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Reactivity of Nitroxyl-Derived Sulfinamides

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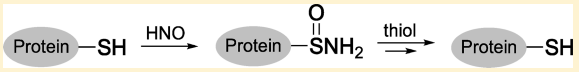
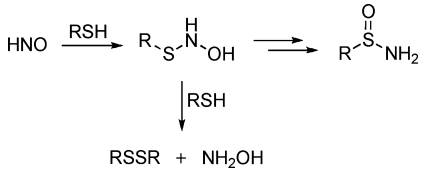
Supporting Information

ABSTRACT: Sulfinamide [$RS(O)NH_2$] formation is known to occur upon exposure of cysteine residues to nitroxyl (HNO), which has received recent attention as a potential heart failure therapeutic. Because this modification can alter protein structure and function, we have examined the reactivity of sulfinamides in several systems, including a small organic molecule, peptides, and a protein. Although it has generally been assumed that this thiol to sulfinamide modification is irreversible, we show that sulfinamides can be reduced back to the free thiol in the presence of excess thiol at physiological pH and temperature. We have examined this sulfinamide reduction both in peptides, where a cyclic intermediate analogous to that proposed for asparagine deamidation reactions potentially can contribute, and in a small organic molecule, where the mechanism is restricted to a direct thiolysis. These studies suggest that the contribution from the cyclic intermediate becomes more important in environments with lower dielectric constants. In addition, although sulfinic acid [$RS(O)OH$] formation is observed upon prolonged incubations in water, reduction of sulfinamides is found to dominate in the presence of thiols. Finally, studies with the cysteine protease, papain, suggest that the reduction of sulfinamide to the free thiol is viable in a protein environment.



Thiol residues are prone to several post-translational modifications under oxidative conditions. These include the formation of disulfide bonds, sulfinamides, and sulfenic, sulfinic, and sulfonic acids.[1,2] Nitroxyl (HNO), the protonated one-electron reduced form of nitric oxide (NO), has been shown to be a potential therapeutic agent for heart failure.[3,4] Moreover, recent reviews highlight the potential uses of HNO in the treatment of alcoholism, vascular dysfunction, and cancer.[5,6] One of the most significant features of HNO is its reactivity toward thiols.[7,8] This reactivity can result in the formation of a disulfide or a sulfinamide depending on the thiol concentration (Scheme 1). In the presence of excess thiol, the end products are disulfide and hydroxylamine, whereas at low thiol concentrations, the product is sulfinamide.

Scheme 1. Reaction of HNO with Thiols



Previous studies have shown that protein cysteine residues are targets of HNO.[9,10] HNO-induced disulfide and/or sulfinamide modifications are observed in papain,[11] glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[12,13] cathepsin B,[14] yeast transcription factor Ace1,[15] ryanodine receptors (RyR),[16] *N*-methyl-D-aspartate (NMDA) receptor,[17] bovine serum albumin (BSA),[18] aldehyde dehydrogenase (AlDH),[19] and phospholamban (PLB).[20] Traditionally, disulfide formation is considered to be reversible, whereas sulfinamide modification has been considered to be irreversible in peptides and proteins.[8,9,11−15,19,20] To date, there have been no reports of sulfinamide reduction under physiologically relevant conditions. Also, recent studies with *tert*-butanesulfinamide indicate that it is unreactive toward dithiothreitol (DTT).[20] Moreover, we are aware of only one example of the reduction of a sulfinic acid in a biological system, which occurs by an ATP-dependent process.[21−23]

Although not investigated thoroughly, the possible reduction of sulfinamides by thiols has been suggested by some recent studies. For example, free thiols have recently been observed upon treatment of peptide sulfinamides with DTT at elevated temperatures.[24] Similarly, the reaction of *N*-phenylbenzenesulfinamide and thiophenol in ethanol produces aniline and diphenyl disulfide, via a direct thiolysis mechanism involving the initial protonation of the sulfinyl group followed by a series of nucleophilic displacement reactions, the first of which gives thiosulfinate and elimination of the amine (Scheme 2).[25] The thiosulfinate is then reduced ultimately to the thiol upon reaction with excess thiophenol.[26]

A well-known process affecting proteins at physiological pH and temperature is the succinimide-mediated deamidation reaction. In this nonenzymatic reaction, the side chain amide linkage in an asparagine residue is hydrolyzed to form a carboxylic acid.[27−29] The mechanism is thought to involve an intramolecular cyclization in which the α-amino group of the carboxyl-side amino acid residue attacks the side chain carbonyl carbon of an asparaginyl residue forming a succinimide intermediate.[27−29] This cyclic intermediate is then hydrolyzed to give the carboxylic acid, either in the L-normal or L-iso peptide (Scheme 3). Consistent with the involvement of the cyclic succinimide intermediate, significant substituent effects

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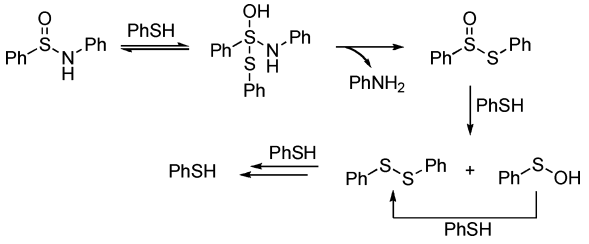
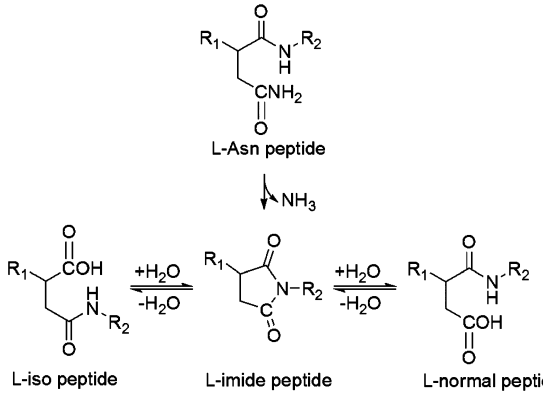
Scheme 2. Direct Thiolysis Mechanism of *N*-Phenylbenzenesulfinamide



Scheme 3. Succinimide-Mediated Deamidation Mechanism



on the C-terminal side of the asparagine residue have been observed.[27] In addition, the effect of the solvent dielectric constant on this reaction has been examined and indicates that the rate of deamidation is significantly reduced in solvents with low dielectric strengths.[30]

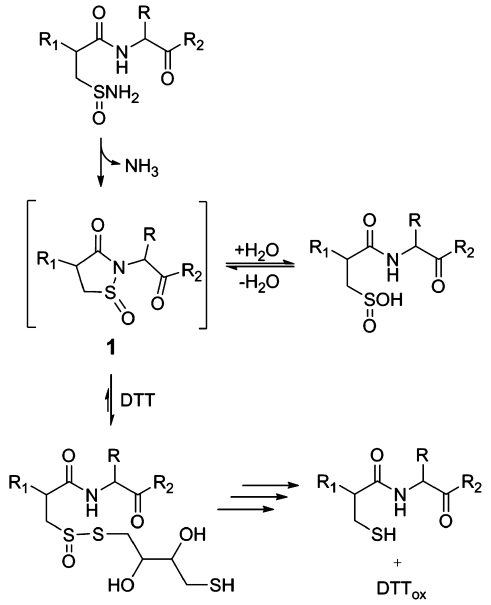
A sulfinamide [RS(O)NH_2] to sulfinic acid [RS(O)OH] conversion has recently been proposed to occur via a mechanism analogous to the succinimide-mediated deamidation reaction, mainly under gel electrophoresis conditions.[24] The mechanism involves the formation of a five-membered succinimide-like intermediate **1** (Scheme 4) with subsequent hydrolysis to yield the corresponding sulfinic acid-containing peptide. Considering the greater nucleophilicity of thiols over water, we hypothesized that the reduction of sulfinamides might be facilitated in peptides via a similar mechanism. To test this hypothesis, we have studied the reactivity of sulfinamides in a small organic molecule, peptides, and a protein. In addition, we have also examined the impact of the solvent dielectric constant on this reactivity.

EXPERIMENTAL PROCEDURES

Reagents. Glutathione (GSH), papain, N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *o*-phthalaldehyde (OPA), *β*-mercaptoethanol (BME), and DTT were of the highest purity available and purchased from Sigma (St. Louis, MO). 2-Phenylethanethiol and 2-(bromoethyl)benzene were purchased from Acros. HPLC grade acetonitrile (ACN) was purchased from Thermo Fisher Scientific (Rockford, IL). The HNO donors, Angeli's salt ($Na_2N_2O_3$, AS) and 2-bromo-*N*-hydroxybenzenesulfonamide (2-BrPA), were synthesized as previously described.[31,32] MilliQ water was used for all purifications and experiments.

Peptide Synthesis and Purification. Synthetic peptides VYPCGA, VYPCLA, VYPNGA, and VYPNLA were synthesized on a Symphony Quartet peptide synthesizer (Protein Technologies Inc., Tucson, AZ) following Fmoc solid-phase peptide synthesis methods.[33] The crude product was dissolved in 0.1% trifluoroacetic acid (TFA) and purified by HPLC (Waters HPLC system equipped with a Delta 600 pump system and a dual-wavelength absorbance detector) on an Apollo C_{18} reverse-phase column using a linear gradient from 5 to 75% ACN with 0.1% TFA over 50 min at room temperature. Peptide fractions were identified by electrospray ionization mass spectrometry (ESI-MS). Pure fractions were pooled and lyophilized, and the purified product was quantified on the basis of the absorbance at 280 nm (ε_{280} = 1490 M^{-1} cm^{-1}).[34] In all cases, the peptides were stored at −20 °C in lyophilized form until they were used.

Scheme 4. Mechanism of Sulfinamide Reduction via a Cyclic Intermediate



Synthesis of 2-Phenylethanesulfinamide. The synthesis was conducted on the basis of the known methods for the synthesis of sulfinamides.[35] Briefly, bis(2-phenylethyl) disulfide was generated from (2-bromoethyl)benzene using thiourea, MnO_2, and Na_2CO_3 as described previously.[36] The disulfide was reacted with 1.5 equiv of *N*-bromosuccinimide (NBS) in methanol to form the corresponding methyl sulfinate. After the mixture had been stirred at room temperature for 3 h, the mixture was diluted with CH_2Cl_2, washed with a saturated $NaHSO_3$ solution, and extracted with a saturated solution of $NaHCO_3$. 2-Phenylethanesulfinamide was formed by reacting methyl 2-phenylethylsulfinate with 2 equiv of butyllithium and 2 equiv of bis(trimethylsilyl)amine in tetrahydrofuran at −78 °C. (**Caution:** *Butyllithium is water-reactive, extremely flammable, and pyrophoric.*) The mixture was then stirred at room temperature for 2 h and the reaction quenched via addition of a saturated solution of NH_4Cl. The mixture was extracted with ethyl acetate (EtOAc) and dried with $MgSO_4$, and the solvent was evaporated under vacuum. The resulting sulfinamide was purified on a silica column by employing a hexane/EtOAc solvent system. The solvent was evaporated under vacuum to yield a white solid: ^{1}H NMR ($CDCl_3$) δ 7.27 (m, 5H), 4.10 (s, 2H), 3.06 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 138.8, 128.8, 128.5, 126.8, 58.7, 29.0; FAB-MS (3-NBA) *m*/*z* calcd 170.06396 [M + H], found 170.06470. The structure of 2-phenylethanesulfina-

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mide was also confirmed by X-ray crystallography (Supporting Information).

Formation of Peptide Sulfinamides by Reaction with HNO. Peptides were dissolved in 10 mM sodium phosphate buffer with 50 μM metal chelator, diethylenetriaminepentaacetic acid (DTPA), at pH 7.4 [or in 10 mM ammonium bicarbonate buffer with 50 μM DTPA (pH 7.4) where indicated] to produce a final concentration of 100 μM and used immediately. Stock solutions of AS were prepared in 0.01 M NaOH, kept in ice, and used within 15 min of being prepared. Stock solutions of 2-BrPA were prepared in ACN and used within 15 min of being prepared. The peptides were incubated with various concentrations of AS or 2-BrPA (as indicated) at 37 °C for 30 min in a block heater. The samples were flash-frozen and lyophilized overnight. The lyophilized samples were stored at −20 °C and used within 1 day.

In the case of ammonium bicarbonate buffer, the peptides were dissolved to produce a final concentration of 250 μM and incubated with 5 mM 2-BrPA as described above. The samples were used immediately without prior lyophilization. Under the conditions used in our experiments, ammonium bicarbonate buffer remained stable over a period of 69 h, as indicated by a pH increase of only 0.7 unit over this time period. For all experiments, the volume of 0.01 M NaOH or ACN introduced was less than 1% of the total sample volume.

Incubations of Peptide in Buffer. Stock solutions of DTT (1 M) were freshly prepared in water and used within 15 min of being prepared. BME was used directly. The sulfinamide-containing peptides were redissolved in water to produce a final peptide concentration of 0.8 mM and a buffer concentration of 82 mM sodium phosphate with 410 μM DTPA (pH 7.4). The samples were aliquoted and incubated in the presence or absence of reducing agents at 37 °C as indicated. Individual aliquots were removed from the samples for analysis at certain time intervals. The peptides were purified and desalted with C_{18} PepClean spin columns and then diluted into 70% ACN with 0.1% TFA for immediate ESI-MS analysis.

Incubations of Peptide in ACN with Buffer or Dioxane with Buffer. Stock solutions of the reducing agents were prepared as described above. The sulfinamide-containing peptides were redissolved in a 50:50 (v/v) ACN/water or 50:50 (v/v) dioxane/water mixture to produce a final peptide concentration of 0.8 mM and a buffer concentration of 82 mM sodium phosphate with 410 μM DTPA. The samples were incubated in the presence of reducing agents at 37 or 55 °C. Individual aliquots were removed from the samples for analysis at certain time intervals. The solvent was then removed under vacuum at room temperature in a Savant Speedvac apparatus. The residue was dissolved in water and prepared for ESI-MS analysis as described above. As a control, in all cases, a side-by-side experiment was conducted in sodium phosphate buffer and the sample was exposed to the same sample preparation steps as the sample in an ACN/buffer or dioxane/buffer mixture.

Incubation of Asn-Containing Peptides. VYPNGA and VYPNLA were dissolved in 50 mM phosphate buffer (pH 7.4). The samples were incubated at 100 °C, and aliquots were taken for analysis at certain time intervals. They were prepared for ESI-MS analysis as described above.

Incubation of 2-Phenylethanesulfinamide and HPLC Analysis. Stock solutions of the reducing agents were prepared as described above. 2-Phenylethanesulfinamide was dissolved in 82 mM sodium phosphate buffer with 410 μM DTPA (pH 7.4) to produce a final sulfinamide concentration of 2.5 mM. The samples were incubated in the presence or absence of reducing agents at 37 °C. Individual aliquots were removed from the samples for analysis at certain time intervals and cooled in ice for 5 min. They were then immediately analyzed by HPLC. All the analyses were performed on an Apollo C_{18} reverse-phase column connected to the HPLC system described above. A linear gradient from 35 to 80% ACN with 0.1% TFA over 35 min was employed at room temperature. The compounds were followed at 220 nm, and peaks were assigned on the basis of co-injection with authentic samples.

For experiments performed in organic cosolvents, 2-phenylethanesulfinamide was dissolved in an 50:50 (v/v) ACN/pH 7.4 phosphate buffer or 50:50 (v/v) dioxane/pH 7.4 phosphate buffer mixture to produce a final sulfinamide concentration of 2.5 mM and a buffer concentration of 82 mM sodium phosphate with 410 μM DTPA. The samples were incubated in the presence or absence of reducing agents at 37 or 55 °C and analyzed as described above.

Mass Spectrometric Analyses. ESI-MS analysis was conducted on a Thermo Finnigan LCQ Deca Ion Trap mass spectrometer fitted with an electrospray ionization source, operating in the positive ion mode with an accuracy of m/z ~0.1. In all experiments, the samples were introduced into the instrument at a rate of 10 μL/min using a syringe pump via a silica capillary line. The heated capillary temperature was 250 °C, and the spray voltage was 5 kV.

DTNB Assay for Quantitation of Free Thiol. Stock solutions of DTT were prepared as described above. The sulfinamide-containing peptides were redissolved in water to produce a final peptide concentration of 0.8 mM and a buffer concentration of 82 mM sodium phosphate with 410 μM DTPA (pH 7.4). The samples were incubated in the presence of reducing agents at 37 °C. Individual aliquots were removed from the samples for analysis at certain time intervals. The peptides were desalted with C_{18} PepClean spin columns to remove excess DTT. The solvent was then removed under vacuum at room temperature, and the residue was redissolved in 10 mM phosphate buffer with 50 μM DTPA (pH 8). The free sulfhydryl content was immediately determined by DTNB titration.[37] In all cases, a peptide sample incubated without AS and exposed to the same sample preparation procedure was employed as a control. The results were corrected for the amount of free thiol in the initial sample and normalized with respect to the indicated control sample.

Detection of Ammonia. 2-Phenylethanesulfinamide was dissolved in 82 mM sodium phosphate buffer with 410 μM DTPA (pH 7.4) to produce a final sulfinamide concentration of 2.5 mM. The samples were incubated in the presence or absence of DTT at 37 °C. Individual aliquots were removed from the samples for analysis at certain time intervals, and the concentration of ammonia was analyzed with a fluorometric assay using OPA.[38] $(NH_4)_2SO_4$ solutions were used as calibration standards. For the standard solutions and incubations conducted in the absence of DTT, the reducing agent was added at the time of the fluorometric assay (which requires DTT). The fluorometric analyses were conducted on a Spex Fluorolog instrument equipped with a 450 W Xe lamp.

Activation of Papain. A 0.5 mg/mL papain solution was prepared by dissolving the lyophilized enzyme in 82 mM sodium phosphate buffer with 410 μM DTPA (pH 7.4). We activated the papain sample by treating it with 2 mM DTT for 1.5 h at room temperature to reconvert its single free cysteine to the sulfhydryl form. The activated enzyme was stored at −20

°C. Before analysis, it was thawed and desalted with Zeba spin desalting columns to remove excess DTT. The papain solution was then diluted to 0.2 mg/mL with sodium phosphate buffer. To overcome batch to batch variation in the actual activity of the reduced papain preparation, all activity results are expressed as a percentage of the indicated control.

Incubation of Papain Solutions and an Activity Assay. A solution of 0.2 mg/mL activated papain in 82 mM sodium phosphate buffer with 410 μM DTPA (pH 7.4) was divided into two portions, and the aliquots were incubated with 0 or 100 μM AS at 37 °C for 30 min. Reducing agents were then added to both solutions to produce the required concentration, as indicated. The samples were incubated at 37 °C, and aliquots were taken from each sample at certain time intervals and immediately analyzed with a 20 min kinetic assay using L-BAPNA as the substrate.[11] In all cases, the sample incubated without AS served as the control sample to correct for the decrease in papain activity caused by long incubations. In some experiments, nonactivated papain, whose single active site cysteine is in a mixed disulfide form,[39] was utilized instead of the activated papain as indicated. All the spectrophotometric analyses were conducted on a Hewlett-Packard 8453 diode array spectrophotometer.

Analysis of the Data. All analyses were conducted in triplicate. Although the absolute amounts of the relevant thiol-containing species cannot be determined from any one of the reported ESI-MS experiments, the changes in the relative amounts of sulfinamide, sulfinic acid, thiol, and disulfide can be determined by calculating the percentage of each species in the total ion count (TIC). Such analyses, in which ratios of ion abundances are compared in different experiments, have been commonly employed as label-free quantification methods in proteomics.[40] In addition, for comparison, absolute yields of thiol were determined by a DTNB assay and were in good agreement with the relative yields determined by ESI-MS. In all cases, the error [standard error of the mean (SEM)] was found to be ±5%.

RESULTS

Formation of Peptide Sulfinamides by Reaction with HNO. We initially investigated the reaction of HNO with the cysteine-containing peptides VYPCGA and VYPCLA to determine the product distribution and to optimize conditions for sulfinamide formation. Both sulfinamide and disulfide modifications are observed upon treatment with HNO, as has been reported in the literature.[7,8] At the concentrations employed, ESI-MS was responsive to the relative concentration of the sulfinamide. As the ratio of HNO donor to peptide was increased, we observed an increase in the relative amount of sulfinamide along with a corresponding decrease in the amount of thiol (Figure 1a,c). As expected, higher yields of disulfide are observed at lower HNO donor:peptide ratios (Figure 1b,d). For 0.1 mM peptide, sulfinamide formation was most efficient with 0.3−1 mM AS. A similar trend is observed upon incubation of GSH with an HNO donor in ammonium bicarbonate buffer, also consistent with previous HPLC studies.[41] (Because of the low molecular weight of GSH, desalting was inefficient and experiments were conducted in ammonium bicarbonate, an MS-compatible buffer.) The higher sulfinamide yield observed with VYPCLA (97%) versus that with VYPCGA (66%) is presumably caused by the presence of the adjacent Leu residue, which inhibits the formation of the peptide disulfide due to steric hindrance (Figure 1b,d).[42−44]

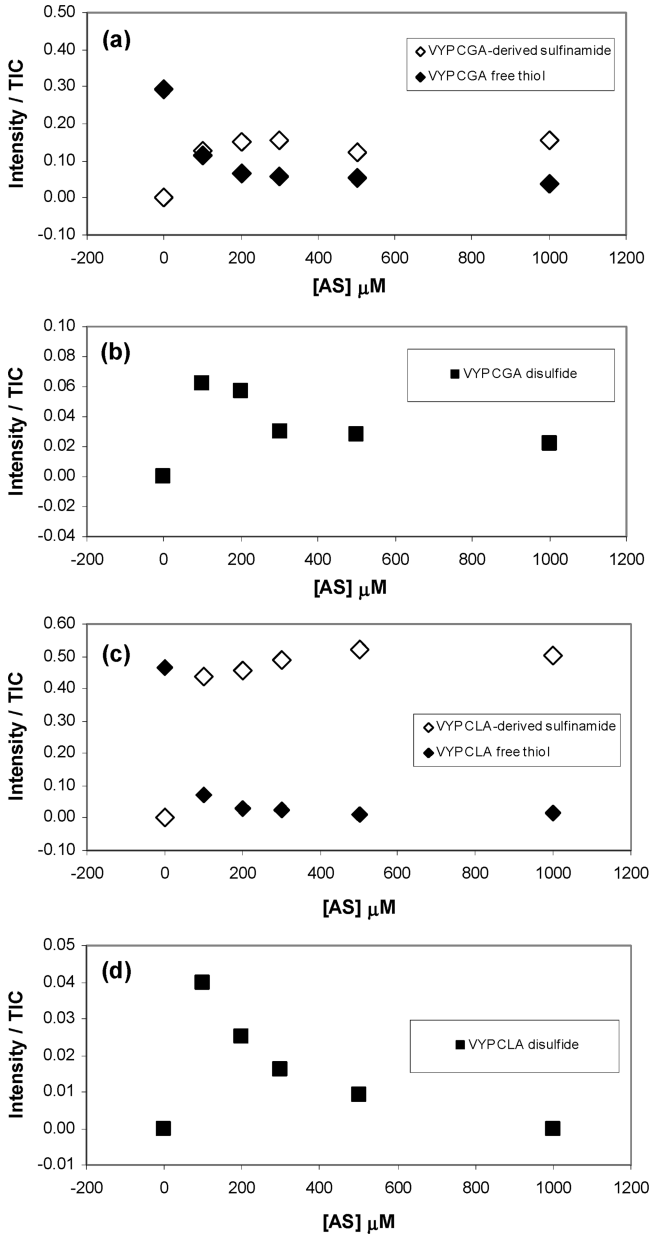

Figure 1. Formation of peptide-derived sulfinamides and disulfides in phosphate buffer. The ratios of sulfinamide, disulfide, and thiol ion abundance to total ion count were analyzed for (a and b) VYPCGA (0.1 mM) and (c and d) VYPCLA (0.1 mM) following incubation with 0−1000 μM AS in 10 mM phosphate buffer with 50 μM DTPA (pH 7.4) at 37 °C for 30 min (SEM of ±5%; $n \geq 3$).

Sulfinamide Reduction. To determine the extent of sulfinamide reduction back to the free thiol at physiological pH and temperature, the VYPCGA-derived sulfinamide [CG-S(O)NH_2] was incubated with 50 mM DTT in phosphate buffer for a total of 26 h and aliquots were analyzed after 0, 1, 6, and 26 h. As observed by ESI-MS (Figure 2a), the ratio of CG-S(O)NH_2 to the corresponding thiol-containing peptide (VYPCGA, CG-SH) decreases significantly after incubation for 26 h. Note that the disulfide-modified peptide, which can be observed in the absence of reducing agents, was not detected in the initial sample because of its immediate reduction by DTT. Thus, we attribute the increase in the amount of free thiol observed following incubation to reduction of the sulfinamide. As one can see in Figure 2a, no other significant changes are

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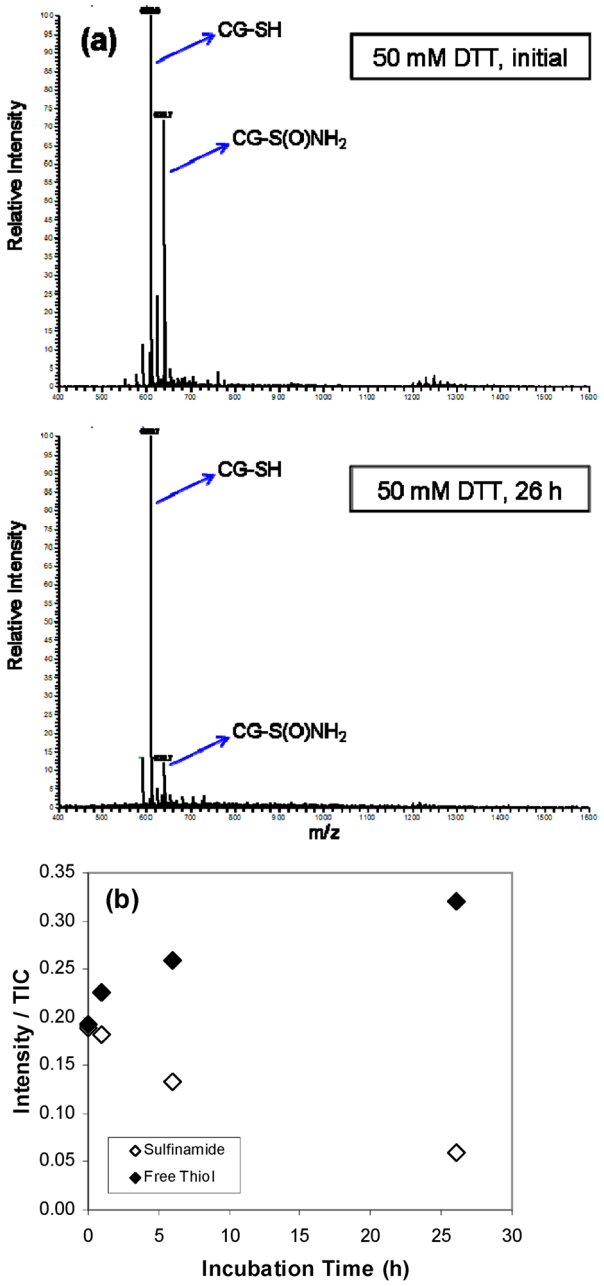

Figure 2. Reduction of the VYPCGA-derived sulfinamide [CG-S(O)NH_2]. VYPCGA (0.1 mM) was treated with 0.3 mM AS to produce the corresponding sulfinamide. The sample was incubated in phosphate buffer at 37 °C in the presence of 50 mM DTT. (a) Representative ESI-MS spectra showing the reduction of CG-S(O)NH_2 to the free thiol (CG-SH). No disulfide was observed in the initial sample because of its immediate reduction by DTT. (b) Ratios of CG-S(O)NH_2 (◇) and CG-SH (◆) ion abundance to total ion count observed by ESI-MS during the reduction of CG-S(O)NH_2 in the presence of 50 mM DTT (SEM of ±5%; $n \geq 3$).

observed in the ESI-MS data. After incubation at 37 °C for 1, 6, and 26 h, 7, 30, and 69% sulfinamide reduction was observed, respectively, with a corresponding increase in thiol (18, 34, and 66%, respectively). As shown in Table 1, the ESI-MS-derived increase in the amount of thiol agrees very well with that determined by a standard DTNB assay.[37] Upon incubation at 37 °C for 64 h, the level of sulfinamide reduction was found to be 86%. In the absence of DTT, no increase in the amount of free thiol was observed upon incubation of CG-S(O)NH_2 at 37 °C.

Table 1. Percent Increases in VYPCGA Free Thiol upon DTT Treatment of VYPCGA-Derived Sulfinamide

time (h)[a]	ESI-MS[b]	DTNB[c]
1	18	22
6	34	36
26	66	64

[a]Incubation time in phosphate buffer at 37 °C in the presence of 50 mM DTT. [b]The ratio of free thiol (CG-SH) ion abundance to total ion count was measured for each incubation time. The percent increase reported was determined by normalization with respect to the initial (t = 0) sample (SEM of ±5%; $n \geq 3$). [c]The percent increase reported was determined by a standard DTNB assay normalized with respect to the initial (t = 0) sample (SEM of ±5%; $n \geq 3$).

Similar results were obtained with 50 mM BME, indicating that the reduction is not specific to DTT and should take place in the presence of thiol-based reducing agents in general (Figure 3 and Supporting Information). Also, with the increase in the BME concentration from 50 to 320 mM, the observed reaction was nearly complete after 26 h.

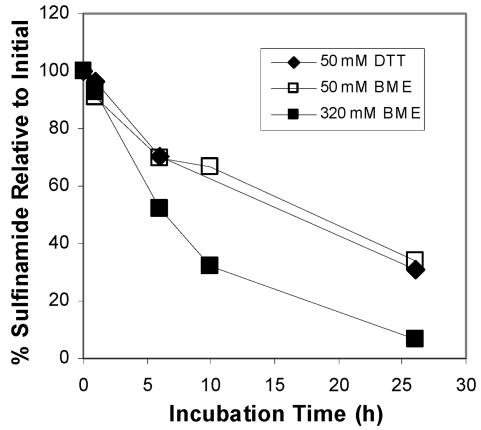

Figure 3. Reduction of the VYPCGA-derived sulfinamide [CG-S(O)NH_2] in the presence of various reducing agents. VYPCGA (0.1 mM) was treated with 1 mM AS to form the corresponding sulfinamide. The samples were incubated with 50 mM DTT (◆), 50 mM BME (□), or 320 mM BME (■) in phosphate buffer at 37 °C. The ratio of CG-S(O)NH_2 ion abundance to total ion count was determined for each incubation time. The percent CG-S(O)NH_2 was determined by normalizing the ion abundance ratios with respect to that detected in the initial peptide sample (SEM of ±5%; $n \geq 3$).

The rate of the protein deamidation reaction is known to be dependent on the nature of the amino acid located on the C-terminal side of the Asn residue. To determine if a similar effect is observed in the reduction of peptide sulfinamides, we utilized the VYPCLA-derived sulfinamide [CL-S(O)NH_2], which contains an adjacent Leu rather than Gly. Again, as observed by ESI-MS (Figure 4), a significant amount of CL-S(O)NH_2 was reduced to the corresponding thiol-containing peptide (VYPCLA, CL-SH) in the presence of DTT. Compared with that of CG-S(O)NH_2, a small decrease in the extent of reduction upon incubation with DTT in phosphate buffer for 26 h was observed (Figure 5). CG-S(O)NH_2 and CL-S(O)NH_2 were reduced by approximately 70 and 60% after 26 h, respectively, suggesting that sulfinamide reduction is not as sensitive to peptide sequence as the deamidation reaction.

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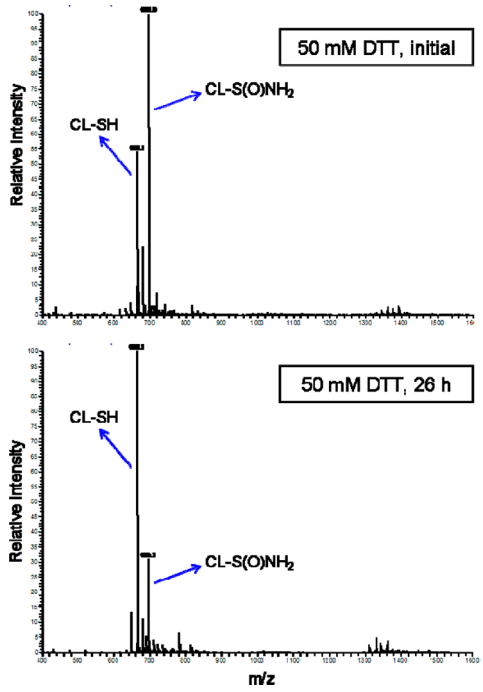

Figure 4. Representative ESI-MS spectra showing the reduction of VYPCLA-derived sulfinamide [CL-S(O)NH_2] to the free thiol (CL-SH). VYPCLA (0.1 mM) was treated with 0.3 mM AS to form the corresponding sulfinamide. The sample was incubated in phosphate buffer at 37 °C in the presence of 50 mM DTT for 26 h. No disulfide was observed in the initial sample because of its immediate reduction by DTT.

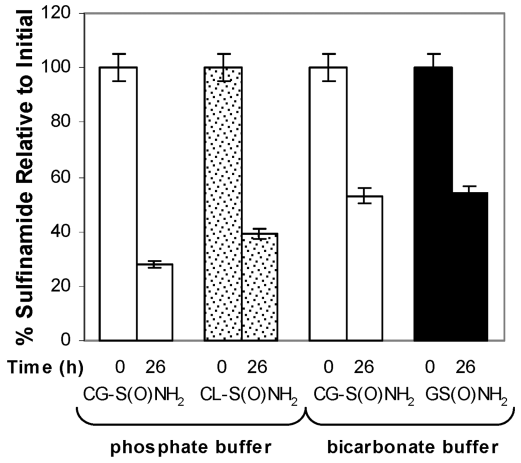

Figure 5. Reduction of the VYPCGA-derived [CG-S(O)NH_2], VYPCLA-derived [CL-S(O)NH_2], and glutathione-derived [GS(O)-NH_2] sulfinamides in phosphate or bicarbonate buffer. The samples were incubated with 50 mM DTT in sodium phosphate or ammonium bicarbonate buffer (as indicated) at 37 °C for 26 h. The ratio of sulfinamide ion abundance to total ion count was determined in each case. The percent sulfinamide was determined by normalizing the ion abundance ratios with respect to that detected in each initial peptide sample (SEM of ±5%; $n \geq 3$).

The reactivities of VYPCGA-derived and GSH-derived sulfinamides were found to be comparable in ammonium bicarbonate buffer (Figure 5). The difference in CG-S(O)NH_2 reduction observed in phosphate (70%) versus bicarbonate (47%) may indicate the importance of buffer conditions, as has been observed in previous deamidation studies.[45]

Sulfinamide Reduction in Peptides versus a Small Organic Molecule as a Function of Solvent Dielectric Constant. To test the hypothesis that sulfinamide reduction is facilitated in the peptides, we conducted experiments with a small organic molecule, which cannot form a "succinimide-like" intermediate **1** (Scheme 4). For this purpose, we employed 2-phenylethanesulfinamide [PE-S(O)NH_2] and compared its reactivity to that of sulfinamides generated by HNO in the VYPCGA and VYPCLA peptides [CG-S(O)NH_2 and CL-S(O)NH_2, respectively]. Also, to probe the reactivity of sulfinamides in hydrophobic environments, we examined the effect of solvent dielectric on these reductions in buffer, a 50:50 (v/v) ACN/buffer mixture, and a 50:50 (v/v) dioxane/buffer mixture, which have dielectric constants of approximately 74, 55, and 34, respectively.[30,46,47]

As shown in Figure 6a, DTT reduction of PE-S(O)NH_2 is much more facile in buffer versus either the ACN/buffer or the dioxane/buffer mixture, demonstrating that the rate of direct thiolysis reaction is substantially inhibited in less dielectric environments. Relative to this PE-S(O)NH_2 result, the reduction of both CG-S(O)NH_2 and CL-S(O)NH_2 is more efficient in the ACN/buffer and dioxane/buffer mixtures (Figure 6b,c). A similar behavior was also observed when the experiments were conducted at 55 °C in the ACN/buffer mixture (Supporting Information). These results indicate that the reduction of peptide sulfinamides involves a second mechanism, implicating the proposed cyclic intermediate **1** (Scheme 4), in addition to the direct thiolysis reaction. Moreover, in the dioxane/buffer mixture, it appears that the reduction of CG-S(O)NH_2 is more efficient than that of CL-S(O)NH_2. This result suggests a substituent effect (Gly vs Leu) on this reaction that becomes more apparent in the dioxane/buffer mixture and may be a further indication of the participation of a cyclic intermediate as has been observed with asparagine deamidation.[27]

Deamidation of Asn-Containing Peptides. The mass difference between a sulfinamide and its corresponding sulfinic acid is 1 Da. To confirm that sulfinamides and sulfinic acids can be distinguished by ESI-MS, we performed initial experiments with the previously studied peptides, VYPNGA and VYPNLA, which are known to undergo deamidation.[27,28,30,48] Upon incubation of these peptides at 100 °C for 1 h, VYPDGA, the corresponding deamidation product of VYPNGA, was detected in significant amounts, whereas no VYPDLA was observed (Supporting Information). VYPDLA, however, was detected following extended incubation at 100 °C for 10 h. These results correlate well with the reported half-lives of 0.15 and 4.9 h for VYPNGA and VYPNLA, respectively.[27] Thus, a 1 Da shift (i.e., the mass difference between an amide and a carboxylic acid) can be observed with our ESI-MS system, making it a viable technique for the analysis of the sulfinamide to sulfinic acid reaction.

Conversion of Sulfinamides to Sulfinic Acids. The time frame for sulfinic acid formation was investigated by incubating CG-S(O)NH_2 in phosphate buffer at 37 °C. As observed by ESI-MS (Figures 7 and 8), slow conversion of CG-S(O)NH_2 to the corresponding VYPCGA-derived sulfinic acid [CG-S(O)-OH] occurs in the absence of reducing agents. No reaction was observed when the incubations were conducted at −20 °C. Interestingly, the reaction was found to be even slower in ammonium bicarbonate buffer (pH 7.4), again indicating the potential importance of buffer conditions.[45]

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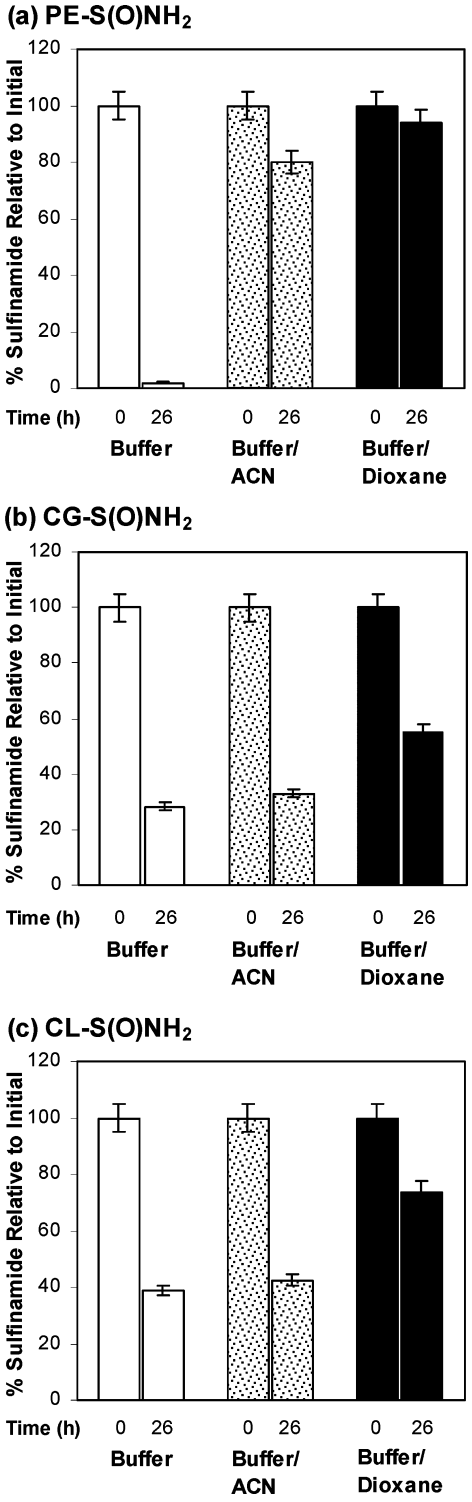

Figure 6. Reduction of (a) 2-phenylethanesulfinamide [PE-S(O)-NH_2], (b) the VYPCGA-derived sulfinamide [CG-S(O)NH_2], and (c) the VYPCLA-derived sulfinamide [CL-S(O)NH_2] as a function of solvent dielectric constant. The samples were incubated with 50 mM DTT in buffer, the ACN/buffer mixture, or the dioxane/buffer mixture at 37 °C for 26 h. The relative amounts of PE-S(O)NH_2 were quantified by HPLC (SEM of ±5%; $n \geq 3$). CG-S(O)NH_2 and CL-S(O)NH_2 were analyzed by ESI-MS by examining the ratios of CG-S(O)NH_2 and CL-S(O)NH_2 ion abundance to total ion count in each case. The percent sulfinamide was determined by normalizing each ion abundance ratio with respect to that detected in the initial peptide sample (SEM of ±5%; $n \geq 3$).

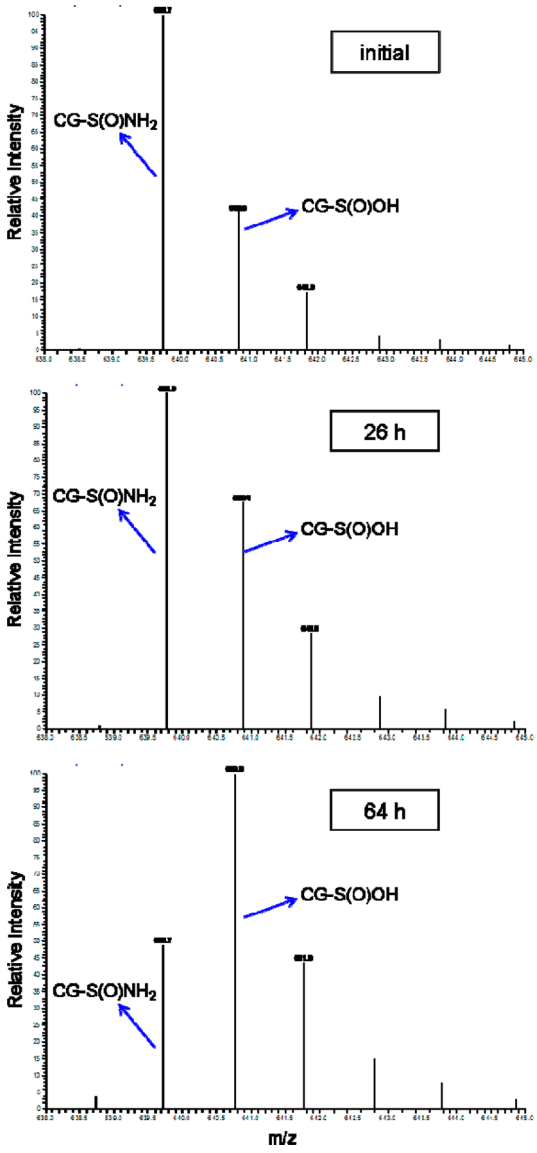

Figure 7. Representative ESI-MS spectra showing the formation of the VYPCGA-derived sulfinic acid [CG-S(O)OH] from the VYPCGA-derived sulfinamide [CG-S(O)NH_2]. VYPCGA (0.1 mM) was treated with 0.3 mM AS to form the corresponding sulfinamide. The sample was incubated in phosphate buffer at 37 °C in the absence of reducing agents. During data analysis, the intensity of the CG-S(O)NH_2 M + 1 isotope was subtracted to determine the actual intensity of the CG-S(O)OH peak.

To confirm the relative rates of sulfinamide reduction (to form thiol) versus hydrolysis (to form sulfinic acid), we conducted analogous experiments in the presence of DTT. Under these conditions, no sulfinic acid is observed, suggesting that there is a competition between the two reactions. Similar results were obtained when HPLC experiments were conducted with PE-S(O)NH_2 in the presence or absence of DTT. These results demonstrate that reduction of sulfinamide by thiols is faster than sulfinamide hydrolysis, as expected.

Detection of Ammonia. The release of ammonia as a byproduct in the reduction of 2-phenylethanesulfinamide was confirmed by a fluorometric assay.[38] When this sulfinamide is incubated in the presence of DTT, increasing concentrations of ammonia are detected corresponding to 53 and 100% reduction after 6 and 26 h, respectively (Figure 9a); no ammonia is

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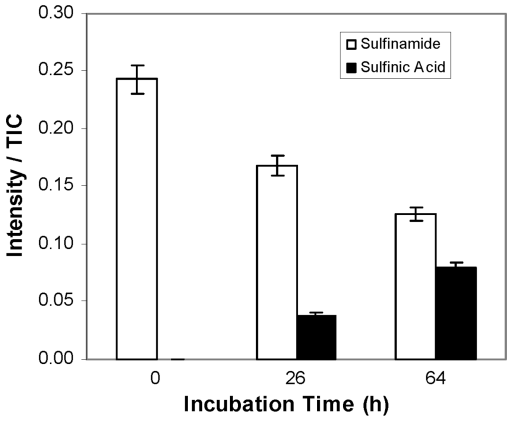

Figure 8. Hydrolysis of the VYPCGA-derived sulfinamide [CG-S(O)NH_2] to the VYPCGA-derived sulfinic acid [CG-S(O)OH]. VYPCGA (0.1 mM) was treated with 0.3 mM AS to form the corresponding sulfinamide, and the samples were incubated in phosphate buffer at 37 °C. CG-S(O)NH_2 and CG-S(O)OH were analyzed by ESI-MS by examining the ratios of CG-S(O)NH_2 (□) and CG-S(O)OH (■) ion abundance to total ion count (SEM of ±5%; $n \geq 3$).

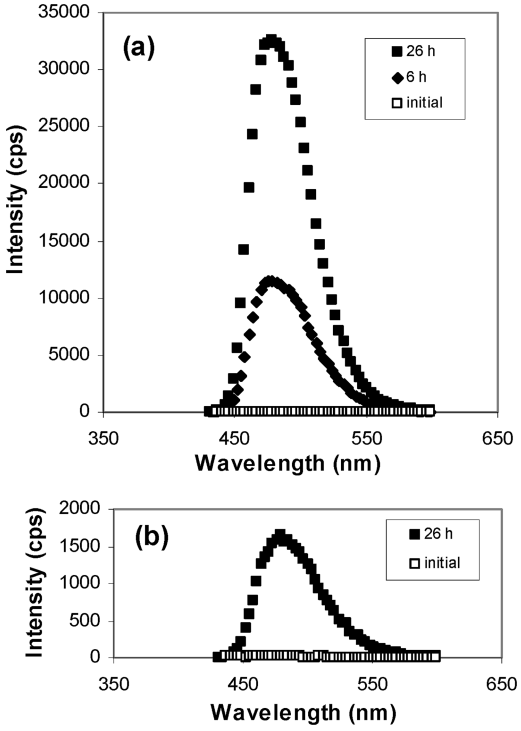

Figure 9. Formation of ammonia detected by an OPA fluorescence assay. A 2.5 mM solution of 2-phenylethanesulfinamide [PE-S(O)NH_2] was incubated at 37 °C for 26 h (a) in the presence or (b) in the absence of 50 mM DTT, and the amount of ammonia was detected in the initial sample (□), the sample that had been incubated for 6 h (◆), and the sample that had been incubated for 26 h (■).

detected in the initial sample. These results are consistent with the results of reduction obtained for 2-phenylethanesulfinamide by HPLC (54% after 6 h and 98% after 26 h). Moreover, Figure 9b shows that a small quantity of ammonia (corresponding to 27% sulfinamide hydrolysis after 26 h) is detected in the absence of DTT, which again corresponds well to the amount of 2-phenylethanesulfinic acid formed in buffer as detected by HPLC (23% after 26 h).

Reduction of Sulfinamide Modification in Papain. HNO targets several enzymes with active site thiol residues, resulting in the loss of enzyme activity.[8,11−14] The cysteine protease, papain, which in its active form has a single free thiol,[49,50] is known to be inhibited by HNO. The mechanism of inhibition has been proposed to be due to the formation of a sulfinamide.[11] In addition, it has been observed that incubation of AS-treated papain with DTT for 1 h results in a small recovery of enzyme activity.[11] To determine whether a sulfinamide modification in a protein can revert to the free thiol, we investigated the reactivity of this modification in AS-treated papain. As seen in Figure 10, the inhibition of papain

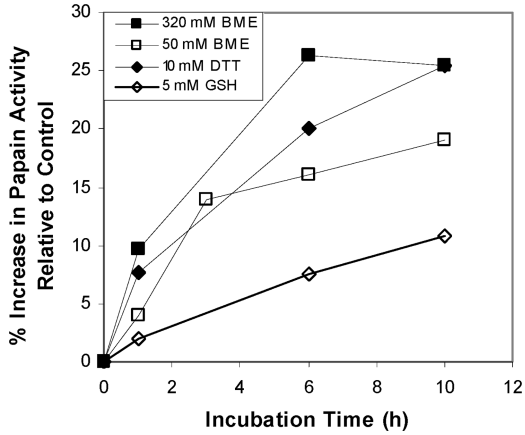

Figure 10. Reduction of the HNO-derived sulfinamide in papain. Previously activated papain (0.2 mg/mL) was treated with 100 μM AS. The samples were incubated with 5 mM GSH (◇), 10 mM DTT (◆), 50 mM BME (□), or 320 mM BME (■) in phosphate buffer at 37 °C. Data are expressed as the percentage of the activity of the control samples (SEM of ±5%; $n \geq 3$). No increase in papain activity was observed in the absence of reducing agents.

activity can be partially reversed upon incubation with thiol reducing agents over 10 h. Moreover, incubation with GSH for 26 and 105 h led to 18 and 44% activity recovery, respectively. Longer incubations were not possible with DTT or BME because of the significant loss of papain activity observed in the control samples.

To rule out the presence of an HNO-induced disulfide and its subsequent reduction, we investigated the time frame for the reduction of disulfide-modified papain. Nonactivated papain was used for these experiments, because its single, active site cysteine is known to exist in a mixed disulfide form rather than the free sulfhydryl form.[39] As expected, nonactivated papain had no significant activity. Upon incubation with 50 or 320 mM BME at physiological pH and temperature, the enzyme was activated in <10 min (Supporting Information). These results support the hypothesis that the increase in activity observed after much longer incubation times with AS-treated papain in the presence of reducing agents is due to the reduction of a sulfinamide.

■ DISCUSSION

Sulfinamide is formed upon reaction of HNO with thiols. Many examples of sulfinamide-modified proteins have been reported, with several of them being enzymes containing critical cysteine residues.[11−14,51] Thiol modification has been shown to have dramatic effects on the activities of these enzymes. Although the reduction of this sulfinamide modification has not been

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studied under physiologically relevant conditions, it has generally been assumed to be stable.[8,11−14,20,51] Our results, employing short peptides as model systems, indicate that the HNO-induced thiol to sulfinamide modification can be reduced in the presence of excess thiol, albeit quite slowly. At physiological pH and temperature, approximately one-third of the sulfinamide-modified peptide is reduced back to free thiol after 6 h. This reduction does not seem to be strongly dependent on the peptide sequence in buffer, but a substituent effect is observed in lower-dielectric constant solvents.

Direct thiolysis (Scheme 2) and a mechanism analogous to the succinimide-mediated deamidation mechanism (Scheme 4) are two plausible pathways. The major difference between the two mechanisms is the involvement of a cyclic intermediate **1**, the formation of which is facilitated by a peptide structure. Intermediate **1** has been proposed in the hydrolysis of peptide sulfinamides to produce the corresponding sulfinic acids.[24] We have observed that under physiological conditions only small amounts of our peptide sulfinamides are converted to the corresponding sulfinic acids after 26 h. Because of the relatively longer half-life of sulfinic acid formation, the reduction of sulfinic acids to thiols is not considered to be a probable pathway.[52,53] This is supported by the fact that no significant sulfinic acid formation is observed in the presence of DTT.

Previously, we have reported that *tert*-butanesulfinamide is unreactive with DTT at room temperature over 12 h.[20] Because *tert*-butanesulfinamide is extremely sterically hindered, we tested the reactivity of a more relevant small organic molecule sulfinamide, 2-phenylethanesulfinamide [PE-S(O)NH_2]. Upon incubation in thiol-containing buffer at 37 °C for 26 h, >90% reduction is observed, demonstrating that direct thiolysis (Scheme 2) is a viable mechanism at physiological pH and temperature. It should also be noted that sulfinic acid formation is not detected upon incubation of 2-phenylethanesulfinamide in the presence of DTT, indicating that direct thiolysis of this sulfinamide is more efficient than the corresponding hydrolysis reaction.

To learn more about the reactivity of sulfinamides, we have also investigated the effect of the solvent dielectric by employing different cosolvents. Our results with PE-S(O)NH_2 indicate that the direct thiolysis reaction is significantly inhibited as the solvent dielectric constant decreases. Moreover, we have shown that in lower-dielectric constant environments peptide sulfinamides are more reactive toward reduction by DTT compared with PE-S(O)NH_2. A possible explanation for the observed difference in reactivity is that in the case of the peptide sulfinamides there is a contribution from cyclic intermediate **1**, which becomes more significant upon inhibition of the direct thiolysis pathway. This explanation is further supported by the observed sequence dependence (analogous to that for the asparagine deamidation reaction[27]) that is manifested only under low-dielectric constant conditions. On the basis of the overall results, it can also be inferred that the solvent dielectric has a larger impact on the direct thiolysis mechanism (Scheme 2) than on the mechanism involving cyclic intermediate **1** (Scheme 4), although both mechanisms are affected. Our results are consistent with a recent computational study in which the energetics and feasibilities of several asparagine deamidation pathways were compared.[54] These computational results indicate that direct hydrolysis is a competitive reaction with the imide-mediated deamidation reaction even in the absence of acid or base catalysis.[54]

Considering that the half-life of proteins varies from <3 min to >20 h in vivo,[55−58] the time scales presented here are relevant to cellular proteins (e.g., for GAPDH, $t_{1/2}$ = 38.1 h).[59] Moreover, our results in lower-solvent dielectric constant media indicate that sulfinamide reduction can take place in hydrophobic as well as hydrophilic regions of proteins. This may also be particularly relevant to HNO-targeted proteins containing cysteines in their hydrophobic domains such as the regulatory protein of the sarcoplasmic reticulum Ca^{2+} pump, phospholamban.[20] Because the irreversible inhibition of proteins with critical thiol residues has been hypothesized to have detrimental effects on lysosomal protein degradation, cell cycle, and energy metabolism at the glycolytic level,[11,14,60] the reduction of a sulfinamide modification is relevant to the use of HNO as a therapeutic agent. It should be noted that the peptides used in this study do not have well-defined secondary structure and that the presence of higher-order structures may very likely affect reaction rates.

Papain is a well-studied cysteine protease containing three disulfide bonds and a single active site cysteine residue,[49,50] which is prone to inhibition presumably due to HNO-induced sulfinamide formation.[11] In a previous study, AS-treated papain was incubated with DTT for a short period of time (1 h) and some recovery in the activity was observed.[11] Because sulfinamide reduction was previously thought to be unlikely, this recovery was attributed to the reduction of a possible disulfide modification. We have observed that the time frames for the reduction of disulfides (<10 min) and sulfinamides (several hours) are significantly different. Although we have not yet directly characterized the modification, on the basis of the results reported here, we suggest that the HNO-derived sulfinamide in papain can be reduced back to free thiol in the presence of reducing agents, indicating the feasibility of this reaction in a protein environment.

■ CONCLUSIONS

We have demonstrated that HNO-derived sulfinamides can revert to free thiols under physiologically relevant conditions. In the presence of reducing agents, this reaction is feasible in small organic molecules, peptides, and proteins. Although both peptide and small organic molecule sulfinamides are susceptible to hydrolysis, this reaction is slower than reduction by thiols. Our results suggest that the mechanism of peptide sulfinamide reduction involves a contribution from a cyclic intermediate, whose relative impact becomes more significant in environments with lower dielectric constants. Considering the broad spectrum of pharmacological effects attributed to HNO,[5,6,60] these findings are relevant to the study of HNO-induced modifications in biological systems.

■ ASSOCIATED CONTENT

Supporting Information

X-ray crystallographic information for 2-phenylethanesulfinamide, ESI-MS spectra for the deamidation of VYPNGA and VYPNLA, and additional information about the reduction of peptides, 2-phenylethanesulfinamide and disulfide-modified papain. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

John P. Toscano is a co-founder, stockholder, and serves on the Scientific Advisory Board of Cardioxyl Pharmaceuticals.

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■ ABBREVIATIONS

ACN, acetonitrile; AS, Angeli's salt; BME, β-mercaptoethanol; 2-BrPA, 2-bromo-*N*-hydroxybenzenesulfonamide; DTPA, diethylenetriaminepentaacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; CG-SH, VYPCGA (free thiol form); CG-S(O)NH_2, VYPCGA-derived sulfinamide; CG-S(O)OH, VYPCGA-derived sulfinic acid; CL-SH, VYPCLA (free thiol form); CL-S(O)NH_2, VYPCLA-derived sulfinamide; GSH, glutathione; GS(O)NH_2, glutathione-derived sulfinamide; HPLC, high-pressure liquid chromatography; L-BAPNA, N_α-benzoyl-L-arginine 4-nitroanilide hydrochloride; NBS, *N*-bromosuccinimide; OPA, *o*-phthalaldehyde; PE-S(O)NH_2, 2-phenylethanesulfinamide; TFA, trifluoroacetic acid; TIC, total ion count.

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