced the flexibility of microtubules in a less extent and preferentially to β-tubulin, as suggested by the reduced deuterium incorporation (192 amide protons) occurred only on β-chain. Local exchange of microtubules was monitored by performing pepsin digestion at a protein to enzyme molar ratio of 1:1 at 0 °C for 4 min. Three sites of contact of β-tubulin with Taxol had previously been determined using photoaffinity labeling, and were confirmed in the present experiments. In addition, a fourth site was found to be protected from deuterium exchange. All other peptides exhibited no significant change in deuterium incorporation after Taxol binding, nor were significant changes observed in peptides derived from α-tubulin, as expected from other biological and biochemical experiments. The data suggest that hydrogen/deuterium exchange coupled to mass spectrometry can be used to study drug binding sites on microtubules.

Radioytic Modification of Amino Acid Side Chains for Quantitative Protein Footprinting

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Hydroxyl radical-mediated protein footprinting with mass spectrometry provides a novel approach for examining the structure and dynamic processes of proteins and large biological complexes not amenable to crystallography and NMR due to difficulties in crystallization and sample limitations. Briefly, protein solutions are exposed to oxidizing radiation, the modified samples are then analyzed by LC/MS after proteolysis to determine the sites and extents of oxidation. The binding interfaces of protein complexes and structural allostery are mapped through examining the changes in solvent accessibility measured by oxidation rates of target peptides.

In the study, the modification and reactivity of common amino acid side chains are investigated for their values as structural probes in protein footprinting. Aqueous solutions of 10–50 mM peptides and amino amides (AA) were irradiated by synchrotron X-rays at National Synchrotron Light Source (NSLS) of Brookhaven National Laboratory or 137-Cs gamma-rays (1300 Rads/min). The irradiated samples were analyzed by ESI-MS, LC/MS and MS/MS, the amounts of modification products were measured, and the reaction rate was obtained by fitting fraction of unmodified sample (y) and exposure time t to the first order reaction equation y = Exp(-kt). Following results were obtained:

1. Aromatic and aliphatic residues. Radiolytic modification of aromatic side chains (Phe, Tyr, Trp) results in hydroxyl attack at the aromatic rings and subsequent one or multiple +16 Da mass shifts. Radiolysis of aliphatic side chains (Leu, Ile, Val, Pro) typically produces hydroxyl group (+16 Da) as the primary product, along with carbonyl (+14 Da) as a minor product.

2. Charged residues. Oxidation of Arg produces a major product with characteristic -43 Da mass change, oxidation of Glu and Asp generates a primary product with characteristic -30 Da mass change. Oxidation of His produces mixed products with -22, -10, +5, and +16 Da mass shifts.

3. Sulfur-containing residues. Cys is oxidized to sulfonic acid (+48 Da), sulfenic acid (+32 Da) and disulfide as primary products. Met is oxidized to sulfone (+16 Da) as primary product with sulfone (+32 Da) and an aldehyde (-32 Da). Disulfide Cys-Cys is cleaved to give rise to sulfonic acid as primary product.

4. Reactivity of different side-chains. Reactivity of amino acid side chains was evaluated based on the rate constants of amino amides normalized to that of internal standard. The reactivity decreases in the order of Cys, Met-Tyr-Trp-Glu-Cys-Pro. Biosynthesis is reduced by adding trace amounts of catalase or a millimolar Met-NH2 or Met-OH buffer immediately after irradiation. Disulfide can be reduced before mass spectroscopic analysis by reducing agents such as TCEP.

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A.39
An Automated Top-down LC/MSn Approach for Identification and Characterization of Unknown Yeast Proteins

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In top-down proteomics the intact protein molecular ions are fragmented inside the mass spectrometer without prior proteolysis, allowing not only their direct identification but also characterization of posttranslational modifications. The probability of a correct identification increases significantly when MS2 fragments are formed that differ by a series of amino acid residues, thus comprising a sequence tag. Here, an automated top-down strategy, including MS3 experiments for efficient sequence tag generation, is described. The mixture of unknown yeast proteins has been separated on-line using reverse-phase HPLC and analyzed using ESI on an LTQ FT mass spectrometer, where the most intense molecular ions were automatically selected, isolated and fragmented. The deconvoluted MS/MS spectra were searched using ProSight PTM search engine to identify three proteins, two of which were found to be modified. Further off-line MS3 analysis pinpointed the location of these modifications. Alternatively, an additional MS3 stage was introduced into the standard top-down experiment to reliably generate sequence tags from MS2 fragments. The experiment was performed with standard and unknown proteins using a stand-alone linear trap mass spectrometer. Initially, the most intense molecular ions were fragmented, followed by dissociation of selected MS/MS fragments. The resulting spectra were automatically processed to identify sequence tags of 8 to 16 amino acids in length. The following hybrid search using both the mass of the MS2 fragment and the MS3 sequence tag provided unambiguous identification of the standard and unknown proteins when searched against a modified human database. This additional MS3 step can be reliably used to generate sequence tags from MS2 fragments, thus greatly improving the confidence of the database retrieval and further characterizing the primary sequence of the protein of interest.

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A Novel Proteomic Approach for High Throughput Membrane Protein Analysis

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Membrane proteins participate in many vital biological processes such as cell-cell communications, cell-environment interactions, signaling pathways and nutrient uptake. There is increasing interest in comprehensive and high through-put analysis of membrane proteins. Mass spectrometry based proteomics has rapidly developed in recent years as a powerful approach for the large scale characterization of proteins. But it is challenging to analyze membrane proteins due to their high hydrophobicity and low solubility. Detergents can be used to improve the solubility of membrane proteins but they interfere with subsequent MS measurement, particularly LC-MS/MS analysis. A novel protocol developed in this study utilizes the advantages of detergents and is friendly with LC-MS/MS. This approach also greatly increases the throughput of membrane protein characterization. Moreover, this protocol can be easily adapted to combine with a stable isotope incorporation technique for protein quantification. This protocol has been employed in several projects in the laboratory to study human diseases such as prostate cancer and diabetes.