ICAT (isotope-coded affinity tag) -based quantification of oxidative post-translational sensitive thiol modifications of H-Ras that accompany its activation

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Introduction

We recently described an approach for identifying and quantifying oxidant-sensitive protein thiols using a cysteine-specific, acid-cleavable isotope-coded affinity tag (ICAT) reagent (Applied Biosystems, Foster City, CA). We are now using this approach to explore the relationship between redox sensitivity of individual cysteine residues and physiologically significant oxidative post-translational modifications, as well as irreversible thiol oxidation by oxidant stress associated with disease. As a proof of principle, we are quantitatively evaluating oxidative post-translational modifications of the recombinant protein H-Ras that accompany changes in its activity.

Methods

H-Ras (1-10 µg) was treated with peroxynitrite ($ONOO^-$, 100 µM) for 5 min. at 37 ^oC in the presence or absence of reduced glutathione. Ras activity was assayed by association with Raf-1 and by GTP/GDP exchange. The $ONOO^-$-treated samples were labeled with heavy ICAT reagent by incubating at 37 ^oC for 30 min. The heavy isotope-labeled protein was mixed in equal amounts with untreated H-Ras that had previously been labeled with light ICAT reagent. The light and heavy labeled proteins were digested with trypsin, desalted, and affinity-purified using an avidin cartridge (Applied Biosystems, USA). The samples were concentrated and dissolved in 0.1% TFA for MALDI TOF MS or 1% formic acid for analysis with a QoTOF LC-MS (Micromass).

Results

The amino acid sequence of H-Ras contains six cysteine residues. Of the six, four (118, 181, 184 and 186) are surface-exposed, as determined by structural, chemical and mutational studies. Although Cys-181, Cys-184 and Cys-186 are known to be modified by prenylation in intact cells, all of the reactive cysteines are potentially oxidized during normal and pathological conditions and this oxidation could alter the cellular function of the protein. We recently demonstrated that Ras was *S*-glutathiolated and activated by oxidants generated from NADPH oxidase in smooth muscle cells stimulated with angiotensin II. This result now makes it imperative to quantify the thiol modifications in the protein associated with its oxidant-mediated activation. The activity of Ras was significantly increased 2- to 3-fold following exposure to peroxynitrite and glutathione, but not to peroxynitrite alone. We are therefore applying our ICAT approach to identify and quantify cysteine modifications that occur upon treatment with $ONOO^-$ in the presence and absence of glutathione. MALDI-TOF MS of the ICAT-labeled peptides of H-Ras showed 15-20 ICAT-labeled peptides with the appropriate 9-Da difference between the light and heavy ICAT-labeled peptides. LC-MS has been carried out to quantify the degree of cysteine oxidation on the basis of the change in signal intensity for the heavy ICAT-labeled peptide. Figure 1 shows pairs of ICAT-labeled, cysteine-containing peptides of H-Ras that span residues Val 103 – Arg 123 and Thr 74 – Lys 88, as confirmed by MS/MS. In the $ONOO^-$-treated samples, the Cys-118 is oxidized

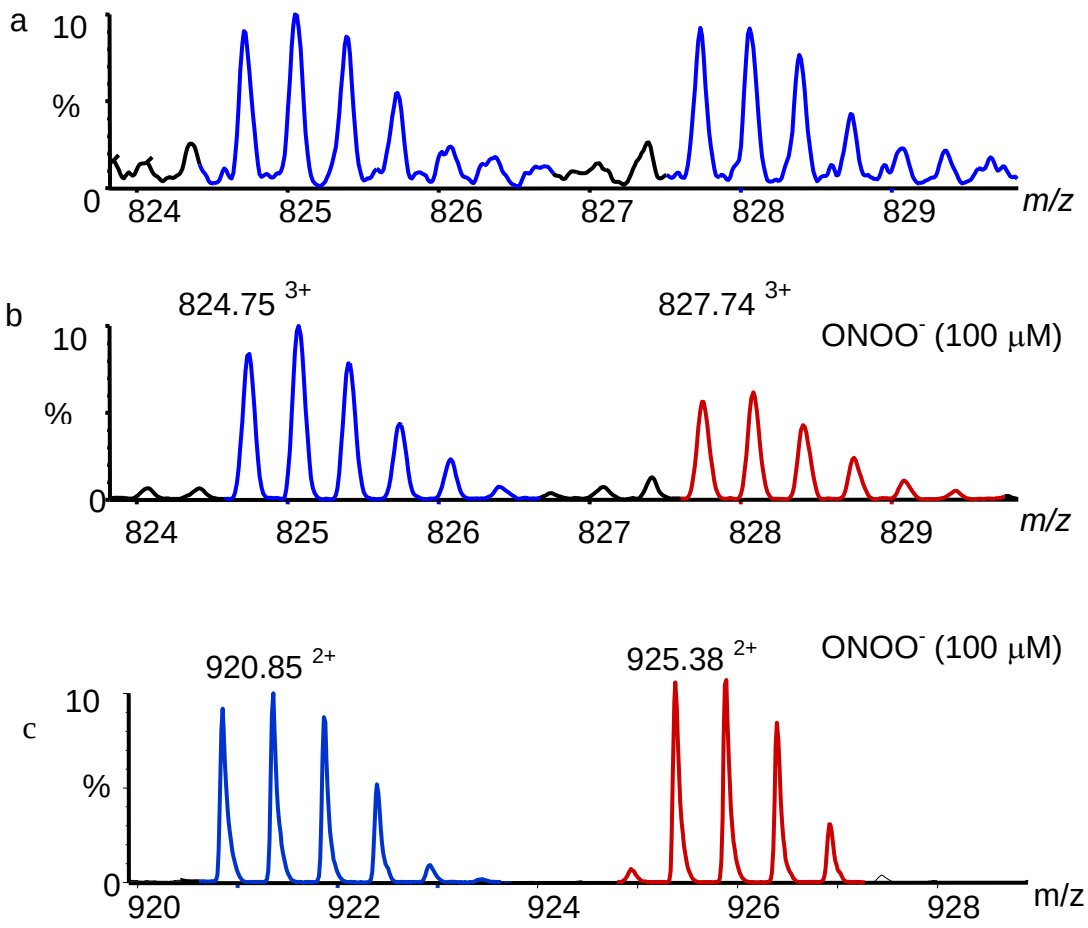

Figure 1: Effect of $ONOO^-$ (100 μM, 5 min, 37 °C) on ICAT labeling of Cys 118 and Cys 80 of H-Ras. MS of ICAT-labeled peptide containing reactive Cys 118 (a&b) and non-reactive Cys 80 (c). Blue line, samples not pretreated with $ONOO^-$. Redline, samples pretreated with $ONOO^-$.

47 % as measured from the change in the intensity for the heavy-labeled peptide (Fig 1b) whereas the non-reactive Cys 80 is not oxidized as indicated by no change in the intensity for the heavy ICAT-labeled peptide (Fig 1c). We anticipate that quantitative evaluation of the extent of modification of individual cysteine residues can be correlated to the activation or inactivation of H-Ras when subjected to reactive oxygen/nitrogen species.

Conclusions:

We have successfully applied our ICAT approach to quantitatively evaluate the oxidative post-translational modifications of the protein H-Ras that accompany changes in its activity.

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