TECHNICAL NOTES

Synthesis of Chemical Probes to Map Sulfenic Acid Modifications on Proteins

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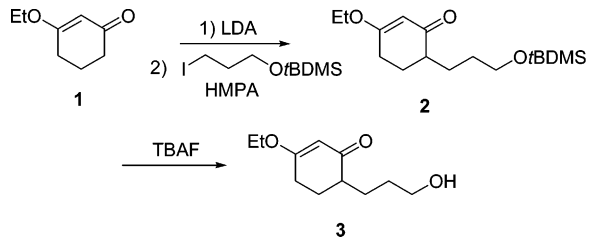
Cysteine sulfenic acids in proteins can be identified by their ability to form adducts with dimedone, but this reagent imparts no spectral or affinity tag for subsequent analyses of such tagged proteins. Given its similar reactivity toward cysteine sulfenic acids, 1,3-cyclohexadione was synthetically modified to an alcohol derivative and linked to fluorophores based on isatoic acid and 7-methoxycoumarin. The resulting compounds retain full reactivity and specificity toward cysteine sulfenic acids in proteins, allowing for incorporation of the fluorescent label into the protein and "tagging" it based on its sulfenic acid redox state. Control experiments using dimedone further show the specificity of the reaction of 1,3-diones with protein sulfenic acids in aqueous media. These new compounds provide the basis for an improved method for the detection of protein sulfenic acids.

INTRODUCTION

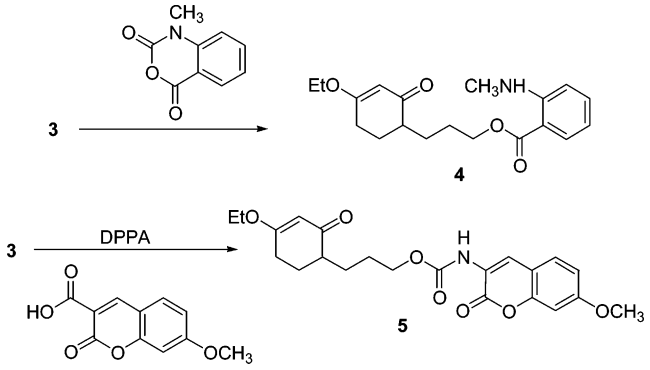
Interest in the identification of cysteine sulfenic acids in proteins by biochemists has grown substantially over the past decade as their biological roles in redox regulation and catalysis within an array of cellular proteins have become better defined (*1*, *2*). Despite their importance, only a limited set of tools to identify these species exist, and most of these are only applicable to in vitro studies of pure, isolated proteins (*3*, *4*). Chemical modification of cysteine sulfenic acids by dimedone (5,5-dimethyl-1,3-cyclohexanedione) provides a useful way to "tag" these species with a specific, irreversible alkylating agent, but the lack of any spectral or affinity label associated with dimedone requires that the detection of this tag be undertaken by mass spectrometry(*4*−*7*). A recently reported method involving biotin tagging of nascent cysteine thiols following cysteine sulfenic acid-specific reduction of cell extracts by sodium arsenite (*8*) has provided a new way to detect and isolate sulfenic acid proteins in a whole cell, proteomics format, although limitations of this method due in particular to cysteine sulfenic acid instability indicate that a rapid trapping and labeling method directed toward the unique chemistry of cysteine sulfenic acid would still be preferable.

To provide a new tool for identifying and isolating sulfenic acid-containing proteins and peptides, we designed and synthesized a functionalized derivative of 1,3-cyclohexadione (**3**, Scheme 1), a dimedone-like compound. Linkage of the alcohol functional group of this derivative to two different fluorophores, isatoic acid and 7-methoxycoumarin, yields fluorescent, sulfenic acid-reactive compounds (**6** and **7**, Scheme 3). Data presented herein confirm the utility of these reagents in specifically and rapidly labeling sulfenic acid groups in proteins. Further, the synthetic methods should be generally useful for linking other types of fluorescent or affinity tags to sulfenic acid-containing proteins for analysis and isolation.

Scheme 1



Scheme 2



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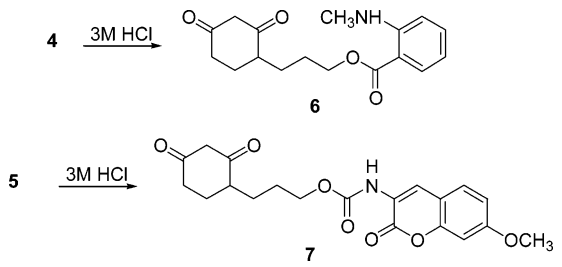
Scheme 3



EXPERIMENTAL PROCEDURES

3-Ethoxy-6-(3-*tert*-butyldimethylsilyloxypropyl)-cyclohex-2-enone (2). To a lithium diisopropylamide (LDA) solution [prepared from diisopropylamine (3.82 mL, 27 mmol) and ^{n}BuLi (7.26 mL of a 2.5M solution in hexanes, 18 mmol) in tetrahydrofuran (THF, 12 mL)[1] at 0 °C] at −78 °C was added 3-ethoxy-2-cyclohexen-1-one (2.64 mL, 18 mmol) in THF (6 mL), dropwise, over 40 min. After stirring for an additional 30 min at −78 °C, hexamethyl phosphoramide (HMPA, 3.16 mL, 18 mmol) was added followed by the dropwise addition of 3-iodo-1-*tert*-butyldimethylsiloxypropane (5.45 g, 18 mmol) in THF (8 mL). The resultant mixture was allowed to warm to room temperature, stirred for 6 h, and then quenched by the addition of water (10 mL). The reaction mixture was then partitioned between dichloromethane (DCM, 100 mL) and sat. NH_4Cl (40 mL). The aqueous phase was extracted with DCM (3 × 50 mL), and the organic phases were combined and washed with brine (50 mL), dried over anhydrous $MgSO_4$, and reduced to dryness. The resultant syrup was purified by flash column chromatography (hexanes/EtOAc 8/2) to yield **2** as a pale yellow oil (2.92 g, 51.2%). R_f 0.21 (hexanes/EtOAc 8/2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.26 (1H, s), 3.83 (2H, q, J = 7.0 Hz), 3.59 (1H, t, J = 6.5 Hz), 3.58 (H, t, J = 6.5 Hz), 2.38 (2H, t, J = 6.2 Hz), 2.20−1.99 (2H, m), 1.85−1.35 (5H, m), 1.31 (3H, t, J = 7.0 Hz), 0.84 (s, 9H), 0.00 (s, 6H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.6, 176.7, 102.3, 64.2, 63.4, 45.0, 30.5, 28.0, 26.4, 26.0 (×2), 18.4, 14.2, −5.2.

3-Ethoxy-6-(3-hydroxypropyl)cyclohex-2-enone (3). To a solution of **2** in THF (20 mL) was added TBAF (23.2 mL of a 1.0 M solution in THF, 23.2 mmol) and NEt_3 (3.2 mL, 23.2 mmol). After stirring at room temperature for 2 h, the reaction was quenched by the addition of water (20 mL) and sat. NH_4Cl (20 mL). The mixture was extracted with DCM (3 × 80 mL), and the combined organic phases were dried over anhydrous $MgSO_4$ and reduced to dryness. The resultant syrup was purified by flash column chromatography with gradient elution (DCM/diethyl ether 1/1 to 3/7) to yield **3** as a pale yellow oil (1.85 g, 100%). R_f 0.37 (hexanes/EtOAc/MeOH 6/3/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.32 (1H, s), 3.89 (2H, q, J = 7.1 Hz), 3.64 (2H, t, J = 6.2 Hz), 2.44 (2H, 2d, J = 7.1 Hz), 2.24 (1H, m), 2.06 (1H, m), 1.92−1.49 (5H, m), 1.36 (3H, t, J = 7.1 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 202.0, 177.2, 102.3, 64.4, 62.6, 44.9, 30.3, 28.4, 26.8, 25.8, 14.3.

3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl 2-(methylamino)benzoate (4). Alcohol **3** (180 mg, 0.89 mmol), NEt_3 (0.56 mL), and catalytic DMAP were added to a solution of *N*-methylisatoic anhydride (220 mg, 0.89 mmol) in dry DMF (2.0 mL) at room temperature. After stirring overnight, the solution was warmed to 65 °C for 3 h. After cooling to room temperature, water was added and this mixture was extracted with ethyl acetate (5 × 50 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, filtered, and evaporated to give the crude product, which was purified by flash column chromatography to afford 87.0 mg (45% yield) of **4** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.85 (1H, m), 7.33 (1H, m), 6.56 (2H, m), 5.27 (1H, s), 4.22 (2H, t, J = 5.2 Hz), 3.85 (2H, q, J = 7.0 Hz), 2.86 (3H, s), 2.39 (2H, t, J = 5.3 Hz), 2.04−1.41 (7H, m), 1.31 (3H, t, J = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.1, 176.8, 168.7, 151.7, 134.7, 131.7, 115.0, 111.3, 110.6, 102.5, 101.9, 64.7, 64.5, 64.3, 44.9, 30.0, 28.2, 26.6, 26.5, 26.4, 14.3; ESI MS *m/z* 354 ($M^+ + Na^+$).

3-(4-Ethoxy-2-oxocyclohex-3-enyl)propyl-7-methoxy-2-oxo-2*H*-chromen-3-ylcarbamate (5). A solution of 7-methoxy-3-carboxycoumarin (335 mg, 1.5 mmol), NEt_3 (1.05 mL, 7.6 mmol), and DPPA (0.36 mL, 1.7 mmol) in benzene (15 mL) was stirred at 65 °C for 4 h. A solution of **3** (260 mg, 1.3 mmol) in benzene (2 mL) was then added and the mixture stirred at 65 °C for 16 h. Upon cooling, water (40 mL) was added and the mixture extracted with DCM (3 × 40 mL). The combined organic phases were washed with sat. $NaHCO_3$ (30 mL) and brine (30 mL), dried over anhydrous $MgSO_4$, and reduced to dryness. The resultant solid was purified by flash column chromatography (×2, gradient elution with DCM/EtOAC (8/2) to DCM/EtOAc/MeOH (8/1/1), and then DCM/MeOH (9/1)) to yield **5** as an off-white solid (270 mg, 49.5%). R_f 0.63 (hexanes/EtOAc 1/2); mp 131−134 °C; ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.22 (1H, s), 7.34 (2H, m), 6.84 (1H, dd, J = 8.6 Hz, 2.4 Hz), 6.79 (1H, d, J = 2.4 Hz), 5.29 (1H, s), 4.18 (2H, t, J = 6.3 Hz), 3.85 (2H, 2q, J = 7.0 Hz), 3.82 (3H, s), 2.42 (2H, 2d, J = 7.1 Hz), 2.22 (1H, m), 2.08 (1H, dq, J = 14.2 Hz, 5.0 Hz), 1.94−1.85 (1H, m), 1.78−1.66 (3H, m), 1.52−1.42 (1H, m), 1.33 (3H, t, J = 7.0 Hz); ^{13}C NMR (75 MHz, $CDCl_3$) δ 201.0, 176.9, 161.1, 158.9, 153.6, 151.3, 128.4, 122.0, 121.9, 113.3, 113.2, 102.4, 100.8, 66.0, 64.4, 55.9, 44.9, 28.3, 26.6 (×2), 26.2, 14.3; ESI MS *m/z* 438 ($M^+ + Na^+$).

3-(2,4-Dioxocyclohexyl)propyl 2-(methylamino)-benzoate (DCP-MAB, compound 6). Compound **4** was stirred in a mixture of THF/3 N HCl (1/1) at room temperature for 3 h, and the mixture was concentrated. The crude product was washed with EtOAc/hexane/MeOH (1/2/0.5) to afford **6** as a white solid: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (1H, m), 7.34 (1H, m), 6.55 (2H, m), 4.22 (2H, t, J = 6.3 Hz), 3.60 (2H, m), 2.84 (3H, s), 2.52−1.50 (9H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 204.3, 203.8, 168.6, 151.6, 134.9, 131.7, 131.5, 115.2, 111.6, 110.5, 64.1, 58.4, 49.1, 39.9, 30.1, 26.4, 26.0, 24.7; ESI MS *m/z* 326 ($M^+ + Na^+$).

3-(2,4-Dioxocyclohexyl)propyl 7-Methoxy-2-oxo-2*H*-chromen-3-ylcarbamate (DCP-MCC, compound 7). Compound **5** (240 mg, 0.58 mmol) was stirred in a mixture of THF/DCM (3/1 v/v, 4 mL) and 3 N HCl (4 mL) for 1 h. The reaction mixture was diluted with water (10 mL) and extracted with DCM (3 × 30 mL). The combined organic phases were dried over anhydrous $MgSO_4$, and reduced to dryness to yield the crude product, which was purified by flash column chromatography (EtOAc/acetone 4/1) to yield **7** as an off-white solid (133 mg, 59.4%). R_f 0.62 (hexanes/EtOAc/MeOH 6/3/1); mp 154−156 °C; ^{1}H

[1]Abbreviations used: TBDMS, *tert*-butyldimethylsilyl; TBAF, tetra-*n*-butylammonium fluoride; amu, atomic mass units; THF, tetrahydrofuran; HMPA, hexamethylphosphoramide; DMAP, 4-(dimethylamino)pyridine; DPPA, diphenyl phosphorazidate; GSNO, *S*-nitrosoglutathione; NMR, nuclear magnetic resonance; LDA, lithium diisopropylamide; DCM, dichloromethane; ESI-MS, electrospray ionization mass spectrometry; NBD chloride, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCP-MAB, 3-(2,4-dioxocyclohexyl)propyl 2-(methylamino)benzoate; DCP-MCC, 3-(2,4-dioxocyclohexyl)propyl 7-methoxy-2-oxo-2*H*-chromen-3-ylcarbamate.

NMR (300 MHz, $CDCl_3$) δ 8.25 (1H, s), 7.42 (1H, s), 7.39 (1H, d, J = 8.7 Hz), 6.88 (1H, dd, J = 8.6 Hz, 2.1 Hz), 6.83 (1H, d, J = 2.1 Hz), 4.23 (2H, t, J = 6.5 Hz), 3.86 (3H, s), 3.44 (2H, d, J = 5.1 Hz), 2.78−2.69 (1H, dt, J = 16.4 Hz, 4.4 Hz), 2.67−2.51 (2H, m), 2.24−2.15 (1H, dq, J = 14.1 Hz, 4.8 Hz), 2.03−1.95 (1H, m), 1.88−1.47 (6H, m); ^{13}C NMR (75 MHz, $CDCl_3$) δ 204.2, 203.8, 161.2, 158.9, 153.5, 151.3, 128.4, 122.0, 121.8, 113.3, 113.2, 100.9, 65.6, 58.5, 55.9, 49.1, 39.9, 26.6, 25.7, 24.8; ESI MS *m/z* 410 ($M^+ + Na^+$), 388 ($M^+ + H^+$); CHN calcd for $C_{20}H_{21}NO_7 \cdot 0.5H_2O$, C: 60.59, H: 5.61, N: 3.53, found C: 61.31, H: 5.56, N: 3.48.

Generation of Pure, Sulfenic Acid-Containing C165S AhpC. The C165S mutant of AhpC was expressed in bacteria and purified in the presence of 5 mM 1,4-dithiothreitol (added to all buffers) essentially as described previously (*9*). Purification of wild type AhpC was also carried out as previously described (*10*). For generation of the sulfenic acid form of C165S AhpC, thawed enzyme in 25 mM phosphate buffer at pH 7, containing 1 mM EDTA, was washed free of the dithiothreitol and transferred into fresh buffer using a PD-10 column (Amersham Biosciences), and pooled fractions were transferred to an anerobic cuvette. The protein solution (160 nmol in 0.60 mL) was made anaerobic by repeated cycles of argon and vacuum over about 30 min, then rapidly titrated with 11 mM hydrogen peroxide to give a maximal absorbance signal at ~367 nm for the sulfenate anion as described previously (*3*, *4*). In subsequent experiments, optimal sulfenic acid formation was observed by adding and mixing the hydrogen peroxide much more slowly (additions of about 0.05 equiv and thorough mixing every ~1 min) and quantitating the sulfenic acid formed using a freshly prepared 2-nitro-5-thiobenzoate (TNB) solution (0.28 mL) and a 60 μL aliquot of the protein removed anaerobically from the cuvette (*3*, *4*). Using this method, ~73% of the protein was converted to the sulfenic acid form after addition of 1.08 equiv of hydrogen peroxide. Protein was kept in the anaerobic cuvette at room temperature for several hours before conducting experiments with no loss in sulfenic acid content.

Labeling of Sulfenic Acid-Containing C165S AhpC for Spectral and Mass Spectrometric Analysis. The pure, oxidized enzyme (32 μL, 6 nmol each) from the anaerobic cuvette was added to argon-flushed 500 μL Eppendorf tubes containing the compound of choice (either dimethyl sulfoxide alone, or dimethyl sulfoxide into which dimedone, 1,3-cyclohexanedione, **6**, or **7** had been dissolved) to give a final concentration of 5 mM for the added reagent in final volumes of 50 μL, and the tubes were flushed again with argon before closing. Other redox forms of C165S AhpC or the wild-type enzyme were also tested for reactivity with one or more of these four compounds. Approximate 100 mM stock solutions of **6** or **7** in dimethyl sulfoxide were standardized using expected extinction coefficients of 5700 M^{-1} cm^{-1} at 353 nm or 25000 M^{-1} cm^{-1} at 334 nm, respectively, for the esterified isatoic acid conjugate or the methoxycoumarin conjugate in methanol (*11*). Samples were incubated at room temperature for 60 min, then transferred to Apollo ultrafiltration devices (30K cutoff, Orbital Biosciences, Topsfield, MA), washed with 5.5 mL of 10 mM ammonium bicarbonate and reconcentrated to about 50 μL, for a total of four washes (> 10^8-fold dilution of the initial small molecule components).

Mass Spectrometric and Spectral Analyses of Adducts with the Sulfenic Acid-Containing Form of C165S AhpC. For mass spectrometric analyses, 60 μL of the ammonium bicarbonate buffer containing 1−2 nmol of labeled or unlabeled protein was submitted to the Mass Spectrometer Facility at Wake Forest University School of Medicine for infusion analysis on a Micromass Quattro II triple quadrupole mass spectrometer equipped with a Z-spray source. Just prior to analysis, samples were diluted 1:1 with acetonitrile and 1% formic acid was added. The data were processed and analyzed using MassLynx Version 3.5.

Labeled protein samples washed (with Apollo concentrators) into potassium phosphate or ammonium bicarbonate buffers were also analyzed for their UV−visible spectroscopic and fluorescence properties using a Beckman DU7500 diode array spectrophotometer or a SLM Aminco-Bowman Series 2 luminescence spectrophotometer, respectively. By comparison with the reported extinction coefficients for each of the free reagents in methanol (5700 M^{-1} cm^{-1} for esterified isatoic acid, and 25000 M^{-1} cm^{-1} for methoxycoumarin) (*11*), the free and presumably protein-bound reagents in neutral pH phosphate buffer exhibited average extinction coefficients of ~4500 and ~21800 M^{-1} cm^{-1}, respectively, for **6** and **7**. These measured values were subsequently used to standardize **6** and **7** and to estimate the labeled protein concentrations.

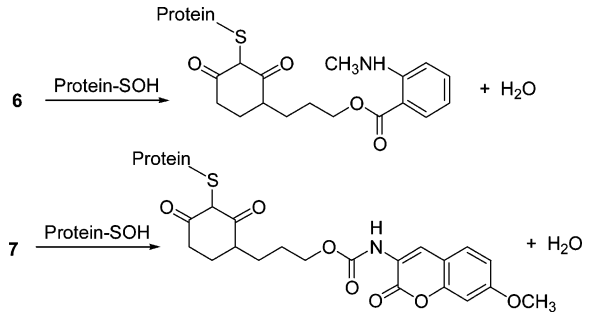
RESULTS AND DISCUSSION

Synthesis. As preliminary biochemical results revealed that 1,3-cyclohexadione reacts in a manner similar to dimedone, the protected version of 1,3-cyclohexadione, 3-ethoxy-2-cyclohexen-1-one (**1**, Scheme 1), was chosen as the initial starting material for the preparation of fluorescently labeled probes. Starting with **1**, which readily hydrolyzes to 1,3-cyclohexadione under acidic conditions, removes any synthetic complications from the highly acidic (pK_a = 5.15) (*12*) active methylene group of dimedone. Alkylation of the anion of commercially available 3-ethoxy-2-cyclohexen-1-one with *tert*-butyldimethylsilyl (TBDMS) (*13*)-protected 3-iodo-1-propanol yields **2** (51% yield, Scheme 1). Treatment of **2** with tetra-*n*-butylammonium fluoride (TBAF) removes the silyl group to give the alcohol (**3**, 100%, Scheme 1). The alcohol group of **3** provides an attachment site for the fluorescent groups.

Condensation of **3** with *N*-methylisatoic anhydride yields the protected 1,3-cyclohexadione derivative (**4**, 45%, Scheme 2). Heating 7-methoxycoumarin-3-carboxylic acid in the presence of diphenyl phosphorazidate (DPPA) and **3** gives the protected derivative (**5**, 50%, Scheme 2), presumably through the Curtius rearrangement of the acyl azide to the isocyanate followed by condensation of **3**. Treatment of **4** and **5** with aqueous HCl cleanly produces 3-(2,4-dioxocyclohexyl)propyl 2-(methylamino)benzoate (**6,** DCP-MAB, Scheme 3**)** and 3-(2,4-dioxocyclohexyl)propyl 7-methoxy-2-oxo-2*H*-chromen-3-ylcarbamate **(7**, DCP-MCC, Scheme 3) in 96 and 59% yield, respectively. Both the ester linkage of **6** and the carbamate linkage of **7** appear generally stable to these acidic deprotection conditions.

Reactivity of New Compounds toward Cysteine Sulfenic Acids in Proteins. To test for the reactivity of **6** and **7** toward protein sulfenic acids, a mutant, cysteine-dependent peroxidase enzyme was used in which the oxidized, sulfenic acid form of the cysteinyl redox center is stabilized, yet accessible (the C165S mutant of AhpC from *Salmonella typhimurium*, a peroxiredoxin) (*3, 9, 13*). Both compounds, dissolved initially in dimethyl sulfoxide and diluted 20-fold to a final concentration of 5 mM in aqueous, neutral pH buffer, gave covalent

Scheme 4



adducts with the sulfenic, but not sulfinic or sulfonic, acid forms of the peroxidase (Scheme 4).

The thiol group of the reduced protein and the oxidized, disulfide-bonded form of wild type AhpC were also unreactive toward these compounds, as confirmed by electrospray ionization mass spectrometry (ESI-MS) of the ammonium bicarbonate-washed proteins following incubations with the labeling agents (Figure 1 and data not shown). As illustrated in Figure 1 and summarized in Table 1, mass spectrometry results were completely consistent with the expected reactivity and products generated with the sulfenic acid-containing protein using dimedone, 1,3-cyclohexadione, and our two new labeling agents (**6** and **7**). Where sulfenic acid and hence adduct generation was substoichiometric due to the rapid addition of excess hydrogen peroxide (converting much of the enzyme to the sulfinic and perhaps sulfonic acid forms), additional peaks for thiol (unoxidized) and hyperoxidized (RSO_2^- and RSO_3^-) protein were observed (Figure 1). Under the conditions used, the sulfenic acid form of the protein either reacts with the reagent or is further oxidized during the workup or analysis of the sample and is therefore not observed. In a subsequent experiment where sulfenic acid formation was measured at ~73%, an equivalent amount of **6** or **7** (74 and 67%, respectively) was incorporated into the protein as observed by ESI-MS (Table 1).

The fluorescence properties of **7** ($\lambda_{ex,max} = 341$ nm and $\lambda_{em,max} = 414$ nm) were unchanged upon reaction with the cysteine sulfenic acid**,** while small shifts in both the excitation and emission wavelength maxima were observed upon adduct formation with **6**, the isatoic acid derivative (from $\lambda_{ex,max} = 353$ nm to $\lambda_{ex,max} = 357$ nm, and from $\lambda_{em,max} = 440$ nm to $\lambda_{em,max} = 430$ nm, Figure 2). This fluorescence difference may allow for the monitoring of the protein reaction with the fluorophore, although apparent bleaching of the fluorophore during measurements makes such experiments technically difficult. Protein adduct amounts assessed by ESI-MS analyses of the intact proteins indicate approximately equal labeling of the protein with the two reagents when 1:1 mixtures of the **6** and **7** are added, suggesting similar rates of reaction for these two compounds. While it is unlikely that all protein sulfenic acids react with these compounds at the same rate due in part to varying accessibility of the reactive group, it is probable that, under denaturing and anaerobic conditions, reaction rates will be similar to those with the model protein sulfenic acid.

Specificity of Dimedone and/or Fluorescent, Cyclohexadione-Derived Reagents toward Sulfenic Acids in Proteins. The known reactivity of the nucleophilic center of dimedone is toward cysteine sulfenic acids and aldehydes (*14, 15*). Amines have also been shown to condense with dimedone (*16*). As described above, control

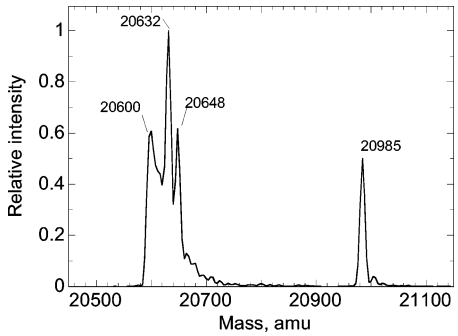


Figure 1. Electrospray ionization mass spectrometry analysis of adducts with the sulfenic acid form of C165S AhpC. The mutant of the bacterial peroxidase AhpC (C165S) containing only the peroxidatic cysteine (Cys46) but not the resolving cysteine (Cys165) that participates in disulfide bond formation was treated with hydrogen peroxide to yield the relatively stabilized sulfenic acid form of the protein under anaerobic conditions, then incubated with DCP-MCC (**7**) to yield the covalent protein adduct. The treated protein sample was concentrated and rediluted four times into 10 mM ammonium bicarbonate buffer using a 30K cutoff Apollo ultrafiltration device (>10^8-fold dilution of the initial small molecule components). Just prior to analysis, samples were diluted 1:1 with acetonitrile, made 1% in formic acid, and then infused at 5 μL/min. The capillary voltage was 3.5 kV; the cone voltage was ramped (30−120 V) and the spectrum scanned from *m/z* 600−1800 in 2.1 s. Eight to twenty scans were acquired for each sample. The data were processed using MassLynx 3.5 to generate the absolute molecular weight of the protein ± 2.1 Da. Shown are the transformed data that represent the relative abundance (shown as both the predicted and observed masses, see Table 1 for more detailed mass information) of four prominent species of C165S AhpC: the protein with the active site Cys46 in the thiol (20600 amu), sulfinic acid (20632 amu), or sulfonic acid (20648 amu) states, or in a covalent complex with **7** (20985 amu). In this case the initial method described in the Experimental Section (based on maximal A_{367} and rapid hydrogen peroxide addition) was used to generate the sulfenic acid form of C165S AhpC, resulting in approximately 1/5 of the protein being stabilized in this redox state at the time of reaction with **7**.

Table 1. Observed and Predicted Masses of Products Following Sulfenic Acid-Directed Labeling of Target Protein (C165S AhpC)

reagent	observed increase in mass of product compared with RSH form[a]	predicted additional mass of product[b]
1,3-cyclohexadione	109.2	110.1
DCP-MAB (**6**)	300.7	301.4
DCP-MCC (**7**)	384.5	385.4
dimedone	137.3	138.2

[a] Mass from electrospray ionization mass spectrometry on a Micromass Quattro II triple quadrapole mass spectrometer of the modified product from which was subtracted the mass for the oxidized sulfinic acid product (RSO_2^-) present in each sample, adjusted 32 amu for the two oxygen atoms. [b] Mass of labeling agent minus 2.016 for loss of hydrogens during adduct formation.

reactions of thiol, disulfide, or hyperoxidized forms of AhpC (wild type or C165S) demonstrated their lack of reactivity toward **6**, **7**, and dimedone based on the lack of ESI-MS-detectable adduct formation. To test for general cross-reactivity of these reagents with other oxidized sulfur-containing functional groups, we tested the reactivity of dimedone, as a model reagent, with a *S*-nitrosothiol and two sulfoxides. Dimedone fails to react with *S*-nitrosoglutathione (GSNO) as judged by absorbance spectroscopy over 1 h at room temperature. Nuclear magnetic resonance (NMR) and spectroscopic and chemical isolation experiments show that dimedone does not

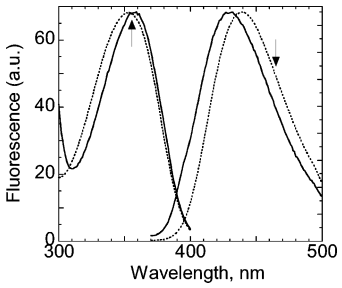


Figure 2. Fluorescence spectra of DCP-MAB (**6**) before (solid) and after (dotted) reaction with cysteine sulfenic acid-containing protein. Labeled C165S AhpC was prepared as described in the Experimental Section with the cysteine sulfenic acid form of the protein incubated anaerobically with 5 mM of **6** for 60 min, then washed free of the unreacted reagent and buffer components into a final buffer of 50 mM Tris-HCl at pH 8 using an Apollo concentrator. The absorbance at 347 nm of this sample was 0.14, and the fluorescence measurements were taken using a semi-microcuvette with the 0.4 mm cuvette width directed toward the excitation beam and the 1.0 mm internal width directed toward the emission detector at 90°. Emission scans (370 to 500 nm) were collected at an excitation wavelength of 353 or 357 nm, respectively, for the free and protein-bound **6**, and excitation scans (250 to 400 nm) were collected at an emission wavelength of 440 or 430 nm, respectively. Arrows indicate excitation and emission wavelengths used in attempts to monitor rates of reaction of cysteine sulfenic acid-containing C165S AhpC with **6**; technical problems were encountered with these measurements, and reproducible rates were not obtained.

react with aqueous solutions of either dimethyl sulfoxide or methionine sulfoxide (Supporting Information).

As reported, dimedone demonstrates reactivity with both aldehydes and amines (*15, 16*). Control reactions show that dimedone reacts with butyraldehyde in the presence of piperidine at 50 °C in aqueous ethanol but fails to react with the same aldehyde at room temperature in the absence of base. In addition, while dimedone condenses with benzylamine to form an imine in organic solvent, no reaction occurs in aqueous ethanol. The failure of **6** and **7** to react with either reduced or oxidized wild type or reduced C165S AhpC proteins also indicates that these compounds do not react with protein amine groups under these conditions. Taken together, these results demonstrate the relative specificity of the reaction of these compounds for sulfenic acids in proteins in aqueous buffers.

CONCLUSIONS

Functionalization of 1,3-cyclohexadione derivatives with an alcohol group and subsequent coupling to fluorophores has successfully generated two sulfenic acid-reactive compounds that specifically incorporate a fluorescent label into a sulfenic acid-containing model protein. Compounds such as these should prove useful in tagging protein sulfenic acids for their detection and isolation from complex protein mixtures in the future.

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Supporting Information Available: Experimental details and NMR data for compounds **2**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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