Application of an Intact Protein Separation Space for PTM Characterization

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Introduction: Post-translational modifications (PTMs) of proteins occur in-vivo both as a function of cellular processes and environmental/oxidative stresses and remain a substantial challenge for characterization. Multiple forms, differential % site occupancy, temporal flux and varied isoforms require non-standard MS and proteomics approaches for complete analyses. Here we describe our application of an intact-protein fractionation workflow in order to maintain protein and PTM integrity throughout the entire analysis scheme. Our workflow allows for MS and MS/MS characterization at the peptide and at the protein level and takes into account the effects of PTMs on search space and data interrogation.

Methods: Protein HPLC was performed with a Beckman Coulter PF2D with chromatofocusing or SEC in the first dimension and rp-C4 or C8 in the second, with UV detection at 214 nm. Fractions were analyzed directly by MS or subjected to trypsin digestion prior to MS and MS/MS. A Bruker Reflex IV mass spectrometer was used for MALDI-TOF MS. LC-MS/MS was performed via Waters QoTOF MS or Thermo LTQ-Orbitrap MS coupled with Waters nanoAcquity UPLCs. Differential UV analysis was via Proteoview (Beckman Coulter). Database searches used Mascot (Matrix Science) and Boston University Protein Identifier Server (BUPID). Protein validation was with the Trans Proteomics Pipeline (ISB). Cluster analyses were performed using open source tools and software written in house. Additional analyses were performed using open source tools and tools written in-house including Software Tool for Rapid Annotation of Proteins (STRAP).

Results: Comparative results were obtained from pilot plasma proteomics projects that emphasized the monitoring of PTMs as a function of cardiovascular disease and oxidative stress. In brief: plasma was obtained from patients and controls, subjected to albumin and IgG depletion. Samples were processed using chromatofocusing followed by reversed phase HPLC. Differential UV analyses indicated a pI shift in a target protein indicative of PTMs. Fractions were collected and subjected to MS analyses. Intact protein MALDI-TOF mass spectra suggested the presence of multiple isoforms as indicated by the change in pI of 1D- fractions and corresponding incremental shift in mass in MALDI mass spectra. Comprehensive sequence mapping of PTMs was then performed on replicate samples of plasma based upon differential MALDI-TOF MS mapping. These same samples were subjected to digestion and PMF analysis. resulting spectra indicated substantial changes in peptide profiles across the sample space indicative of isoforms and PTMs. Fractions of interest, based upon preliminary MALDI results were then subjected to LC-MS/MS and database search using Mascot and Error Tolerant search strategies and an iterative and recursive search strategy. All proteomics results were then processed via the TPP. From 8739 MS/MS results; 2005 unique peptides were observed yielding 1774 unique proteins (1203 single hits) resulting in 44 conclusive identifications (0.05).

The protein separation space allowed for assignments of PTMs as exemplified in Figure 1 where multiple forms of W oxidation were observed on a peptide obtained from serotransferrin.

We observed that protein abundances did not significantly change across different fractions between patient and control groups.

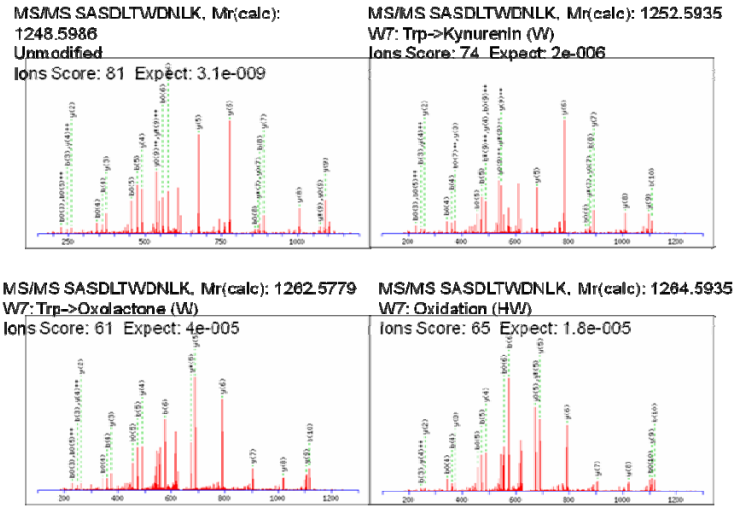

Figure 1. MS/MS Identification of W-PTMs on a peptide from serotransferrin.

However, substantial numbers of PTMs were identified via ET and directed PTM searching. Each protein contained numerous sites of modification and the PTMs were observed in multiple forms. Remarkably, patient groups contained more PTMs than controls.

Example results shown here in Figure 2 indicate that several amino acids of serotransferrin were highly susceptible to modification independent of the sample class and that global observation of modifications increased in the disease class. Several forms of PTMs occupied these select sites including multiple forms of oxidation, di-oxidation, tri-oxidation, cyano, kynurenin, nitro, crotonaldehyde, hydroxykynurenin , oxolactone, and carbamyl. It is noted that not all susceptible amino acids were modified.

Figure 2. AA Sequence of serotransferrin showing location of PTMs

Furthermore, the location of the residue was deemed to be strategically important. Those near the surface of the tertiary structure were more actively modified. This is indicated in Figure 3 where 3 site-specific locations of the surface amino acids, W460, W479, C374 were extensively modified via redox PTMs solely as a function of the study. Of note is that W479 appears in close proximity to metal binding site and that modification of this site may result in a decrease in functional activity.

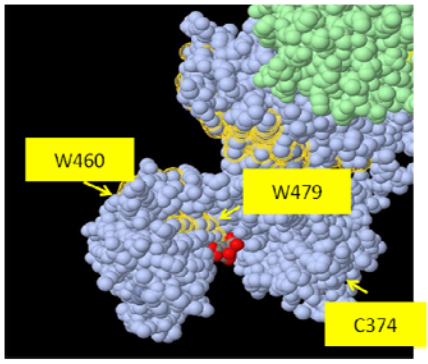

Figure 3. Mapping of select PTMs on the crystal structure of serotransferrin.

Conclusions: The aim of this work is the development of an analytical platform to identify and quantify changes in both protein expression and changes in protein PTMs by employing a significantly different strategic approach from that commonly used in MS based proteomics. The platform is based upon intact protein purification and fractionation using common HPLC technology. This gel-free separation scheme may be operated using one or more orthogonal dimensions to expand the separation space to address complex biological samples. Coupling HPLC with MS via MALDI-MS in an off-line manner allows for multiplexing and provides for flexibility in the experimental design and analytical pipeline. Off-line coupling may be facilitated by using novel multiplexed sample preparation approaches. Further analysis via capillary- and nano-LC-MS/MS is feasible for additional select fraction interrogation. Advanced software based data interrogation schemes may be employed which afford multidimensionality in analyses and allow for the application of advanced statistical search methods for PTM identification and localization. Absolute and relative protein abundance, differential analysis and primary characterization may be obtained from a combination of UV and MS data streams at both the protein and peptide level using this label-free approach. Further characterization may be obtained by both PMF and MS/MS database searches which may be run individually or in a combined and iterative fashion. We observed large numbers of PTMs occurring on multiple amino acids in abundant and medium abundance plasma proteins. We could correlate these findings directly to samples from patients who had been subjected to different types of oxidative stress. Multiple forms of oxidation were predominantly observed, as well as various PTMs known to be generated by formation of adducts via free radical mechanisms.

In summary, by maintaining an intact protein separation space and integration the results from multiple forms of data interrogation and analyses, we improved our ability to identify and compare oxidative stress-induced PTMs present in clinically relevant plasma samples.

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