Examining Protein Conformations in Mixtures by Hydroxyl Radical Surface Mapping with High Resolution Mass Spectrometry

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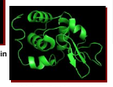
OVERVIEW

- Structural characterization of proteins in complex biological mixtures is an important analytical and biological challenge for MS-based proteomics.
- Previous work has shown surface mapping with hydroxyl radicals can quickly and accurately characterize individual protein structure,[1] but this technique has not been tested in mixtures.
- The goal of this study is to rapidly and accurately elucidate the structures of discrete proteins in a simple protein mixture using hydroxyl surface mapping with LC-MS/MS and high resolution FTICR-MS.
- Important factors considered in this study are:
  - Dynamic range in comparison of measurements
  - Mass accuracy to reduce ambiguity
  - Oxidation site determination

INTRODUCTION

- Protein structure and function are intimately related.
- Current structural techniques such as NMR and XRC are very powerful for protein structure determination, but require extensive sample purification and are limited in the scope of proteins which can be analyzed.
- MS has more versatile capabilities which can be applied to structural studies of assorted proteins in mixtures.
- Using MS, we have previously demonstrated a surface mapping technique that rapidly and accurately probes the structures of known and uncharacterized individual proteins using hydroxyl radicals.[2]
- In this study, we have extended this approach to the examination of proteins of varying structure and mass ranges in a simple mixture:

Bovine Serum Albumin



Chicken Egg Lysozyme

β-lactoglobulin

- Our experimental approach consists of:
  - *ESI-FTICR MS*
    - To determine oxidation level of intact proteins and measure peptide mass accuracy
  - *LC-MS/MS*
    - To pinpoint sites of oxidation on specific residues in peptides

EXPERIMENTAL

Sample Preparation:

- Simple protein mixtures composed of bovine serum albumin, β-lactoglobulin, and chicken egg lysozyme were dissolved in Tris-$CaCl_2$ buffered solution at equimolar concentrations. An individual BSA control was also prepared in the same manner.
- Hydroxyl radicals were generated *in situ* from 254nm UV-irradiation of hydrogen peroxide at concentrations of 5% and 10% of the total solution. Samples were allowed to react in 100 ul titer plates to expose more surface area. The hydroxyl labeling reaction was allowed to proceed for five minutes and was then quenched in one minute with the addition of 1nmol catalase.
- Oxidized samples and native controls were then denatured and reduced with 6M Guanidine, 10mM DTT and digested with trypsin.
- Samples were spun at 10,000rpm to remove debris and then desalted via solid phase extraction to prepare for MS analysis.

ES-FTICR MS Analysis:

- Proteins and peptides were examined using high resolution ESI-FTICR MS (IonSpec) to obtain high mass accuracy and to verify oxidation sites obtained in LC-MS/MS data.
  - Oxidation levels were measured with proteins.
  - High mass accuracy measurements were made with peptides.

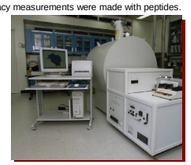


Figure 1 A high resolution (9.4 Tesla) IonSpec ESI-FTICR MS.

LC-MS/MS Analysis:

- Tryptic peptides from all native and oxidized samples were analyzed via 2-hour one-dimensional LC-MS/MS with C18 reverse phase column using an Ultimate HPLC (LC Packings) and an LCQ-DECA XP quadrupole ion trap mass spectrometer (Thermo Finnigan).

Figure 2 A Thermo Finnigan quadrupole LC-MS setup.

Data Analysis:

- Normal and oxidized peptides from the LC-MS dataset were identified using a new peptide search algorithm, DBDigger.[3]
  - DBDigger can identify multiple modifications (i.e. multiple oxidations) on the same residue.
  - Oxidized peptides are listed in an html viewer called **DTASelect**.
- LC-MS spectra were searched against a database containing the sequences of the four protein species as well as sequences of common contaminants like keratin and species with similar sequences, i.e. human serum albumin.

RESULTS AND DISCUSSION

BSA

Native | Oxidized

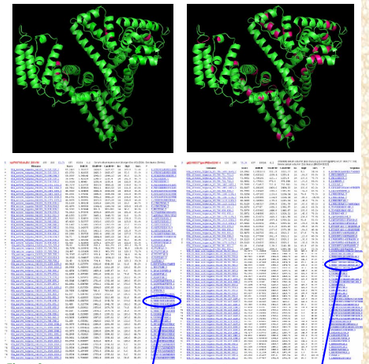


Figure 3a Partial list of peptides identified for unoxidized BSA.

Figure 3b Partial list of peptides identified for oxidized BSA.

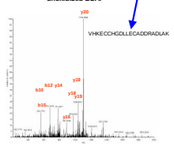


Figure 4a MS/MS of unoxidized BSA peptide VHKECCHGDLLECADDRADLAK.

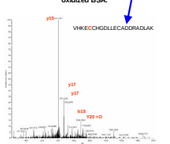


Figure 4b MS/MS of oxidized BSA peptide VHKECCHGDLLECADDRADLAK.

- A BSA control was run to test the level of background oxidation, which was low, but non-zero.
- 84% sequence coverage was obtained for each sample.
- 9 of 35 cysteines were oxidized.

Figures 3a & b show select DTASelect peptide search results identifying oxidations.

Figures 4a & b show MS/MS spectra of peptide VHKECCHGDLLECADDRADLAK for the native and oxidized BSA samples, where C is oxidized .

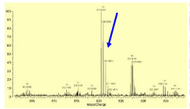


Figure 5 FTICR spectrum of oxidized peptide VHKECCHGDLLECADDRADLAK.

Figure 5 shows the FTICR spectrum verifying the oxidized BSA peptide shown above.

Protein Mixture

Bovine Serum Albumin | β-lactoglobulin | Lysozyme

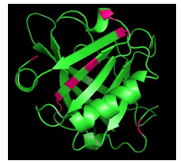


Figure 6a Surface oxidized BSA: oxidations in red, potential oxidations in dk. Blue, sequence coverage in lt. blue.

Figure 6b Surface oxidized β-lactoglobulin.

Figure 6c Surface oxidized lysozyme.

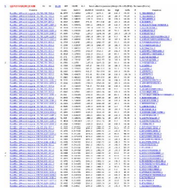


Figure 7a Select DTASelect search results for oxidized residues in BSA.

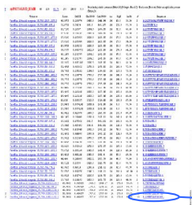


Figure 7b Select DTASelect results for oxidized residues in β-lactoglobulin.

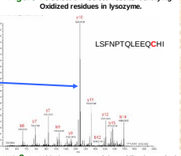


Figure 7c Select DTASelect results identifying Oxidized residues in lysozyme.

- The proteins were in equimolar concentrations with percent sequence coverage of 93.5% for BSA, 82.1% for β-lactoglobulin, and 97.7% for lysozyme.
- 17 of 35 cysteines were oxidized for BSA, 1 of 5 for β-lactoglobulin, and 7 out of 8 for lysozyme.

Figures 6a, b, & c show sequence coverage (light blue), potential oxidation sites (dark blue) and measured oxidations (red) for BSA, β-lactoglobulin, and lysozyme based on solvent accessibility.

Figures 7a, b, & c display DTASelect results from DBDigger searches for oxidations identified on individual residues in peptides.

Figure 8 shows the MS/MS of oxidized terminal β-lactoglobulin peptide LSFNPTQLEEQCHI, where C is triply oxidized.

Figure 9 displays the high resolution FTICR spectrum of the β-lactoglobulin peptide shown above, verifying the accurate mass of the oxidized peptide.

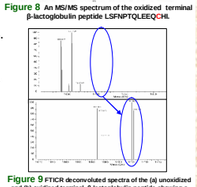


Figure 8 An MS/MS spectrum of the oxidized terminal β-lactoglobulin peptide LSFNPTQLEEQCHI.

Figure 9 FTICR deconvoluted spectra of the (a) unoxidized and (b) oxidized terminal β-lactoglobulin peptide showing a mass shift from the addition of a hydroxyl group.

Solvent Accessibility

- Theoretical oxidation sites were determined based on solvent accessibility of residues.
  - GETAREA 1.1[4] was used to determine the relative solvent accessibility of residues.
    - The higher the solvent accessibility rating, the more likely the residue would be surface oxidized.
  - Oxidation rate also contributed to the predicted oxidation of the residues.
    - The order of reactivity with hydroxyl residues is as follows: Cys > Trp/Tyr > Met > Phe > His > Ile > Leu[2,5]
- An excerpt from the GETAREA readout for lysozyme is displayed below.
  - Solvent accessibility for each residue is based on the sidechain information.
  - The cysteines are underlined and all are oxidized in the lysozyme sample except for Cys76, which was not covered in the overall sequence coverage of the protein.
  - Although each of the cysteines has a relatively low accessibility score, i.e. they are not very solvent exposed, they are each oxidized (except for Cys76). This result is likely due to over-oxidation of lysozyme under the 10% H_2O_2 experimental conditions.
  - Since solvent accessibility values are influenced by various factors, they were used as a general guide in deciding theoretical oxidation sites for surface mapping in the native state.

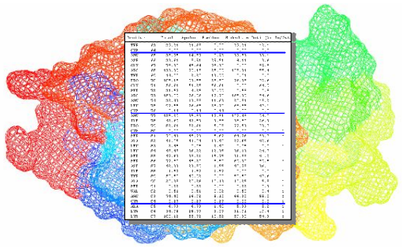


Figure 10 GETAREA excerpt showing solvent accessibility per residue on lysozyme .

Surface Mapping Results

- The comprehensive oxidation results for each protein are listed in Table 1 below.

Table 1	Protein Mixture		
	BSA	β-lactoglobulin	Lysozyme
Oxidized Cysteines	17	1	7
Total Cysteines	35	5	8

Table 1 Total number of oxidized cysteines found for each protein.

- Based on the MS data obtained, oxidized cysteines were measured for each protein in the mixture, demonstrating a successful initial step for our technique for hydroxyl surface mapping in mixtures.
- Several considerations were noted after this early study of mixtures:
  - Generous sequence coverage was obtained for all the proteins, showing how well we can identify peptides from each protein even with the competition between the smaller proteins in the presence of larger BSA.
  - Oxidation levels affect proteins differently depending on their size.
    - The 10% oxidation over-oxidized the relatively small lysozyme, but was reasonable for the larger BSA.
  - The main challenge is to identify which specific residues on the peptide are oxidized.
    - This requires manual inspection of the MS/MS spectra of all putative DBDigger oxidation assignments.

CONCLUSIONS

- We found that MS can be used to identify normal and oxidized peptides in a mixture through a combination of LC-MS and FTICR-MS.
- Each protein in the sample mixture had over 82% sequence coverage, indicating a comprehensive identification and analysis of the protein sequences.
- Protein samples were kept in the native state as much as possible for accurate surface mapping.
  - Tris buffer used to keep proteins at neutral pH
  - Low H_2O_2 concentration prevented unfolding
- DBDigger was useful in identifying native vs. oxidized states of peptides and recognizing multiple oxidations on single residues.
- The native samples for both BSA and the mixture showed some low, non-zero background oxidation which was negligible in the oxidized samples.
- The 10% H_2O_2 concentration was a little strong for the smaller proteins, which will help us to determine our experimental conditions for future work.
- Cysteines were shown to be reasonable probes for studying dynamic range in surface mapping techniques because of their strong reactivity with hydroxyl radicals.
- Future work will focus on more complex mixtures to study dynamic range capabilities and continue efforts toward confidently identifying exact locations of oxidations on peptides.

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