An approach is described 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). The approach is based on the fact that only free cysteine thiols are susceptible to labeling by the iodoacetamide-based ICAT reagent, and that mass spectrometry can be used to quantitate the relative labeling of free thiols. To validate our approach, creatine kinase with four cysteine residues, one of which is oxidant-sensitive, was chosen as an experimental model. ICAT-labeled peptides derived from creatine kinase were used to evaluate the relative abundance of the free thiols in samples subjected (or not) to treatment with hydrogen peroxide. As predicted, hydrogen peroxide decreased the relative abundance of the unmodified oxidant-sensitive thiol residue of cysteine-283 in creatine kinase, providing proof of principle that an ICAT-based quantitative mass spectrometry approach can be used to identify and quantify oxidation of cysteine thiols. This approach opens an avenue for proteomics studies of the redox state of protein thiols. Molecular & Cellular Proteomics 3:273–278, 2004.

Regulation of cellular homeostasis through post-translational modification of proteins is one of the major responses to oxidative and nitrosative stress (1). Proteins containing cysteine thiol groups are particularly susceptible to oxidation by free radicals, electrophilic molecules, and nitric oxide donors (2, 3). 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 and represents not only a major mechanism of normal cell signaling or those affected by disease, this report describes a method to identify and quantify oxidant-sensitive protein thiols by mass spectrometric peptide fingerprinting.

The procedure is based on the fact that oxidized Cys residues are not susceptible to modification by iodoacetamide (IAM)1 analogues (7). Isotope-coded affinity tag (ICAT) reagents that are IAM analogues have been used extensively in quantitative proteomics to evaluate the abundance of expressed proteins (8). The ICAT approach is based on affinity tag targeting of free cysteines in proteins that are labeled after the proteins are isolated under strong reducing conditions. This study was designed to determine the feasibility of using the acid-cleaveable IAM-based ICAT reagent (catalogue no. 4337335; Applied Biosystems, Foster City, CA) to quantitate the extent of thiol oxidation under nonreducing conditions. The principle of the ICAT approach described here is that only free thiols are susceptible to modification by the IAM moiety of the ICAT reagent. When exposed to oxidants, oxidant-sensitive cysteine thiols are expected to be oxidized, thereby decreasing the labeling by the ICAT reagent. The decrease in specific labeling of oxidant-sensitive cysteines can then be quantified from the ratio of ICAT labeling, and the cysteine residue can be identified from the liquid chromatography (LC) mass spectrometry (MS) analysis of the proteolyzed peptides. In this study, after exposing equivalent protein samples to hydrogen peroxide or control conditions, the side chains of cysteinyl residues in proteins were derivatized with either the light (12C) or heavy (13C) isotope form of the acid-cleavable IAM-based ICAT reagent (Fig. 1). The two samples were combined and enzymatically cleaved to generate peptide fragments. The peptides were desalted by subjecting to purification through a cation exchange cartridge followed by affinity isolation of their relative sensitivity to oxidation. As a step in the development of a proteomic approach to identify post-translational modification of Cys residues in proteins involved in redox signaling or those affected by disease, this report describes a method to identify and quantify oxidant-sensitive protein thiols by mass spectrometric peptide fingerprinting.

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tagged peptides using an avidin cartridge. The isolated peptides were separated and analyzed by capillary LC (capLC)-tandem MS (MS/MS). The quantitation and sequence identification was carried out with automated multistage MS.

Creatine kinase, which catalyzes the reversible transfer of the $\gamma$-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, Cys283, is known to have a redox-sensitive thiol that plays an important role in regulating catalytic activity of the enzyme (9).

Hydrogen peroxide, which is generated in all aerobic organisms as a result of normal cellular metabolism (10, 11), was used as the oxidant in this proof of principle demonstration that the ICAT labeling under nonreducing conditions followed by mass spectral analysis could be used to measure relative abundance of oxidized thiols on specific proteins.

**EXPERIMENTAL PROCEDURES**

**Hydrogen Peroxide Treatment**—Creatine kinase (100 $\mu$g), from rabbit skeletal muscle (Roche, Mannheim, Germany), in 50 mM Tris (pH 7.1), was incubated with hydrogen peroxide (1 $\mu$M to 1 mM) at room temperature for 10 min. The reaction was terminated by the addition of catalase from bovine liver (Sigma, St Louis, MO) to a final concentration of 0.1 $\mu$g/ml.

**Biotinylated IAM (BIAM) Labeling of Either Untreated or Hydrogen Peroxide-treated Creatine Kinase Analyzed by Streptavidin Blot**—Creatine kinase (100 $\mu$g) in 100 $\mu$l of 50 mM Tris, pH 7.1, either untreated or previously treated with hydrogen peroxide, was incubated with BIAM (40 $\mu$M) at room temperature in the dark for 30 min. The labeling reaction was terminated by addition of $\beta$-mercaptoethanol to a final concentration of 20 mM. The protein samples were subjected to SDS-PAGE, and the separated proteins were transferred to polyvinylidene fluoride membrane. Proteins labeled with BIAM were detected with horseradish peroxidase (HRP)-conjugated streptavidin (Pierce, Rockford, IL) and electrochemiluminescence reagent (Amersham Biosciences, England, UK).

**ICAT Labeling of Creatine Kinase Either Untreated or Pretreated with Hydrogen Peroxide**—Creatine kinase (100 $\mu$g) in 100 $\mu$l of 50 mM Tris, pH 7.1, was incubated with the acid-cleavable $^{12}$C and $^{13}$C ICAT reagent using the Applied Biosystems protocol at 37 °C for 2 h. The pH at which labeling was performed in this study was kept the same as that at which the oxidation by $\text{H}_2\text{O}_2$ was done to avoid any potential differences in the products that might be introduced by changing the pH. After labeling for 2 h, the labeled protein was subjected to digestion with trypsin by incubating at 37 °C for 10–14 h. The tryptic peptides were purified using a cation exchange cartridge to remove excess labeling reagent. The desalted peptides were affinity purified using an avidin affinity cartridge provided by Applied Biosystems and were then dried and suspended in the cleavage reagent to release the peptides from the acid-cleavable linker by incubating at 37 °C for 2 h. The acid-cleaved peptides were dried and
then suspended either in 0.1% trifluoroacetic acid (TFA) for matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS or 1% formic acid for capLC-electrospray ionization (ESI) MS/MS experiments. Because the recommended labeling protocol was carried out under nonreducing conditions, some oxidation of ICAT-labeled peptide was observed (12). Milder conditions for labeling including lower temperature, shorter duration, and labeling in the dark can reduce this oxidation and are therefore recommended.

Capillary High-performance LC (HPLC)-ESI MS/MS of ICAT-labeled Peptides—Capillary HPLC with tandem electrospray mass spectrometry was performed using a capillary HPLC system (CapLC, Waters Corp., Bedford, MA) coupled to a quadrupole orthogonal time-of-flight mass spectrometer (Q-TOF API US; Micromass/Waters Corp.) equipped with a Pico Tip Sprayer, NanoLockSpray, and Z-Spray source. Affinity-purified ICAT-labeled peptides were dissolved in 1% formic acid at a concentration of ca. 1 pmol; injection volumes were 1 µl. Sample preconcentration and desalting were performed on-line using a peptide trap cartridge (Michrom BioResources, Inc., Auburn, CA) in line with the auto-sampler and column. Separation was on a 300-µm × 15-cm capillary column packed with Vydac C18 phase (5 µm, 300 Å). A linear gradient was used to elute peptides into the mass spectrometer at 2 µl min⁻¹: 5–65% B over 55 min (A: 95% H₂O, 5% acetonitrile (ACN), 0.1% formic acid, 0.001% TFA; B: 5% H₂O, 85% ACN, 10% 2-propanol, 0.1% formic acid, 0.001% TFA). Columns were washed and re-equilibrated between LC experiments. ESI was carried out at 2.8 kV, with the ion source temperature at 80 °C, and 22 V cone voltage. Mass spectra were acquired in the positive-ion mode over the range m/z 400–1600. NanoLockSpray was performed by constant infusion of renin substrate hexadecapeptide (Sigma) 10⁻⁶ M 60% ACN at 0.2 µl min⁻¹. Mass accuracy was

**Fig. 2.** Effect of hydrogen peroxide (1 mM) on BIAM labeling of creatine kinase as assayed by HRP-conjugated streptavidin blot analysis described in "Experimental Procedures."

**Fig. 3.** LC-MS of ICAT-labeled peptides. a, Total ion chromatogram for the LC-ESI-MS/MS of the ICAT-labeled tryptic peptides from creatine kinase. The numbers on the top of the peaks refer to the retention time of the molecular ion selected for fragmentation. b, Molecular ion regions in the mass spectra of ions eluted between 16.60 and 17.40 min; the peptides identified as ICAT-labeled peptides are indicated by asterisks.
within 10 ppm and resolution was above 1:10,000 (fwhm). MS/MS of the three most-abundant MS precursor ions with intensities >25 counts was acquired. MS/MS collision energies were dependent on the mass and charge of the precursor and ranged from 16 to 40 volts. Ar was the collision gas at 2 x 10⁻⁵ Torr in the cell. MS/MS spectra were acquired for 4 x 1 s scans over the range m/z 100–1600. Mass Lynx 4.0 and Protein Lynx Global Server 2.0 (Micromass/Waters Corp.) were used for data analysis.

RESULTS AND DISCUSSION

The reactivity to oxidants of Cys residues within proteins is variable and dependent upon the local charge microenvironment, which determines their pKₐ value. The pKₐ value depends on the charge interactions of the thiol with both positively and negatively charged side chains of nearby amino acids. Because the cysteine thiolate anion is more readily oxidized than is the cysteine sulfhydryl group (Cys-SH), only the relatively few protein Cys residues having low pKₐ values are susceptible to oxidation under physiological conditions (13). Given the fact that oxidized cysteine residues do not react with sulfhydryl reagents such as BIAM, a decreased extent of labeling with BIAM after exposure to hydrogen peroxide would be expected (7). The extent of BIAM labeling can be readily measured by SDS-PAGE and blot analysis of the biotinylated proteins with HRP-conjugated streptavidin and chemiluminescent detection. A preliminary study showed less BIAM labeling of creatine kinase after treatment with hydrogen peroxide at pH 7.1 (Fig. 2). This is an indication that certain cysteines are oxidized upon treatment with hydrogen peroxide. The experiments using the ICAT approach described here were designed to identify which cysteines of creatine kinase are sensitive to oxidation by hydrogen peroxide and to quantify the amount of cysteine oxidized at the susceptible position(s).

The total ion chromatogram for the LC-MS/MS analysis of ICAT-labeled tryptic peptides from creatine kinase is shown in Fig. 3a. The region of the summed mass spectrum of peptides eluting between 16.6 and 17.4 min that contains the signals from the peptide with the redox-sensitive Cys²⁸³ (m/z 519.92 and m/z 522.94, z = 3 and m/z 779.43 and m/z 783.90, z = 2) is shown in Fig. 3b. The peptides identified as ICAT-labeled peptides are indicated by asterisks and have the expected mass differences of m/z 3 and 4.5, respectively, for the triply and doubly charged ions as predicted by the 9-Da mass difference introduced by the nine ¹³C atoms in the ICAT (14). MS/MS analysis was performed on the two peptides, one

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**Fig. 4. Effect of hydrogen peroxide on ICAT labeling.**

*Fig. 4. Effect of hydrogen peroxide on ICAT labeling. a, MS of the ICAT-labeled peptide (peptide 1) containing reactive Cys²⁸³. b, The MS of ICAT-labeled peptide 2 containing the nonreactive Cys¹⁴⁶. Blue line, Samples not pretreated with hydrogen peroxide before labeling; red line, samples pretreated with hydrogen peroxide (0.1 and 1 mM) before labeling; i, no hydrogen peroxide treatment; ii, hydrogen peroxide (1 mM)-treated sample labeled with heavy ICAT; iii, hydrogen peroxide (1 mM)-treated sample labeled with light ICAT; iv, hydrogen peroxide (100 µM)-treated sample labeled with light ICAT.*
eluting at 16.6 to 17.4 min (peptide 1), which contains the reactive Cys283, and the second one eluting from 11.5 to 12.6 min (peptide 2), which contains the nonreactive Cys146. The molecular ion regions in the mass spectra of these two cysteine-containing peptides are shown in Fig. 4. For peptide 1, labeling with ICAT is dramatically reduced after 1 mM hydrogen peroxide treatment (Fig. 4a). As estimated from the peak areas of the single ion chromatogram reconstructed from MS of the peptide, 92% of Cys283 in peptide 1 was oxidized by 1 mM hydrogen peroxide. Experiments with varying concentrations of hydrogen peroxide from 1 μM to 1 mM showed that 1 and 10 μM hydrogen peroxide had no measurable effect on ICAT labeling of Cys283, whereas 100 μM hydrogen peroxide caused a 38% reduction in labeling (Fig. 4a). For peptide 2, the labeling with ICAT was not affected by hydrogen peroxide, as shown by the equivalent intensity of the MS peaks of the light and heavy ICAT-labeled peptides (Fig. 4b). With 1 mM hydrogen peroxide treatment, the ratio of reduced to oxidized Cys283 thiols in peptide 1 was 10.6, whereas in contrast for the nonreactive Cys146, the ratio of reduced to oxidized thiols was 1.2.

The collision-induced dissociation (CID) spectrum (Fig. 5a) recorded from the doubly charged precursor ion with \([M+2H]^2+\) \(m/z\) 779.41 identified as the creatine kinase peptide spanning residues Val280–Arg292 containing Cys283 with the reactive thiol.

The b and y ions (15, 16) in MS2 showed that this peptide spans residues Val280–Arg292 of creatine kinase and includes Cys283. The acid-cleaved ICAT modification adds 227 Da to the mass of the Cys283 as expected. This creatine kinase peptide is derived by a chymotrypsin-like digestion at the C-terminal tyrosine of the trypptic peptide that spans residues Ala267–Arg292. Peptide 2 corresponds to a trypptic peptide of creatine kinase that spans residues Gly139–Arg148 as shown by MS/MS (Fig. 5b).

This ICAT approach has the potential to be superior to previously described methods using fluorescein-conjugated IAM (17) or \([3H]\)iodoacetic acid (18), for quantifying redox-sensitive cysteine thiols, because 1) it does not require gel separation of proteins, and 2) it accomplishes simultaneous identification and quantification of the involved peptides. Because this method uses an ICAT reagent, it can be used for proteomic screens. The approach shown here can be em...
employed to quantitate oxidation of specific cysteines in the presence and absence of oxidants, a matter of interest because there is variable sensitivity of reactive cysteines toward oxidants. When it is known which proteins and which cysteines are susceptible to oxidation in a particular normal sample, the abundance and site of oxidized cysteines may be analyzed in samples of tissues and cells exposed to different oxidant stresses and disease. Additionally, we intend to use this approach to understand the relationship between redox sensitivity of cysteines and physiologically significant post-translational modifications such as S-glutathiolation and S-nitrosation, as well as irreversible thiol oxidation by oxidant stress associated with disease. Finally, this method takes advantage of a potential vulnerability in the usual use of the ICAT approach. That is, if cysteines are irreversibly oxidized in an experimental sample, then ICAT analysis may falsely indicate a decrease in protein abundance. Knowledge of which proteins in a normal sample have cysteines that are susceptible to oxidant modifications should help investigators to avoid this misinterpretation when control and diseased samples are analyzed.

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