

Identification of oxidant-sensitive cysteines in proteins by mass spectrometry using an isotope-coded affinity tag approach

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Novel Aspects: Development of ICAT (isotope-coded affinity tag) approach to analyze post-translational modification of protein thiols upon oxidative stress.

Introduction: Redox regulation through post-translational modification of proteins is one of the major cellular responses to oxidative and nitrosative stress. Proteins that contain thiol groups are particularly susceptible to oxidation and may represent important targets in oxidative signaling. Cysteine (Cys) is an oxidative target because of the reactivity of the thiol group, which is susceptible to modification by free radicals, electrophilic molecules, and nitric oxide donors. The reactivity of Cys residues within proteins is variable and dependent on the local charge microenvironment. One or more reduced thiol groups are essential for the function of many proteins. Oxidation of these critical thiol groups can increase or decrease the activity of these proteins. Thus it is essential to identify those proteins containing oxidant-sensitive Cys residues. Towards our aim to develop a global approach to identify post-translational modification of Cys residues in proteins involved in redox signaling or affected by disease, we are developing a method to identify proteins with oxidant reactive Cys residues by mass spectrometric peptide fingerprinting. For this purpose we have employed a cysteine specific binding, by biotinylated iodoacetamide (IAM) or by an acid cleavable IAM-based ICAT reagent (Applied Biosystems, USA) to quantitate thiol oxidation.

Methods: The procedure is based on the fact that oxidant-sensitive Cys residues with low pK_a are selectively modified at pH 6.5 by iodoacetamide and that oxidized Cys residues are not susceptible to modification with IAM.¹ These modifications at Cys residues can be followed using mass spectrometry. Creatine kinase, which catalyzes the reversible transfer of the γ -phosphate group of ATP to the guanidine group of creatine, was chosen as our experimental model. Creatine kinase has four Cys residues, among which only one is known to have a redox-sensitive thiol which plays an important role in regulating catalytic activity of the enzyme. Alkylation of creatine kinase using iodoacetamide was carried out at pH 6.5 (50 mM MES) and pH 8.5 (20 mM Tris). The modified protein samples were desalted using a reverse phase C4 guard column. An aliquot of this sample was treated with trypsin and analysed using MALDI-MS on an Applied Biosystems QSTAR Pulsar *i* quadrupole/orthogonal acceleration TOF mass spectrometer or Bruker MALDI-TOF REFLEX. The experiments were also repeated with biotinylated iodoacetamide (BIAM) for comparison because the biotinylated analogue will be used in future experiments for isolation of labeled proteins in complex mixtures. An IAM-based acid cleavable ICAT reagent was used in order to test if this approach was adaptable to quantitative redox proteomics. The protein creatine kinase was initially treated with 1 mM hydrogen peroxide at pH 7.1 (50 mM Tris) for 10 minutes. The reaction was terminated by the addition of 0.1 ng/ml of catalase. Following this, the protein reduced thiols were labeled with either heavy or light ICAT reagent. The labeled protein was mixed with an equal amount of protein labeled with either heavy or light ICAT reagent but with no hydrogen peroxide treatment. Following tryptic digestion overnight at 37 °C, the peptides were desalted using a cation exchange cartridge (Applied Biosystems, USA) to remove excess labeling reagent. The desalted peptides were affinity purified using an avidin cartridge (Applied Biosystems, USA) to capture only the labeled peptides. The affinity-purified peptides were cleaved from the avidin matrix with the acid reagent, as per the manufacturer's (Applied Biosystems, USA) protocol. After cleaving the linker, the samples were concentrated by Speedvac and dissolved in 1% formic acid to analyse by μ LC-MS (Micromass).

Results: The amino acid sequence of creatine kinase from the database showed that there are four Cys residues. MALDI MS analysis of creatine kinase tryptic digest before and after alkylation with IAM showed a peptide at $[M+H]^+$ m/z 2870.36 and 2927.38, respectively. The 57.02 u shift corresponded to alkylation of the Cys residue of the tryptic peptide Ala123 – Arg321. Because this peptide is modified at low pH 6.5, the Cys residue in the peptide is expected to be oxidant-sensitive at physiological pH. When we used biotinylated iodoacetamide (BIAM), we observed a peptide at $[M+H]^+$ m/z 3196.54 which corresponded to alkylation by BIAM at the Cys residue of the same peptide. From these two independent results, Cys282 which was modified at pH 6.5 can be identified as oxidant-sensitive at physiological pH. Aliquots of similar samples are being treated with Asp-N, Glu-C, and Lys-C and analysed using MALDI MS to generate peptide fingerprints to identify other Cys containing peptides. We intend to use this approach for complex mixtures by using an IAM based ICAT (isotopically coded affinity tag) reagent² in order to understand multiple redox-sensitive proteins. As a preliminary measure, protein extract from rat vascular smooth muscle cells exposed to the thiol oxidant, diamide, was modified with biotinylated IAM, and the modification was monitored by streptavidin blot. The results showed that upon exposure to diamide there is a significant decrease in BIAM modification. This indicates that certain Cys residues (so called oxidant-sensitive Cys residues) are oxidized by diamide. The ICAT reagent was tested on creatine kinase exposed to H₂O₂ X mM. The results shown in Figure 1 show that the extent of ICAT labeling was decreased upon oxidation with H₂O₂ compared to

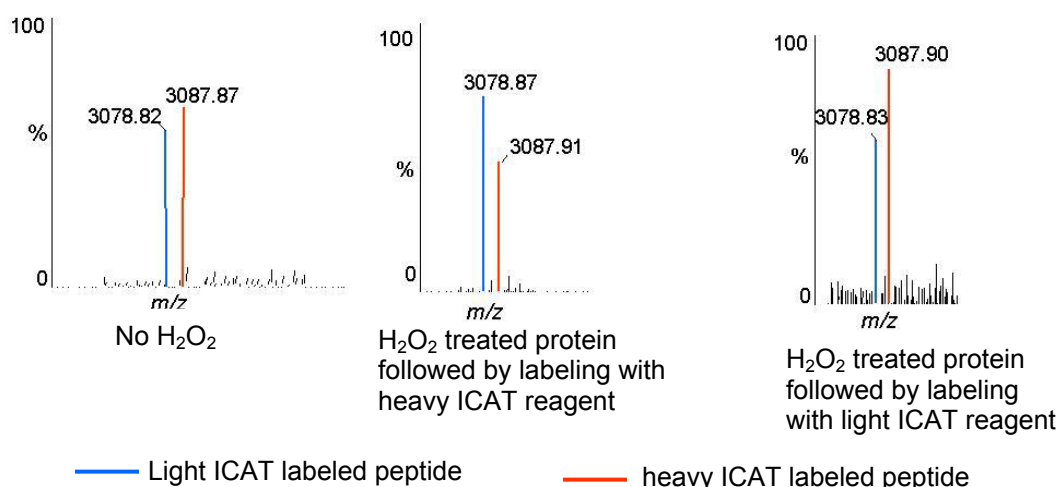


Figure 1: Reduction in ICAT labeling of oxidant sensitive cysteine containing tryptic peptides from creatine kinase upon oxidation with H₂O₂

the control samples. Quantitation of the peak areas from the reconstructed ion chromatogram for the respective peptide ion is in progress in order to determine quantitative change in thiol oxidation. This approach will be applied to complex mixtures from disease models to identify proteins which contain oxidant-sensitive cysteines. Further, we intend to use this approach to specifically correlate the redox sensitivity to the physiologically significant post-translational modifications like S-glutathiolation and S-nitrosation.

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