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Biophysical and Proteomic Characterization Strategies for Cysteine Modifications in Ras GTPases

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Abstract

Cysteine is one of the most reactive amino acids and is modified by a number of oxidants. The reactivity of cysteines is dependent on the thiol pK_a ; however, measuring cysteine pK_a values is nontrivial. Ras family GTPases have been shown to contain a free cysteine that is sensitive to oxidation, and free radical-mediated oxidation of this cysteine has been shown to be activating. Here, we present a new technique that allows for measuring cysteine pK_a values using a fluorescent detection system with the molecule 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F). In addition, we also describe how to generate several oxidants. Lastly, we describe several mass spectrometry-based experiments and the necessary adjustments to the experiments to detect cysteine oxidation.

Keywords

Cysteine pK_a ; Oxidation; Ras GTPases; Mass spectrometry; Reactive oxygen and nitrogen species

1 Introduction

One of the most reactive amino acids in proteins is cysteine, which can undergo a variety of different posttranslational modifications and several different types of oxidative reactions [1]. In fact, thiol oxidation plays a key role in regulating redox homeostasis and protecting the cell during oxidative stress [2–4]. Moreover, many genes that respond to redox stress show altered regulation by reactive oxygen and nitrogen species (ROS and RNS) [5]. Some of the common end products of cysteine oxidation are disulfide bond formation, mixed disulfide bond formation with glutathione (glutathiolation), and nitrosation, as well as sulfenic, sulfinic, and sulfonic acid [6]. Many redox-sensitive proteins contain cysteine residue(s) that has an altered pK_a . The microenvironment surrounding the cysteine determines the pK_a of the thiol side chain. However, cysteine pK_a values are difficult to reliably predict as several factors influence pK_a , including solvent exposure, hydrogen bond formation, and charge-charge interactions [7]. The pK_a of L-cysteine in water has been measured to be ~ 8.5 –9, and the thiolate form (RS^-) is approximately tenfold more reactive than the thiol form (RSH) [8]. Thus, redox-regulated proteins often contain reactive cysteines with altered pK_a values that are close to or lower than physiological pH.

Critical cellular enzymes that regulate redox homeostasis include peroxiredoxins (reduce peroxide), thioredoxins (reduce oxidized peroxiredoxin), and glutathione peroxidases (reduce peroxide using the cellular glutathione pool), which act through mechanisms involving cysteine oxidation. The pK_a of the active-site cysteines have been shown to be markedly reduced in each of these classes of enzymes; for example, the active-site cysteine pK_a has been shown to be approximately 5–6 in 2-Cys peroxiredoxins [9], the active-site cysteine in *Escherichia coli* thioredoxin was measured to be between 7.1 [10] and 7.5 [11], and the pK_a of the active-site cysteine in glutathione peroxidase has been estimated to be 7.2 [12].

The work in our lab is centered on the redox regulation of Ras superfamily GTPases. We have shown that the activity of a subset of Ras and Rho GTPases can be regulated through redox-sensitive cysteines [13–15]. Ras GTPases, in particular, have received a great deal of interest in the field of redox biology. There are four distinct Ras genes in the human genome, H-, K- (1A and 1B), and N-Ras, which differ primarily in their carboxyl-terminal regions. They encode small, 21-kDa guanine nucleotide-binding proteins that function as molecular switches to modulate signaling pathways that control cell growth, differentiation, and apoptosis [16]. This is achieved by cycling between the inactive (“off”) GDP-bound and active (“on”) GTP-bound states. Given the high affinity interaction between Ras and its nucleotide ligands (GDP and GTP) as well as the slow intrinsic rate of GDP dissociation and GTP hydrolysis, two classes of modulatory proteins regulate the activation state of Ras proteins. One class of proteins that activate Ras is guanine nucleotide exchange factors (GEFs), which promote exchange of GDP for GTP. Analogous to guanine nucleotide exchange factors, redox agents have been shown to stimulate nucleotide exchange and alter the activity of Ras proteins through reaction with cysteine 118 (Cys¹¹⁸), which is located in a conserved guanine nucleotide-binding motif [17]. We have found that only redox agents capable of thiyl radical formation, such as NO₂[•], can modulate Ras activity [18]. Thiyl radical formation at Cys¹¹⁸ facilitates guanine nucleotide exchange in Ras by promoting oxidation and the subsequent dissociation of the bound guanine base [19]. In cells, where the GTP/GDP ratio is in excess, this can lead to exchange of GDP for GTP and result in Ras activation. Importantly, this mechanism of Ras regulation has been shown to play a role in Ras-mediated tumorigenesis and tumor maintenance [20].

Rho GTPases are members of the Ras superfamily, and like Ras GTPases, function as molecular switches to regulate cellular growth. However, they also regulate cell motility and oxidant regulation [21]. We have previously shown that a subset of Rho GTPases can be regulated through redox modification [22]. These GTPases contain a distinct, reactive cysteine that is conserved in ~40 % of Rho GTPases and renders them sensitive to oxidation and oxidative modification in cells [23].

Although several methods to determine cysteine pK_a values have been employed in the field [7], we have recently developed a fluorescence-based method to measure cysteine pK_a values [24]. Using 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F), a compound that specifically reacts with the thiolate form of the cysteine side chain, we have been able to measure the pK_a of reactive thiols in Ras and Rho family GTPases. In Ras, Cys¹¹⁸ is the only solvent accessible cysteine in the core catalytic domain (Ras residues 1–166). Thus,

Ras provides an excellent system to demonstrate the use of the ABD-F strategy for measuring pK_a values of cysteine thiol side chains.

When coupled with mass spectrometry (MS), this method provides an analytical platform for the detection and quantification of redox modifications in Ras family GTPases and other proteins. The bottom-up MS method we've employed [25] requires proteolytic digestion with trypsin followed by LC-MS analysis by electrospray ionization (ESI). The accurate identification of peptides has become a routine practice with the availability of a vast number of programs and search engines that assist in assigning peptide sequences to mass spectra using probabilistic prediction algorithms [26]. In addition, the intensity, or spectral count information, can be used for the relative or absolute quantification of peptides associated with site-specific protein modifications.

Herein, we describe in detail biophysical and proteomic approaches to detect cysteine modifications in Ras GTPases, as well as the preparation and handling of ROS and RNS. These methods include:

1. Cysteine pK_a determination using 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F).
2. Generation of cysteine-modifying redox-active compounds.
3. $\text{NO}_2^\bullet$ generation and NONOates.
4. Quantitative mass spectrometry including

Isotope-coded affinity tag-labeling for relative cysteine quantification.

Quantification of the cysteine oxidation.

Differential quantification of cysteine oxidation.

Differential thiol trapping to determine reversible oxidation of cysteine residues.

Filter-aided sample preparation.

2 Materials

2.1 ABD-F Buffers and Reagents

1. Components: 4-fluoro-7-aminosulfonylbenzofurazan (ABD-F), black, flat-bottom, non-coated plates, and protein spin concentrators.
2. Reducing buffer: 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 8.0, 5 mM MgCl_2 , 30 mM NaCl, 200 μM diethylene triamine pentaacetic acid (DTPA), and 5 mM dithiothreitol (DTT; added day of use).
3. ABD-F modification buffer: 15 mM 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6.5; (*see* Note 1), 5 mM MgCl_2 , 30 mM NaCl, and 200 μM DTPA.

¹pH 6.5 was selected because low pH values help maintain cysteine residues in the thiol (–SH) state. Higher pH buffers (>8.0) allow cysteines to populate the thiolate (–S[–]) state, which makes them more susceptible to air oxidation.

4. pH screen buffer (for a pH range between 5.5 and 8.5): 100 mM MES, 100 mM HEPES, 5 mM MgCl₂, and 200 μM DTPA (titrate each pH value individually).

2.2 Nitrosation Reagent (CysNO/GSNO) Generation

1. Solution I: 50 mM L-cysteine (or reduced glutathione) in 120 mM HCl.
2. Solution II: 50 mM NaNO₂.
3. Solution III: 40 mM ammonium sulfamate.
4. CysNO dilution buffer: 100 mM HEPES, pH 7.5, 5 mM MgCl₂, and 1 mM DTPA.

2.3 Nitric Oxide-Releasing Agents (NONOates)

1. All NONOate compounds (available from Cayman Chemicals).
2. 4,5-Diaminofluorescein (DAF-2).

2.4 Mass Spectrometry Techniques

2.4.1 Isotope-Coded Affinity Tag (ICAT)-Labeling for Relative Cysteine Quantification

1. Strong cation-exchange (SCX) PolySULFOETHYL A column.
2. Isotope-coded affinity tag (ICAT) reagents, including a cation-exchange cartridge and avidin affinity cartridge.
3. Micro-concentrator/spin-filter device.
4. C18 spin column.

2.4.2 Quantification of the Cysteine Oxidation

1. Ammonium bicarbonate (ABC) buffer: 200 mM NH₄HCO₃.
2. ABC + Iodoacetamide (IAM): 200 mM NH₄HCO₃ and 5 mM IAM.
3. Phosphate buffer: 100 mM Na₂HPO₄, pH 7.4, 50 mM NaCl, and 5 mM MgCl₂.
4. Phosphate-buffered saline (PBS): 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, and 2.7 mM KCl.
5. BIAM-elution buffer: 50 Na₂HPO₄, pH 7.2, 2 mM D-biotin, and 150 mM NaCl.

2.4.3 Differential Quantification of Cysteine Oxidation

1. Tris buffer: 50 mM Tris, pH 8.5, 25 mM NaCl, and 5 mM MgCl₂.
2. SCX buffer A: 10 mM KH₂PO₄, pH 3.0, and 20 % (vol/vol) acetonitrile (ACN).
3. SCX buffer B: 10 mM KH₂PO₄, pH 3.0, 350 mM KCl, and 20 % (vol/vol) ACN.

2.4.4 Differential Thiol Trapping to Determine Reversible Oxidation of

Cysteine Residues—Denaturing alkylation buffer (DAB): 200 mM Tris, pH 8.5, 8 M urea, 0.5 % (wt/vol) SDS, and 10 mM EDTA.

2.4.5 Filter-Aided Sample Preparation (FASP)

1. Denaturing buffer: 0.1 M Tris, pH 8.5, and 8 M urea.
2. Iodoacetamide (IAM) buffer (should be made day of use): 0.1 M Tris, pH 8.5, 50 mM IAM, and 8 M urea.

3 Methods

3.1 Cysteine pK_a Determination Using 4-Fluoro-7-aminosulfonylbenzofurazan (ABD-F)

1. Determine the amount (in moles) of protein that will be required to perform the assay. The assay as described is performed in a 96-well plate and requires fluorescence detection at 513 nm. Each well should contain approximately 5 or 10 μ M protein and be performed in triplicate at each pH value (technical repeats). To determine the pK_a accurately, a pH range must be performed with adequate coverage. As ABD-F fluorescence intensity is sensitive to the site of modification, it is important to ensure similar buffer conditions across the pH range selected.
2. Calculate the quantity of protein required; for example, 12 pH values performed in triplicate requires approximately 72 nmol of protein to perform the assay (200 μ l/well; 10 μ M/well $\times$ (36 wells $\times$ 200 μ l) = 72 nmol required).
3. Reduce the protein with dithiothreitol (DTT) or dithiobutylamine (DTBA) (*see* Note 2) to increase the yield of protein that can react with ABD-F. For adequate reduction, exchange the protein into a buffer containing 5 mM (or greater) DTT. Prepare the reducing buffer (minimum pH of 8.0) and exchange the protein into the buffer (using Amicon Ultra concentrators). As a subset of oxidative reactions (sulfenic and sulfonic acid) will not be reversed by DTT (*see* Note 3), this step serves to increase the percentage of protein that can be modified by ABD-F and enhances the signal-to-noise ratio of the assay.
4. While the protein is being buffer exchanged, prepare the ABD-F modification buffer. To remove the dissolved oxygen (*see* Note 4), sparge the ABD-F modification buffer with an inert gas, such as N_2 , for 30 min.
5. Incubate the protein in reducing buffer for at least 60 min on ice for complete reduction.

²To modify proteins with redox-active reagents, it is important that the thiol moieties of the protein be in the reduced state. ABD-F will not react with oxidized cysteines and will result in reduced fluorescence yield. Further, oxidation of a thiol to sulfenic acid (-SOH) can result in disulfide bond formation. Thus, disulfide bonds and further oxidation states can readily occur when the protein is in the sulfenic acid state regardless of the thiol pK_a . If the protein is in the sulfinic (-SO₂H) or sulfonic (-SO₃H) state, the protein cannot be reduced and will be less reactive to ROS and RNS. By maintaining the protein in a reduced state, the reactivity will be dependent on the thiol/thiolate state of the protein.

³DTT works most efficiently at pH values greater than 8.0. This is because the reducing agent is effective when one of the thiol moieties is in the thiolate form. The pK_a of the DTT thiols are ~9.2 and 10.1 [44]. Mercaptoethanol (β -ME) is not optimal as it can stably modify thiols, which will reduce reactivity with ABD-F. Tris(2-carboxyethyl)phosphine (TCEP), which is a phosphine-reducing agent, appears to be efficient at reducing disulfides; however, other oxidation states, such as sulfenic acid (-SOH), are not efficiently reduced by TCEP according to our observations. A new reducing agent, dithiobutylamine (DTBA), functions similarly to DTT and has a thiol pK_a that is approximately one unit lower [45], which increases the reactivity of DTBA at physiological pH values. However, this reducing agent needs to be generated in house and is currently unavailable for purchase.

⁴The buffer should have all oxygen removed as this will aid in preventing air oxidation over time, especially when studying thiols with altered pK_a values, as the thiolate (RS⁻) is more reactive than a thiol (RSH).

6. Buffer exchange the protein into the sparged ABD-F modification buffer using Amicon Ultra concentrators (use appropriately sized membrane pores). This step removes reducing agents from the sample. It is critical that at least three rounds of exchange are performed to efficiently remove reducing agent from the sample (*see* Note 5).
7. Determine the protein concentration.
8. Set up the 96-well plates. The excitation wavelength for ABD-F is 389 nm and emission wavelength is 513 nm. Set up a “dummy tray” that contains half of the volume of the final reaction (100 μ l). In the “dummy tray,” add ABD-F to a final concentration that is double the intended concentration (add 2 mM ABD-F in 100 μ l). As the reaction proceeds through second-order kinetics, it is critical that all wells contain exactly the same amount of protein and ABD-F; therefore, the rate of modification is related to the thiol/thiolate state of the cysteine. In a separate 96-well plate, replicate the pH screen of the “dummy tray,” but add 20 μ M protein in 100 μ l to each well (*see* Note 6).
9. Using a multichannel pipette, quickly remove the solution from the “dummy tray” and place the solution into the respective wells of the test plate to initiate the ABD-F reaction. Quickly start the plate reader for data collection.
10. Data analysis (*see* Note 7). Use the linear portion of the initial slopes to calculate the rate of fluorescence generation over time (Fig. 1a, b). Plot the determined rates of fluorescence generation versus pH and fit the resulting sigmoidal curve to a Boltzman sigmoidal equation (GraphPad Prism). This equation is used to calculate the pK_a for a single site-specific modification (*see* Note 8; Fig. 1c).
11. After the fluorescence data has been collected, determine the site(s) modified by ABD-F (*see* Note 9). A straightforward method is to employ liquid chromatography-mass spectrometry (LC-MS) on trypsin-digested samples. We have also found that cysteine-to-serine mutants can be used to confirm modification sites if the mutation does not alter the protein structure and/or ABD-F reactivity.

⁵ABD-F reacts with free thiols in solution. Thus, reducing agents that act through a free thiol (e.g., DTT, β -ME, and DTBA) will generate fluorescence signal (false reactivity) and interfere with pK_a measurements. Therefore, these reagents cannot be present during data collection.

⁶A final concentration of 10 μ M protein is sufficient to give a good signal-to-noise ratio for most proteins. In general, a 100:1 ABD-F:protein ratio is sufficient.

⁷Although the curve-fitting approach outlined quantifies the initial linear slopes, collecting data at longer times will aid in verifying whether equivalent amounts of protein and ABD-F were added to each well (*see* Fig. 1a).

⁸This method is most effective when using a protein that only has one solvent accessible cysteine because the reactivity of other cysteines will complicate data analysis.

⁹If the protein of interest contains multiple solvent accessible cysteines, the assay can be modified to determine whether the protein has a cysteine with an altered pK_a . Using a substitution mutant of the suspected redox-sensitive cysteine (Cys-to-Ser (or Ala) mutation), perform the assay essentially as described but at one pH value (pH 6.5 is recommended such that non-redox-sensitive cysteines that do not have altered pK_a values do not result in high background signal). Thus, the cysteine mutant serves as a control and provides the background level of ABD-F modification in the protein.

3.2 Generation of Cysteine-Modifying Redox-Active Compounds (CysNO/GSNO Generation)

As the reactions of ROS and RNS with proteins are diverse and often complex, this section will focus on the generation and quantification of various redox-active compounds. Subheading 3.4 will describe mass spectrometry (MS) approaches to quantify and determine the sites of modification.

1. Prepare solution I and solution II on the day of use (*see* Notes 10 and ¹¹).
2. Mix 100 μ l of solution I with 100 μ l of solution II and place into a foil-covered 1.5-ml Eppendorf tube. Allow the reaction mixture to sit in the dark for 10 min. These reactions are highly light sensitive; therefore, care should be maintained to keep all reactions in the dark.
3. Add 20 μ l of 40 mM ammonium sulfamate to remove unreacted nitrate and allow the reaction to proceed in the dark for an additional 2 min. This protocol assumes an $\sim$ 85 % efficiency of the reaction (although published reports suggest an efficiency as high as $\sim$ 90 % [27]) and adds a sufficient concentration of sulfamate to remove all remaining NO₂.
4. Dilute the thiol-NO (i.e., CysNO or GSNO) with 880 μ l (fourfold) of CysNO dilution buffer. While this buffer can be altered to fit the needs of the individual researcher, the high concentration of the buffer component prevents changes in pH, reduces metal contaminants, and increases the half-life of the thiol-NO in solution [28, 29].
5. Measure the concentration of the generated thiol-NO using absorbance at 336 nm. The molar absorptivity for CysNO and GSNO is 900 M⁻¹ cm⁻¹. Alternatively, the molar absorptivity of 16.8 M⁻¹ cm⁻¹ at 543 nm can be used for concentration determination (*see* Note 12).

3.3 NO₂[•] Generation and NONOates

While CysNO represents a good nitrosation agent that can modify protein thiols through non-radical oxidative (two-electron) chemistry, the activity of Ras GTPases are uniquely sensitive to free radical oxidation. Free radical agents, such as NO₂[•], can react with a solvent accessible thiol in Ras to produce a thiyl radical. Through electron transfer, the bound guanine nucleotide becomes oxidized, which results in enhanced guanine nucleotide dissociation and enhanced guanine nucleotide exchange (activation) [13]. Thus, Ras activity is modulated by free radical agents capable of thiyl radical formation but not through non-radical oxidation. However, this radical-mediated mechanism may not be unique to Ras GTPases. Increasing evidence suggests that in many cases, non-radical-mediated thiol

¹⁰As reported in Grossi et al. [27], the pH of the reaction is critical for generating optimum nitroso-thiol content; therefore, after solution I and II are mixed, the solution pH should be approximately 1.7–2.0.

¹¹In general, any volume and concentration of nitroso-thiol is possible. The protocol explained in detail here is scaled to meet the needs of our experiments. Thus, this protocol will yield approximately 1,100 μ l of 8 mM thiol-NO.

¹²An alternative is to use the Saville assay [46] to measure thiol-NO concentration. However, this assay detects NO₂⁻ as well as the thiol-NO, which could lead to error if the free NO₂⁻ in solution has not been reacted with the thiol or sulfamate. In our experience, the absorbance at 336 nm is reliable.

oxidation reactions may be too slow to be biologically relevant [30]. Thus, in this section, we describe the preparation and handling of NO₂• for use in radical-mediated protein modification.

3.3.1 NO₂• Gas Generation

1. NO₂• gas can be purchased from various companies; however, it can just as easily be generated. Obtain a small reaction vial and add a small piece of copper wire (~100 mg or less) and cover the vial with a rubber stopper that is airtight.
2. Deplete the oxygen from the vial using an inert gas, such as N₂.
3. Inject in a small volume of nitric acid. Allow the reaction to proceed for 5–10 min. Nitrogen dioxide radicals are formed by the following equation:

The reaction proceeds by the above reaction pathway if a brown gas is generated. This reaction should be performed in a hood as NO₂• gas is highly toxic.

4. Estimate the concentration of NO₂• gas that will dissolve in solution using Henry's Law. Henry's Law states that the amount of a gas that will dissolve in solution is directly proportional to the partial pressure of the gas in equilibrium with the liquid. The coefficient (*k*) for NO₂• is needed for these calculations. From a table containing these coefficients, a range of 1,800–2,500 mol/l/atm can be obtained [31, 32]. As the density for NO₂• gas (2.62 g/cm³) is heavier than N₂ gas (0.808 g/cm³), one can assume that the relative gas concentration is 100 % NO₂• at the bottom of the vial. The coefficient needs to be converted to units of atm/mol/l by taking the reciprocal of the coefficient. Thus, a range of 1.2–3.4 × 10⁻² atm/mol/l is obtained.
5. Prepare a reaction vial with the protein. Seal the reaction vial and deplete the oxygen using N₂ or another inert gas. The headspace remaining will be critical; thus, the volume remaining in the reaction vial after the addition of the protein sample needs to be determined.
6. Add NO₂• gas to the reaction vial. The amount of gas added to the sealed reaction vial will not likely affect the pressure in the reaction vial significantly; therefore, the pressure is estimated to be 1 atm. However, the percent of NO₂• added to the vial relative to the remaining headspace must be calculated to determine the percent of NO₂• gas in the gas mixture. Thus, if 100 µl of pure NO₂• gas is added to a reaction vial with 1 ml head space (inert N₂ gas), then the total concentration of NO₂• gas will be 10 % (thus, 0.10 × 1.00 atm = 0.10 atm NO₂• gas in this example).
7. Using the equation $c = p/k$, where *c* is the concentration, *p* is the partial pressure (from **step 8**, 0.10 atm), and *k* is the inverse of Henry's law coefficient (from **step 4**, using an average of 2.3 × 10⁻² atm/mol/l), approximately 4.35 mol/l NO₂• gas will dissolve in liquid in a sealed reaction vial.

3.3.2 NONOates—NONOates, such as the compounds listed in Table 1, are compounds that release NO[•] in solution in a pH and time-dependent manner over seconds, minutes, or hours.

There are 8 NONOate compounds available. All NONOates are relatively stable at alkaline pH (approximately 24 h at 0 °C) and are water soluble. However, at pH 5, the release of NO[•] is nearly instantaneous. In addition, each NONOate has a characteristic UV absorbance value. Relevant information on NONOates is listed in Table 1.

1. Suspend the desired amount of NONOate in 10 mM NaOH to minimize NO[•] release (*see* Note 13 and Fig. 2).
2. To measure NO[•] release in the selected buffer, 4,5-diaminofluorescein (DAF-2) can be used. DAF-2 specifically binds to NO[•] and undergoes a change in fluorescence upon reaction. DAF-2 has excitation and emission wavelengths of 485 and 538 nm, respectively. Approximately 10 μM DAF-2 is recommended. DAF-2 has a detection limit of 5 nM at neutral pH (*see* Note 14).

3.4 Quantitative Mass Spectrometry

The cysteine labeling procedure known as isotope-coded affinity tag (ICAT) [33] was the first stable isotope-based chemical labeling method used in quantitative proteomics. The ICAT approach was designed primarily for the semiquantitation of proteins expressed in two cellular states by labeling proteins with heavy and light cysteine-reactive tags. The trypsin-digested peptides that correspond to the differential labels are identified using MS, and the relative signal intensities are used for quantitative comparison (*see* Fig. 3).

Proteomics-based methodologies have recently been developed to study redox regulation in proteins, also known as the redoxome [34, 35]. A number of stable isotope labeling methods exist for measuring cysteine oxidation by MS. The majority of these methods fall into two main categories: (1) cysteine oxidation measured directly by comparing the loss of oxidized peptide as a function of oxidants by the relative signal intensities of cysteine-containing peptides labeled with light and heavy stable isotope reagents, such as ICAT, biotinylated-ICAT [36, 37], or biotinylated iodoacetamide (BIAM) [35], and (2) cysteine oxidation measured indirectly by thiol-blocking, selective reduction, and reversible modification using thiol probes that detect a gain in signal due to thiol oxidation, which is defined as oxICAT (*see* Fig. 4) [38]. In the context of measuring redox modifications, ICAT and other types of cysteine-reactive tags allow for the accurate quantification of cysteine-containing peptides

¹³An inherent disadvantage to using NONOates is that they generate NO[•]. In the case of Ras, NO₂[•] is required for thiyl radical production. However, NO[•] can react with O₂ to form NO₂[•] as well as other reaction products. A further disadvantage is that once NO₂[•] is generated, it can react with NO[•] released by the NONOate to produce other reaction products. A common reaction product is N₂O₃, which can nitrosate cysteines at an approximate rate of $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 2) [47]. In Ras reactions with DEA NONOate, we observed that only a fraction of Ras reacts with NO₂[•] to induce nucleotide dissociation and a sizable fraction of Ras can be nitrosated by N₂O₃. However, nitrosation of Ras through a non-radical pathway does not alter Ras activity [48, 49]. Modification by N₂O₃ is only likely under the reaction conditions used in vitro with purified proteins; however, in cells, this oxidation pathway is not favored.

¹⁴One can verify the dependence of the NO[•] species generated on protein activity when using NONOates by adding in PTIO (2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), which is a selective NO[•] scavenger. This control will allow the user to verify whether free radical byproducts cause the observed effects, as opposed to a competing reaction with the NONOate breakdown products.

[39]. Here, we describe new techniques for detecting and quantifying cysteine oxidation using mass spectrometry-based methods, including isotope-coded affinity tag-labeling for relative cysteine quantification, quantification of the cysteine oxidation, differential quantification of cysteine oxidation, differential thiol trapping to determine reversible oxidation of cysteine residues, and filter-aided sample preparation (*see* Note 15).

3.4.1 Isotope-Coded Affinity Tag (ICAT)-Labeling for Relative Cysteine Quantification

1. Generate proteins from cellular extracts and measure total protein using the bicinchoninic acid (BCA) assay.
2. Supplement the buffer with 0.1 % sodium dodecyl sulfate (SDS) before modification with the ICAT reagents (*see* Note 16).
3. Incubate the modified and unmodified samples at 37 °C with the acid-cleavable ¹²C (light) or ¹³C (heavy) ICAT reagents in the absence of reducing agent using the protocol supplied by the manufacturer.
4. After 2 h of incubation, mix the light and heavy ICAT-labeled protein samples at a stoichiometric ratio of 1:1, and trypsin digest the mixture by incubating at 37 °C overnight in ABC buffer.
5. Purify the trypsin-digested peptides using a cation-exchange cartridge to remove excess labeling reagent. The desalted peptides are affinity purified using the avidin affinity cartridge. Dry the peptides and suspend in the cleavage reagent to release the peptides from the acid-cleavable linker by incubating at 37 °C for 2 h. Dry the acid-cleaved peptides and suspend in 0.1 % formic acid for LC-MS analysis (*see* Notes 17 and ¹⁸ for data analysis, *see* Notes 19 and ²⁰ for alternative approaches).

3.4.2 Quantification of the Cysteine Oxidation—The biotinylated iodoacetamide (BIAM) approach can be used to study oxidation of exposed cysteines under controlled redox conditions by quantifying differential BIAM labeling. The redox chemistry includes

¹⁵All of the strategies described in Subheading 3.4 rely on the loss of the analytical signal upon cysteine modification (oxidation). Most cysteine residues are maintained in their reduced state in cells, measuring glutathiolation directly using chemical labeling significantly impacts the sensitivity and dynamic range of (oxidation) detection.

¹⁶Buffers with low concentrations of SDS or urea allow for more complete labeling of the unfolded protein by the ICAT reagents.

¹⁷All buffers should be prepared in HPLC grade water for optimum performance. All reversed-phase LC-MS buffers should be prepared in LC-MS grade water.

¹⁸All peptides containing cysteine residues will contain heavy and light pairs of ICAT labels, and their relative intensities are measured using a full MS scan. The relative intensity, ratio = $[L^{int}/H^{int}]$, is used to determine the relative levels of modification.

¹⁹A similar strategy to ICAT has been demonstrated to measure cysteine oxidation due to NO[•] through the use of light and heavy *n*-ethylmaleimide (NEM) reagents that are generally used to protect sulfhydryls. We believe that this strategy could be adapted to study glutathiolation as well because the ICAT reagents can be easily replaced by NEM and d5-NEM. More recently, other stable isotope labeling reagents have become available, such as Cys-TMT (Thermo Fisher Scientific; San Jose CA). The use of Cys-TMT allows for an analytical work-flow to quantitatively assay free cysteine residues and determine the extent of modification under six biologically distinct conditions in a single mass spectrometry experiment. The multiplexing is particularly useful for studies involving time-course measurements.

²⁰An alternative strategy compared to the methods described in Subheading 3.4 is to introduce a reduction step to remove reversible cysteine modifications. Protein modifications can be measured indirectly by the following steps: (a) alkylation of free sulfhydryl groups on cysteine residues; (b) reduction of glutathione adducts by glutaredoxin (Grx), which does not affect other cysteine modifications; and (c) blocking all nascent sulfhydryl groups with an irreversible labeling reagent, such as IAM or NEM. The advantage of this method is that glutathione-specific cysteines will be quantitatively assayed. This methodology is more sensitive due to a net increase in the analytical signal for measuring glutathiolation, which is advantageous because most cysteine residues have low levels of glutathiolation in cells.

the reversible oxidation of cysteine residues by the addition of H₂O₂ or other oxidants as well as reduction with reducing agents to reduce oxidized cysteines.

1. Generate oxidized proteins.
2. Dissolve the proteins in ABC buffer containing 5 mM iodoacetamide (IAM) and incubate at room temperature in the dark for 30 min to complete the modification of accessible Cys residues with IAM.
3. Centrifuge the samples at 15,000 × *g* for 1 h, dialyze the supernatant against phosphate buffer, and divide the sample into two equal parts.
4. Dilute the two equal samples to 5 ml with phosphate buffer.
5. Add 250 µl of 100 mM DTT. Similarly, incubate the second control sample with 250 µl phosphate buffer.
6. Incubate both samples at room temperature for 30 min and treat with 275 µl of 100× BIAM for 30 min in the dark at room temperature. Remove excess BIAM by overnight dialysis against ABC buffer.
7. Load the sample on an avidin affinity cartridge followed by incubation for 20 min at room temperature.
8. Wash the column with 1–4 volumes of PBS, collect the flow-through, and wash the column further with PBS until the absorbance at 280 nm returns to baseline.
9. Elute the BIAM-modified proteins with four bed volumes of BIAM-elution buffer.
10. Trypsin digest the proteins overnight at 37 °C. Purify peptides using a PepClean desalting column according to the manufacturer's protocol and analyze the peptides by LC-MS analysis.

3.4.3 Differential Quantification of Cysteine Oxidation

1. Incubate oxidized and reduced protein samples (200 µg) in 100 µl of Tris buffer with light and heavy acid-cleavable ICAT reagents, respectively, at 37 °C for 3 h in the absence of reducing agents.
2. Volumetrically mix the light and heavy-labeled proteins at a 1:1 ratio and buffer exchange the samples against ABC buffer.
3. Digest the samples with trypsin by overnight incubation at 37 °C.
4. Lyophilize the digested peptides and suspend in either affinity loading buffer for direct avidin affinity purification or fractionate by reconstituting the dried peptides in SCX buffer A.
5. Fractionate the peptides using HPLC and a strong cation-exchange (SCX) PolySULFOETHYL A column with a step gradient of SCX buffer A to SCX buffer B.
6. Mix the individual fractions from SCX with equivalent amounts of affinity loading buffer and load onto an avidin affinity cartridge. Dry the avidin affinity purified

peptides using a lyophilizer and suspend in the cleavage reagent to release the ICAT-labeled peptides from the acid-cleavable linker by incubating at 37 °C for 2 h.

7. Dry the peptides obtained by acid cleavage and suspend in 0.1 % formic acid for LC-MS/MS analyses.

3.4.4 Differential Thiol Trapping to Determine Reversible Oxidation of Cysteine Residues

1. After purifying the oxidized protein sample, precipitate the protein with 10 % trichloroacetic acid (TCA).
2. Centrifuge the TCA precipitates ($13,000 \times g$, 4 °C, 30 min) and wash the pellet with 500 µl of ice-cold 10 % TCA and 200 µl of ice-cold 5 % TCA.
3. Dissolve the pellet in 80 µl of denaturing alkylation buffer (DAB) and the contents of one vial of cleavable heavy ICAT reagent dissolved in 20 µl of ACN.
4. Incubate the sample at 900 rpm for 1 h at 37 °C in the dark. To remove the light ICAT reagent, precipitate the proteins with 400 µl of chilled (20 °C) acetone for 4 h at 20 °C. After centrifugation ($13,000 \times g$, 4 °C, 30 min), wash the pellet twice with 500 µl of chilled acetone.
5. Dissolve the protein pellet in a mixture of 80 µl of DAB, 1 µl of 100 mM DTT, or other selected reducing agents.
6. Add the contents of one vial of cleavable light ICAT reagent dissolved in 20 µl of ACN.
7. Incubate the sample at 900 rpm for 1 h at 37 °C in the dark.
8. Perform trypsin digestion of the ICAT-labeled peptides, enrichment on streptavidin columns, and cleavage of the biotin tag according to the description in Subheadings 3.4.1 and 3.4.2.
9. Subject the sample to LC-MS/MS analysis.

3.4.5 Filter-Aided Sample Preparation (FASP)—There are two major strategies for converting proteins extracted from biological material to generating peptides suitable for MS-based proteome analysis: SDS-PAGE separation and in-gel digestion or in-solution digestion. The SDS-PAGE separation method reduces sample complexity by removing sample contaminants, such as detergents, salts, DNA, and other nonprotein compounds. The in-gel digestion approach is generally less efficient compared to in-solution digestion; however, when sample amounts are not limiting, this technique has shown wider usage amongst biologists for generating peptides. More recently, the filter-aided sample preparation (FASP) method has been introduced as it removes high levels of salts, SDS, and other contaminants prior to in-solution digestion [40–43]. In the FASP protocol, all protein cleanup steps, enrichment, and tryptic digestions are performed in a single micro-concentrator/spin-filter device. The technique can be quite useful for performing fast sample cleanup of in vitro-purified proteins where no further separation is required.

1. Mix 30 μ l of sample with 200 μ l of denaturing buffer in a YM10 or YM30 (depending on protein size) spin column and centrifuge for 15 min at $14,000 \times g$.
2. Add an additional 200 μ l of denaturing buffer to a YM10 or YM30 spin column and centrifuge for 15 min at $14,000 \times g$ (*see* Note 21).
3. Discard the flow-through.
4. Add 100 μ l of IAM buffer and mix on a shaker at 600 rpm for 1 min.
5. Incubate for 20 min at 25 °C (*see* Note 22).
6. Centrifuge for 10 min at $14,000 \times g$.
7. Add 100 μ l of denaturing buffer to the spin column and spin for 15 min at $14,000 \times g$. Repeat this step two additional times.
8. Add 100 μ l of ABC buffer and spin as in **step 7**. Repeat this step 2 additional times.
9. Add 16 μ l of 0.1 μ g/ μ l trypsin solution (trypsin should be diluted with ABC buffer) to the micro-concentrator/spin-filter device (*see* Note 23 for an alternative approach).
10. Remove old collection tubes from the spin column and replace with fresh collection tubes before centrifuging for 10 min at $14,000 \times g$.
11. Add 40 μ l of ABC buffer and centrifuge for 10 min at $14,000 \times g$ to collect peptides.

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²¹A YM30 filter (MW cut off of 30 kDa) is used to improve filtration speeds, whereas denaturants assist on-filter retention of proteins less than 30 kDa. However, the use of YM10 filters would be a conservative approach for ensuring the retention of lower molecular weight proteins/polypeptides (approximately 5–8 kDa range).

²²As we are studying cysteine oxidation in the sample prior to alkylation, the reducing step is omitted. Alkylation is performed to prevent disulfide exchange from scrambling the oxidative modifications to other cysteine residues that are exposed upon trypsin digest.

²³Incubate overnight at 37 °C or incubate for 1–5 min using microwave irradiation as described in the GOFast method [50]. An alternative approach uses endoprotease Lys-C (1:100 enzyme/protein ratio) for 4 h at 37 °C prior to trypsin digestion to improve the overall peptide digestion efficiency.

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Fig. 1. ABD-F modification of H-Ras^{WT} over a selected pH range. **(a)** H-Ras^{WT} (10 μM) was reacted with ABD-F (1 mM) over a period of 6 h at the indicated pH values. Data was collected every minute, and each reaction was performed in triplicate (error bars removed for clarity). Absolute fluorescence values are presented. In systems containing one modifiable cysteine, the total fluorescence values will plateau at a similar value if given sufficient time. The inset shows the relative reactivity of H-Ras^{C118S}, which was performed under identical conditions as H-Ras^{WT}. In this graph, it is apparent that little to no

modification of cysteines occurred in Ras^{C118S}. **(b)** The initial rates of ABD-F modification are used for pK_a determination. Here, the initial data are presented (zoomed in from **a**), and the initial rate was determined using GraphPad Prism; linear curve fitting was performed. Error bars are removed for clarity. **(c)** The observed rates of modification are plotted against pH. Each data point represents the rate of modification determined from the curve fitting in **(b)**. The data were fit to a Boltzmann Sigmoidal distribution to determine the pK_a. Control data (see inset in **a**) show that only Ras^{C118} is modified by ABD-F as the Ras^{C118S} control shows no reactivity. However, Ras^{Cys118} does not have an altered pK_a. Hence, the maximal rate of modification could not be obtained as Ras becomes unstable at high pH values. Therefore, the pK_a is an estimate

Fig. 2.

The reaction profile of $\text{NO}^\bullet$ generated from NONOates. The two most important reactions with $\text{NO}_2^\bullet$ are with free thiols (*black* pathway) and free $\text{NO}^\bullet$ (*red* pathway). The rate for $\text{NO}_2^\bullet$ with free thiol (RSH) is $2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ [51, 52]. For the reaction of $\text{NO}_2^\bullet$ with $\text{NO}^\bullet$ to form N_2O_3 , the rate is $1.1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [51, 53]. While this reaction is readily reversible, the reaction kinetics in vitro will favor the nitrosation of thiols through the reaction $\text{N}_2\text{O}_3 + \text{RSH} \rightarrow \text{RSNO}$ (in *red*, rate of $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ and an autohydrolysis rate with water of $4.75 \times 10^7 \text{ s}^{-1}$). However, in an in vivo system, the pathway in *black* will likely be the major route of protein oxidation [13]. While the end product of the pathway in *red* is identical to the pathway in *black* (free radical mediated), the reactions involving thiyl radical species result in unique regulation in Ras family GTPase activity. The gray reaction pathway is less likely to occur because it relies on three bimolecular reactions ($2(\text{NO}^\bullet + \text{NO}^\bullet \rightarrow \text{NO}_2^\bullet)$ and $\text{NO}_2^\bullet + \text{NO}_2^\bullet \rightarrow \text{N}_2\text{O}_4$), whereas the *red* and *black* pathways require only two bimolecular reactions

Fig. 3. Generalized ICAT-based relative quantification schematic for measuring cysteine peptides. ICAT allows for the relative quantification of samples from two separate conditions, such as quantification of protein expression levels under different cellular conditions. However, if one omits the reducing steps and adds an oxidation step to one of the conditions, then this approach can be adapted to study oxidation of proteins in cellular tissue (described in detail in the acid-cleavable ICAT with H_2O_2 approach). Each sample can be subjected to a technical replicate by reverse labeling to ensure reproducibility. Furthermore, ICAT reagents can be swapped out for other stable isotope-labeled tags, such as BIAM or Cys-tandem mass tag reagents

Fig. 4.

The oxICAT schematic for measuring cysteine oxidation. This method measures reversible cysteine oxidation. Here, the protein is denatured and labeled in one step, which labels all non-oxidized and buried cysteines with the heavy label, and is completed by reducing the oxidized species and labeling with the light isotope. Thus, if several experiments are performed by varying levels of oxidant exposure, one can estimate the level of oxidation (relative reactivity) of cysteines in a protein. A gain in signal intensity of the light isotope-labeled signal is an indirect measure of reversible thiol oxidation. Note that a technical or biological replicate analysis can be performed by reversing the order of the ICAT labels with the heavy isotope representing the oxidized species

Table 1

Specifications and use of NONOate compounds

Name	Cas#	Efficiency (mol NO [•] per NONOate)	Half-life at 37 °C; 22–25 °C	λ_{max} (nm)	ϵ (M ⁻¹ cm ⁻¹)
DETA NONOate	146724-94-9	2	20 h; 56 h	252	7,640
Spermine NONOate	136587-13-8	2	39 min; 230 min	252	8,500
Prol1 NONOate	N/A	2	1.8 s; N/A	252	8,400
DPTA NONOate	146724-95-0	2	3 h; 5 h	252	7,860
DEA NONOate	372965-00-9	1.5	2 min; 16 min	250	6,500
PAPA NONOate	146672-58-4	2	15 min; 77 min	252	8,100
MAHMA NONOate	146724-86-9	2	1 min; 2.7 min	250	7,250
Sulpho NONOate [54–56]	61142-90-3	0	7 min; 77 min	252	8,100

All NONOate compounds currently available and the CAS# (Prol1 NONOate currently has no CAS#) are listed. The efficiency represents the number of moles NO[•] released per mol of NONOate compound. The half-life information is provided at both temperatures. The λ_{max} is the wavelength where concentration of the compounds can be taken using a UV spectrophotometer, and ϵ is the corresponding extinction coefficient