

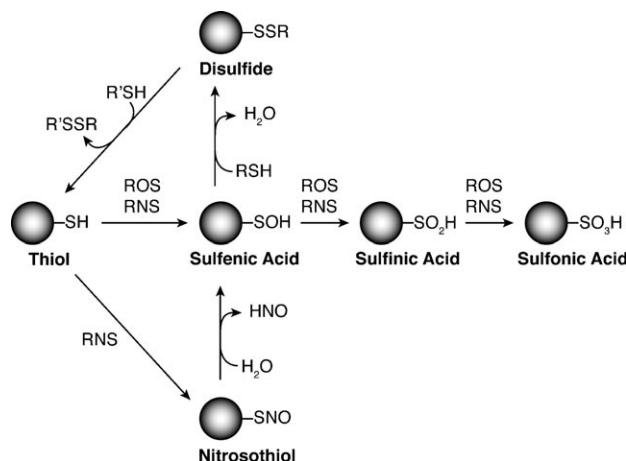
Quantification of Protein Sulfenic Acid Modifications Using Isotope-Coded Dimedone and Iododimedone**

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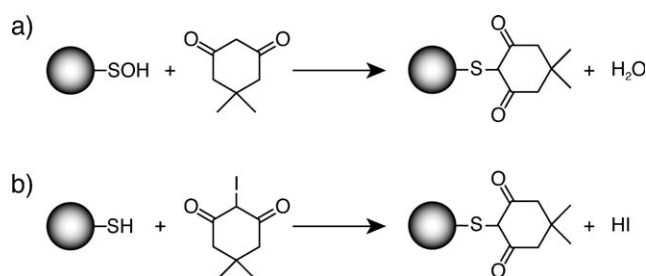
Dedicated to Professor Carolyn R. Bertozzi on the occasion of her 45th birthday

Since its discovery almost 40 years ago,^[1] S-hydroxylation (–SOH) of cysteine thiol side chains at active and allosteric sites within proteins has emerged as a central post-translational modification.^[2] At present, more than 200 transcription factors, signaling proteins, metabolic enzymes, proteostasis regulators, and cytoskeletal components that undergo sulfenic acid modification have been identified.^[3] Like phosphorylation, S-hydroxylation can be a dynamic and reversible post-translational modification whereby hydrogen peroxide (H₂O₂) and other reactive oxygen species (ROS) generated during the “oxidative burst” that accompanies many receptor-mediated signaling processes react with a thiolate anion to form sulfenic acid, and a family of enzymes reduces these modifications (or subsequent disulfides) in proteins (Scheme 1).^[4] Although protein sulfenic acids are often transient and labile, S-hydroxylation is significant in physiological and pathophysiological events. For example, it has been shown that this modification plays an essential role in eukaryotic H₂O₂ sensing,^[5] T-cell activation,^[6] enzyme catalysis,^[7] as an intermediate in disulfide formation,^[8] and correlates with disease states.^[9]

Given the rapidly expanding interest in this field, a number of strategies have been developed to detect protein S-hydroxylation.^[10] Chemical probes based on the selective reaction between 5,5-dimethyl-1,3-cyclohexanedione (dimedone) and sulfenic acid are particularly well suited to monitor this modification in proteins under biological conditions (Scheme 2a). Using dimedone-based reagents, many S-hydroxylated proteins have been observed by immunoblot and identified by mass spectrometry (MS);^[3] however, in vivo sulfenylation levels are virtually unknown. Knowledge regarding the degree or extent of S-hydroxylation within proteins is essential for understanding the function and



Scheme 1. Oxidation states of protein cysteines that are implicated in biological function. Both reactive oxygen and nitrogen species (ROS/RNS) can oxidize the thiol side chain of cysteine to sulfenic acid, which may be stabilized by the protein microenvironment or go on to form other reversible (disulfides and glutathione conjugates) and largely irreversible (sulfinic and sulfonic acid) species.



Scheme 2. Chemoselective reactions of cyclic 1,3-diketone derivatives. a) S-alkylation of a sulfenic acid by dimedone. b) S-alkylation of a thiol by iododimedone.

regulation of this oxidative post-translational modification. In addition, the ability to quantify S-hydroxylation should help overcome a major hurdle in the field—namely, the prioritization of proteins within the sulfenome selected for further characterization and functional analysis.

Current methods permit the relative abundance of oxidized or reduced forms to be estimated for *intact* proteins using dimedone or 2-nitro-5-thiobenzoic acid (the reduced form of Ellman's reagent) and MS.^[11] Nonetheless, this general approach is limited because it: 1) assumes that different protein species will ionize with equal efficiency, 2) cannot analyze individual sites of modification within a

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protein, and 3) is not applicable to peptide-based proteomic strategies. Herein, we describe a new method that overcomes these issues based on a class of reagents termed isotope-coded dimedone and iododimmedone (ICDID) for the quantitative analysis of protein sulfenic acid modifications.

Our general strategy to quantify protein S-hydroxylation is motivated by the use of isotope-labeled reagents in functional proteomics.^[12] In the present case, the key challenge is to devise a set of probes that generate chemically identical proteins, but differ in the specific mass of their label depending on the original redox state of the cysteine residue. Since the selectivity of dimedone for protein sulfenic acids is well established, our initial goal was to transform this cyclic 1,3-diketone into a thiol-reactive probe. From literature examples of thiourea S-alkylation by 1,3-dicarbonyl halides,^[13] we reasoned that installation of iodine at the 2-position of the dimedone scaffold would furnish the requisite trapping reagent (Scheme 2b; see also Figure S1 in the Supporting Information).

With this plan in mind, we prepared iododimmedone according to established procedures,^[14] and characterized its reactivity with low-molecular weight biomolecules by NMR spectroscopy and LC-MS under simulated physiological conditions. We initially studied the chemical stability of iododimmedone. To this end, the reagent was prepared in PBS/D₂O/10% [D₆]DMSO (pH 6, 7, or 8; PBS = phosphate-buffered saline; DMSO = dimethylsulfoxide) and ¹H NMR spectra were collected over a 24 h period. In all cases, iododimmedone was stable at room temperature and no degradation was observed (Figure S2). Next, we examined whether iododimmedone would react with glutathione (GSH), a biologically relevant thiol-containing tripeptide. LC-MS analysis of the reaction revealed a unique *m/z* 446 signal corresponding to the expected alkylation product (Figure S3) and verified further by ¹H chemical shifts of the cysteine β-protons in GSH [[D₆]DMSO, 400 MHz: δ = 2.7 (dd, 1H), 2.6 ppm (dd, 1H)]. Finally, we tested iododimmedone for potential cross-reactivity with a range of other biological nucleophiles, including the primary ε-amine of the lysine side chain; no undesired reaction was observed (Figure S4).

Next, we tested the ability of iododimmedone to modify cysteine residues within double (C64S C82S) and single (C64S) mutant forms of recombinant glutathione peroxidase Gpx3 from yeast.^[5] Gpx3 protein was reduced with dithiothreitol (DTT) and treated with iododimmedone or DMSO alone. The resulting ESI mass spectra acquired for intact Gpx3 demonstrate selective and quantitative formation of the expected single and double protein–dimedone adducts (Figure 1a and Figure S5). Having confirmed thiol-selectivity in a prototype protein, we investigated the peroxide dependence of dimedone or iododimmedone labeling of C64S C82S Gpx3, which harbors a single reactive cysteine at position 36 (C36). Mutant Gpx3 was untreated or oxidized with 0.25 to 2 equivalents of H₂O₂ and then incubated with dimedone or iododimmedone. Samples were resolved by SDS-PAGE and analyzed by Western blotting using an anti-dimedone antibody (Figure 1b).^[9] Dimedone-treated Gpx3 exhibited a

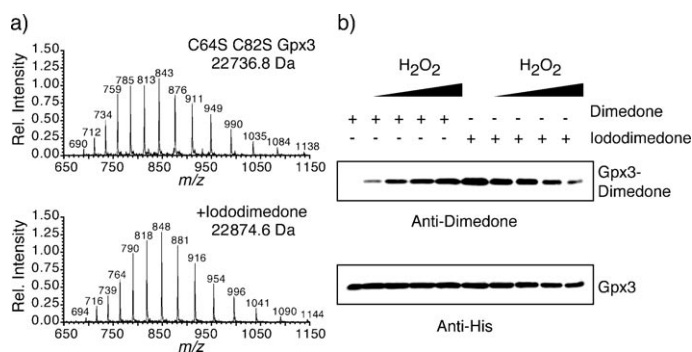
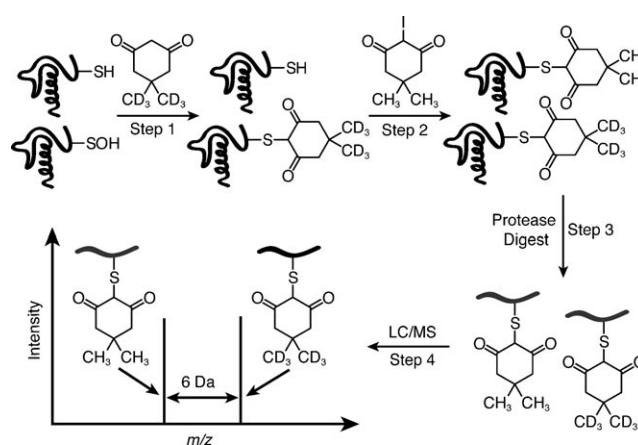


Figure 1. ESI-LC/MS and Western blot analysis shows that iododimmedone selectively labels the thiol group of C36 in C64S C82S Gpx3. a) The molecular weight of mock-treated C64S C82S Gpx3 is 22736.8 Da corresponding to intact, unmodified protein (top spectrum). The molecular weight of C64S C82S Gpx3 incubated with iododimmedone is 22874.6 Da (bottom spectrum), corresponding to C64S C82S Gpx3 with a single dimedone adduct ($\Delta m = 138$ Da). b) C64S C82S Gpx3 (50 μ M) untreated or oxidized with H₂O₂ (12.5, 25, 50, or 100 μ M) followed by dimedone (20 mM) or iododimmedone (20 mM). The resulting samples were analyzed by Western blot using an antibody that recognizes the protein–dimedone adduct (top panel). Total protein content was determined by an anti-His antibody (bottom panel).

peroxide-dependent increase in adduct formation. The exact opposite trend was observed among reactions that contained iododimmedone owing to oxidation of C36 and the concomitant loss of the thiol groups, as expected.

With qualitative results demonstrating the feasibility of our approach in hand, we prepared an isotopically labeled version of dimedone ([D₆]dimedone) to distinguish between the reaction product of a sulfenic acid and a thiol (i.e., $\Delta 6$ Da). Overall, the method we envisioned for ICDID includes the following sequential steps (Scheme 3): 1) sulfenic acids are derivatized with [D₆]dimedone, 2) excess reagent is removed and free thiols are labeled with iododimmedone, 3) protein samples are proteolyzed, and 4) the resulting peptides are separated and analyzed by LC-MS. The extent or fraction of sulfenic acid modification at a particular cysteine residue can be determined by dividing the heavy-isotope labeled peak by the sum of the heavy and light-



Scheme 3. The ICDID strategy to quantify protein sulfenic acid modifications.

isotope labeled peak intensities in the mass spectrum. As a proof-of-concept, we applied ICDID toward the quantification of sulfenic acid modifications within Gpx3 and the glycolytic enzyme, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), as detailed below.

We first investigated the approach with C64S C82S Gpx3, which contains a single cysteine, C36. During the oxidative part of the catalytic cycle, C36 thiolate reduction of H_2O_2 leads to sulfenic acid formation. For these experiments, C64S C82S Gpx3 was untreated or oxidized with 0.25 to 2 equivalents of H_2O_2 and incubated with $[\text{D}_6]$ dimedone. Small molecules were removed and the resulting protein was treated with iododimmedone. The labeled protein was digested with trypsin and tagged peptides were identified and quantified by LC-MS. Figure 2a shows the peroxide-dependent changes observed for the molecular ions corresponding

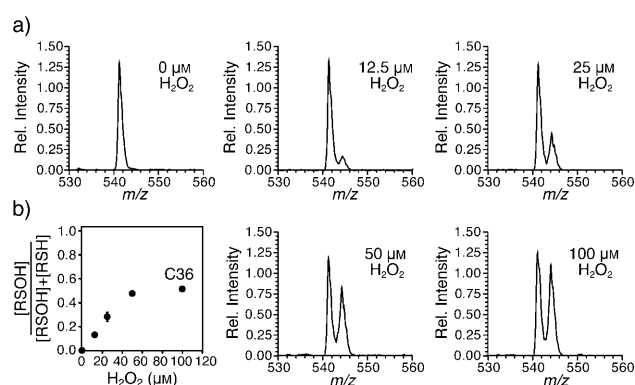


Figure 2. Quantifying sulfenic acid modification of C64S C82S Gpx3. a) C64S C82S Gpx3 (50 μM) was untreated or oxidized with H_2O_2 and incubated with $[\text{D}_6]$ dimedone (20 mM). Small molecules were removed and the resulting protein treated with iododimmedone (50 mM). Labeled protein was digested with trypsin and tagged peptides were identified and quantified by LC-MS. The molecular ions corresponding to m/z of 541 and 544 are consistent with the mass of peptide 36–43 modified by iododimmedone (representing the thiol form) or $[\text{D}_6]$ dimedone (representing the sulfenic acid form), respectively. b) Fraction of sulfenic acid detected at C36 (●) plotted as a function of H_2O_2 concentration. The extent of sulfenic acid formation was determined by dividing the heavy-isotope labeled peak by the sum of the heavy- and light-isotope labeled peak intensities in the mass spectrum (i.e., $[\text{RSOH}]/([\text{RSH}] + [\text{RSOH}])$). Error bars represent $\pm$ s.d. calculated from duplicate experiments.

to m/z of 541 and 544, which represent the masses of peptide 36–43 labeled by iododimmedone (i.e., the thiol form) and $[\text{D}_6]$ dimedone (i.e., the sulfenic acid form), respectively. Figure 2b shows the fraction of C36 present in the sulfenic acid form plotted as a function of peroxide concentration.

Several features of the ICDID strategy are immediately apparent from the data reported in Figure 2. First, the fraction of sulfenic acid modified peptide 36–43 parallels the increase in H_2O_2 up to 50 μM . Second, at stoichiometric oxidant concentration (i.e., 50 μM) approximately one-half of Gpx3 C36 is present in the sulfenic acid form. Accurate quantification of sulfenic acid modification requires that the

$[\text{D}_6]$ dimedone and iododimmedone tagging reactions proceed to completion. In this regard, we note that no change in the extent of peptide labeling was observed when the concentrations of probes or the length of incubation were increased, indicating that the labeling reactions were complete. Third, no further increase in the fraction of sulfenic acid modification at C36 was observed at 100 μM H_2O_2 . Since intermolecular disulfide formation was not observed in MS of intact Gpx3, and it is well established that the catalytic cysteine of thiol peroxidases is prone to hyperoxidation in the presence of $\geq 100 \mu\text{M}$ oxidant,^[15] a likely explanation is that excess H_2O_2 promotes oxidation of the sulfenic acid at C36 to sulfinic acid (which is not a target for dimedone or iododimmedone). Consistent with this proposal: 1) the sulfinic acid modified form of Gpx3 peptide 36–43 was observed at 100 μM H_2O_2 , and 2) simulated oxidation of C36 accurately reproduces experimental observations (Figure S6).

To investigate the ability of ICDID to monitor sulfenic acid modification at distinct sites within a protein we performed the analogous set of experiments with C82S Gpx3. This mutant protein harbors C36 and a second cysteine residue at position 64 (C64), which is characterized by a high pK_a .^[16] Like C64S C82S Gpx3, the fraction of sulfenic acid modified peptide 36–43 paralleled the increase in H_2O_2 up to 50 μM (Figure S7). Compared to the single mutant protein, the overall extent of oxidation was twofold lower. The structure homology model of Gpx3 indicates that C64 is located near C36, but biochemical studies show that these residues do not form a disulfide bond.^[5,16] Thus, it is possible that C64 alters the active-site environment at C36 and increases its propensity for hyperoxidation. The molecular ion corresponding to m/z of 639 represents the reaction product of iododimmedone and C64 within peptide 58–67, whereas no $[\text{D}_6]$ dimedone-tagged peptide 58–67 was observed (Figure S7). The absence of sulfenic acid modification at C64 is in excellent agreement with earlier studies of Gpx3, and indicates that S-hydroxylation can be monitored at discrete sites within a single protein.

Finally, we investigated sulfenic acid modification in GAPDH. This enzyme is well known to undergo sulfenic acid modification at its active-site cysteine (C149).^[1,3,9] However, in contrast to Gpx3, the reactive cysteine in GAPDH is significantly less susceptible to hyperoxidation.^[17] For these experiments, GAPDH was untreated or oxidized with 0.1 to 2 equivalents H_2O_2 and incubated with $[\text{D}_6]$ dimedone. Small molecules were removed, the resulting sample treated with iododimmedone, and reaction products were analyzed as illustrated in Scheme 3. Figure 3a shows the peroxide-dependent changes observed for the molecular ions corresponding to m/z of 992 and 995, which represent peptide 143–159 tagged by iododimmedone and $[\text{D}_6]$ dimedone, respectively. Figure 3b shows the fraction C149 (or C244) present in the sulfenic acid form plotted as a function of peroxide concentration. No sulfenic acid modification of C149 within peptide 143–159 was detected in the absence of H_2O_2 , as expected. The addition of H_2O_2 led to an increase in the fraction sulfenic acid modification at C149, which was determined as 1.0 at 50 μM oxidant. By contrast, the cysteine residue within peptide 232–245, C244, showed no

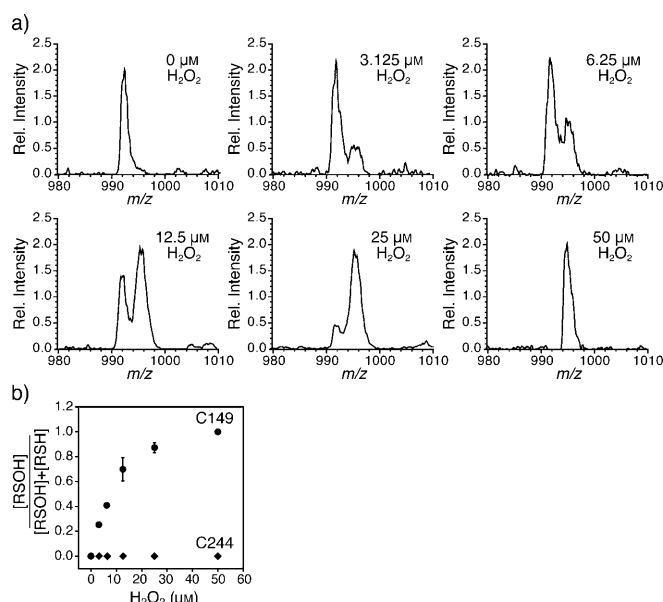


Figure 3. Quantifying sulfenic acid modification of GAPDH. a) GAPDH (25 μM) was untreated or oxidized with H_2O_2 and incubated with $[\text{D}_6]$ dimedone (10 mM). Small molecules were removed and the resulting protein treated with iododimedone (20 mM). Labeled protein was digested with trypsin and tagged peptides were identified and quantified by LC-MS. The peaks corresponding to m/z of 992 and 995 represent the reduced peptide and the sulfenic acid modified peptide, respectively. b) The fraction of sulfenic acid formation at C149 (●) and C244 (◆) plotted as a function of H_2O_2 concentration (see Figure S8 for MS data corresponding to peptide 232–245). Error bars represent $\pm$ s.d. calculated from duplicate experiments.

$[\text{D}_6]$ dimedone labeling in the presence of H_2O_2 (Figure S8), in accordance with earlier studies of thiol reactivity in GAPDH.^[1,3,9]

To summarize, we have presented ICDID, a new technique that enables quantification of protein sulfenic acid modifications. Using several prototypes, we show that this approach permits S-hydroxylation to be monitored at individual cysteines within a single protein and is amenable to peptide-based proteomic strategies. Another key feature of this approach is that it allows the occupancy of the site of modification to be determined. In some proteins, conversion of sulfenic acid to other oxoforms may preclude absolute quantification. However, our findings indicate that this phenomenon is readily diagnosed by peroxide-dependence studies in cases where the fraction of sulfenic acid modification levels off below one. Moreover, we anticipate that this technology can be extended to monitor changes in protein disulfides by the inclusion of an additional chemical reduction step.^[18] Going forward, the ICDID approach should provide a widely applicable means for the quantitative profiling and a comparison of thiol redox status in physiological and pathological conditions associated with oxidative stress. Ongoing efforts are focused on extending this approach to profile

in vivo sulfenylation levels and dynamics, particularly in growth factor pathways and cancer models.

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- [18] For example, an additional chemical reduction step could be included after step 2 in Scheme 3. The resulting free thiols could then be labeled by iododimedone (or an isotopic version of the iododimedone reagent) and further processed. At any given cysteine, the fraction present in the thiol, sulfenic acid or disulfide state could be calculated by comparing data obtained using the modified procedure to data obtained from samples processed without the additional reduction and alkylation steps.