Reactive Sulfur Species: Kinetics and Mechanism of the Hydrolysis of Cysteine Thiosulfinate Ester

ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · OCTOBER 2007

Impact Factor: 3.53 · DOI: 10.1021/tx700168z · Source: PubMed

Reactive Sulfur Species: Kinetics and Mechanism of the Hydrolysis of Cysteine Thiosulfinate Ester

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Received May 16, 2007

The kinetics and mechanisms of the hydrolysis of cysteine thiosulfinate ester ($CyS(=O)SCy^{x-}$, x = 0–2) have been investigated by stopped-flow spectrophotometry between pH 6 and pH 14. The rate-limiting reaction of hydroxide is observed for pH < 13. More complicated kinetics are observed above pH 13, where the hydrolysis of $CyS(=O)SCy^{2-}$ can be fast relative to subsequent reactions. The eventual products of hydrolysis are a 1:1 molar ratio of cystine (CySSCy) and cysteine sufinic acid ($CySO_2H$) under all reaction conditions. The rate of hydrolysis is dependent upon the proton state of $CyS(=O)SCy^{x-}$. Furthermore, cysteine thiosulfonate ester ($CyS(=O)_2SCy$) was observed as an intermediate during the hydrolysis of $CyS(=O)SCy^{x-}$ at lower pH. $CyS(=O)_2SCy$ eventually hydrolyzes to give stoichiometric amounts of CySSCy and $CySO_2H$. However, $CySO_2H$ is observed under some conditions for which hydrolysis of $CyS(=O)_2SCy$ is relatively slow, thus suggesting multiple hydrolysis pathways for $CyS(=O)SCy^{x-}$. The mechanism up to the rate-limiting step is proposed to be as follows: $CyS(=O)SCy^0 = H^+ + CyS(=O)SCy^-$, $pK_{a3} = 7.32$; $CyS(=O)SCy^- = H^+ + CyS(=O)SCy^{2-}$, $pK_{a4} = 7.92$; $CyS(=O)SCy^0 + OH^- \rightarrow$ products, $P_0k_0 = (5.0 \pm 0.01) \times 10^3\ M^{-1}\ s^{-1}$; $CyS(=O)SCy^- + OH^- \rightarrow$ products, $P_1k_1 = 60 \pm 18\ M^{-1}\ s^{-1}$; and $CyS(=O)SCy^{2-} + OH^- \rightarrow$ products, $P_2k_2 = 0.36 \pm 0.01\ M^{-1}\ s^{-1}$, where P_x is a constant ($1 \le P_x \le 3$) that accounts for the partitioning between the possible hydrolysis pathways and the stoichiometries of their net reactions.

Introduction

Although it has been previously assumed that the one-electron couple between thiol and disulfide is the only redox chemistry of biological relevance, it has been recognized for more than 30 years that cysteine residues in proteins can be "overoxidized" beyond the disulfide form under conditions of oxidative stress (*1*). More recently, chemical and crystallographic evidence has been introduced for functional protein sulfenic acid moieties for native proteins, for example, nicotinamide adenine dinucleotide (reduced form) (NADH)[1] peroxidase (*2*), NADH oxidase (*3*), nitrile hydratase (*4*), both classes of methionine sulfoxide reductases (*5*), and certain peroxiredoxins (*6–10*). Furthermore, sulfenic acid derivatives have been implicated in the redox status regulation of certain cellular functions. In contrast to the relatively stable sulfenic derivatives of cysteine that have been identified in proteins, the parent compound remains elusive. In fact, of the common oxidized derivatives of cysteine (Scheme 1), only cysteine sulfenic acid (CySOH)[2] has not been isolated. With the exception of a few derivatives that are stabilized through steric hindrance, H-bonding, or conjugation (*11–16*), sulfenic acids are generally considered to be transient species (*17*). One reason that CySOH exhibits only fleeting existence is that its condensation to give the corresponding cysteine thiosulfinate ester, CyS(=O)SCy, is a facile process:

Scheme 1. Nomenclature and Oxidation States of Common Derivatives of Cysteine

	CySH	CySOH	$CySO_2H$	$CySO_3H$
ox state	-2	0	+2	+4
name	thiol	sulfenic acid	sulfinic acid	sulfonic acid
	CySSCy	CySSCy (O)		CySSCy (O, O)
ox state	-1/-1	+1/-1		+3/-1
name	disulfide	thiosulfinate ester		thiosulfonate ester

$$2CySOH \rightleftharpoons CyS(=O)SCy + H_2O \quad (1)$$

The CySOH/thiosulfinate ester equilibrium is unique amongst acid/anhydride equilibria in that the anhydride is thermodynamically favored over the acid.

From a biological perspective, thiosulfinate esters are known to induce redox modification of proteins (*18, 19*), and there are several natural products that contain thiosulfinate moieties. The most often studied thiosulfinate ester is allicin (Scheme 2), one of the components of garlic (*20–22*) that is believed to exhibit antibacterial (*23–26*), antifungal (*27–30*), antithrombotic (*22, 31, 32*), cholesterol-lowering (*33, 34*), antineoplastic (*31, 35–38*), and hepatoprotective (*39*) activities. Another example of a naturally occurring thiosulfinate is leinamycin (Scheme 2), an antineoplastic compound that was first isolated from a strain of *Streptomyces* (*40*). The antibiotic and cytotoxic properties of allicin and leinamycin are often associated with their abilities to react rapidly with thiols, which is a chemical property that

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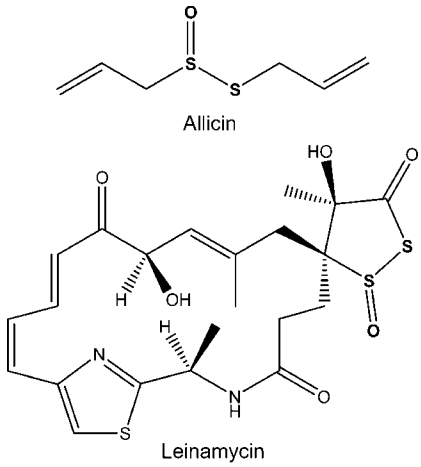
[1] Abbreviations: CySH, cysteine acid; CySOH, cysteine sulfenic acid; CySSCy, cystine; CyS(=O)SCy, cysteine thiosulfinate ester; $CyS(=O)_2SCy$, cysteine thiosulfonate ester; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; EI, electron impact; iP, inorganic phosphate ($H_3PO_4 + H_2PO_4^- + HPO_4^{2-} + PO_4^{3-}$); NADH, nicotinamide adenine dinucleotide (reduced form); PMT, photomultiplier tube; SVD, singular-value decomposition; TOF MS ES+, time-of-flight electrospray (positive ion mode) mass spectrometry.

[2] References to compounds without charges are inclusive of all acid/base derivatives.

10.1021/tx700168z CCC: $37.00
Published on Web 09/01/2007

Scheme 2. Biologically Active Thiosulfinate Esters



is shared by all thiosulfinate derivatives, including the cysteine derivative that is the subject of the present study. It is noteworthy that allicin is unstable with respect to hydrolysis, and this is apparently why the enzyme alliinase (EC 4.4.1.4) and the precursor alliin (*S*-allyl-L-cysteine sulfoxide) are compartmentalized in intact garlic cloves, and allicin is only produced upon commingling of the enzyme and the precursor when the cloves are damaged (crushed). Allicin occurs exclusively in plants, and comparatively little is known about the formation and possible actions of thiosulfinate esters in animals. However, redox cascades that include CyS(=O)SCy have been proposed to play a role in human health (*18, 41–43*). To garner a better understanding of the chemical properties of CyS(=O)SCy in aqueous media, we report herein the kinetics and mechanism of its hydrolysis. This is apparently the first mechanistic study of the hydrolysis of a thiosulfinate ester at physiologic pH.

Materials and Methods

Reagents. All chemicals were ACS-certified grade or better. Water was doubly distilled in glass. Solutions of NaOH, mostly free of CO_2 contamination, were quantified by titration with potassium hydrogen phthalate or standardized HCl or $HClO_4$ solutions using phenolphthalein as an indicator. $HClO_4$ and HCl were standardized against bicarbonate. The buffer solutions were prepared from the solids K_3PO_4, $NaH_2PO_4{\cdot}H_2O$, Na_2HPO_4, and $Na_3PO_4{\cdot}12H_2O$; the ionic strength was adjusted with $NaClO_4$; and the pH/pD was adjusted with NaOH, NaOD, $HClO_4$, or DCl. L-Cystine (CySSCy), L-cysteine, L-cysteic acid monohydrate, L-cysteinesulfinic acid monohydrate, peracetic acid, deuterium chloride (35 wt % solution in D_2O), NaOD (40 wt % solution in D_2O), $NaClO_4$, and K_3PO_4 were used as received from Sigma-Aldrich. $NaH_2PO_4{\cdot}H_2O$, Na_2HPO_4, and $Na_3PO_4{\cdot}12H_2O$ were used as received from Mallinckrodt. Deuterium oxide (99.9%) was obtained from Cambridge Isotope Laboratories. The synthesis of CyS(=O)SCy was accomplished using a published procedure (*44*). ^{1}H NMR of the CyS(=O)SCy showed some CySSCy impurity (ca. 15 %).

Synthesis of Cysteine Thiosulfonate Ester, $CyS(=O)_2SCy$. $CyS(=O)_2SCy$ was synthesized by the hydrolysis of CyS(=O)SCy (104 mM) in 0.2 M DCl. When 99% of the CyS(=O)SCy was hydrolyzed, the product distribution was 42 mM $CyS(=O)_2SCy$, 74 mM CySSCy, and 13 mM $CySO_2H$ (based on the integration of the ^{1}H NMR spectrum). The extra 19 mM CySSCy was derived from an impurity in the CyS(=O)SCy starting material. Aliquots of 8 M NH_4OH were added to the reaction mixture at 1 °C to precipitate the product as a white solid (*45*). The final pH of the solution was ca. 4. The precipitate was isolated by filtration and dried under vacuum. The composition of the solid was determined by ^{1}H NMR (after dissolution in 1.0 M DCl) to be 68% CySSCy and 32% $CyS(=O)_2SCy$.

pH/pD Measurements. The $[OH^-]$ for the unbuffered solutions was determined by acid–base titration against standardized HCl or standardized $HClO_4$ solutions. The $[H^+]$ of the buffered solutions was determined with an Orion-Ion Analyzer EA920 using an Ag/AgCl combination pH electrode. The ionic strength was kept constant at 1.0 M for all H_2O solutions [$NaClO_4$ + $HClO_4$/NaOH + inorganic phosphate (iP) + borate + TRIS]. To obtain the $[H^+]$ or $[OH^-]$ of the buffered solutions from the measured pH values, all pH measurements were corrected for the "Irving factor" (*46*) and the ionic product of water (pK_w) that was measured by titration of a 1.0 M $NaClO_4$ solution by a standardized 0.1 M NaOH (in 1.0 M $NaClO_4$) solution. pD measurements in D_2O were made using the same pH electrode by adding 0.4 units to the measurement. (*47*).

NMR Studies. ^{1}H NMR spectra were recorded with a Varian XL-300 spectrometer at 20 (±0.5) °C. Deuterated buffers were prepared from D_2O solutions of anhydrous K_3PO_4 by adding DCl, by dilution of a 40 wt % NaOD solution with D_2O, or by dilution of a 35 wt % DCl solution with D_2O. The chemical shifts (ppm) were referenced to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS, $\delta = 0.015$ ppm).

UV Spectroscopy. Electronic spectra were measured using a HP 8452A diode array spectrophotometer using quartz cells with calibrated 1 mm, 2 mm, and 1 cm path lengths at 20 °C or the monochromator of the HI-TECH SF-61 DX2 stopped-flow instrument with a Xe arc lamp at 18 °C.

Mass Spectrometry. A VG ZAB SE two-sector mass spectrometer was employed to measure the electron impact (EI) spectra, and a Micromass Q-TOF hybrid mass spectrometer was used to measure the time-of-flight electrospray mass spectra of CyS(=O)SCy and $CyS(=O)_2SCy$ in positive ion mode (TOF MS ES+). These MS spectra were consistent with the respective formulations.

Measurement of the Acid Dissociation Constants of CySSCy and CyS(=O)SCy. The two ammonium acid dissociation constants of CySSCy were measured by potentiometric titration using an Orion-Ion Analyzer 8103 Ross with an Ag/AgCl combination pH electrode that was refilled with 1.0 M NaCl (to avoid possible precipitation of $KClO_4$ inside the electrode). The electrode was calibrated using potassium-borate/carbonate (pH 10.00) and potassium-hydrogen-phthalate (pH 4.00) buffers. Gilson micropipettes were used for the base additions. The titrant, an 80.9 mM $HClO_4$ (in 1.0 M $NaClO_4$) solution, was standardized with a 0.1 M NaOH solution. The titration was performed in a 1.0 M $NaClO_4$ solution to maintain ionic strength, at 25.0 °C. The acid dissociation constants, which were calculated from the potentiometric titration data (58 data points) that were obtained at 1.97 mM concentration (50 mL) in the pH range of 6.2–11.7, were evaluated using the program PSEQUAD (*48*).

The two ammonium acid dissociation constants of CyS(=O)SCy were measured by determining the change in the UV spectra and the ^{1}H NMR spectra as a function of pH at 20 °C and $I = 1.0$ M ($NaClO_4$). To minimize decomposition of the CyS(=O)SCy stock solution, a pH jump method was used to carry out these experiments. A CyS(=O)SCy stock solution (0.6 and 40 mM for the UV and the ^{1}H NMR experiments, respectively) in $[HClO_4] = 0.5$ M and $[NaClO_4] = 0.5$ M was rapidly mixed with a buffer solution ([iP] = 0.1 M, [borate] = 0.1 M, [NaOH] = 0.5 M, and $[NaClO_4]$ = 0.4–0.6 M yield a $I = 1.0$ M solution at the desired pH after mixing), and the spectra were recorded within 10 min or 20 s for the ^{1}H NMR and UV spectra, respectively. No significant decomposition of the CyS(=O)SCy stock solution at $[HClO_4] = 0.5$ M was observed in 6 h. The adiabatic temperature increases that were associated with the pH jump were determined experimentally to be less than 1 °C. The pH (after the pH jumps) was adjusted by adding aliquots of $HClO_4$ (9.1 M) or NaOH (3.1 M) to the buffer solution before mixing. The pH values of the solutions that were produced after mixing were determined immediately after the spectra were recorded. For the UV measurement, the conditions after mixing were as follows: $[CyS(=O)SCy]_0 = 0.3$ mM, [CySSCy] = 0.048 mM (an impurity), [iP] = 0.05 M, [borate] = 0.05 M, $I = 1.0$ M, and pH = 3.04–11.10. The UV spectra of the

appropriate amount of CySSCy and buffers were also measured for each data point under similar conditions. These UV spectra were subtracted as background, a baseline correction was applied, and the acid dissociation constants were obtained by fitting the data using SPECFIT 3.0.36 global analysis software (Spectrum Associates, Chapel Hill, NC). For the ^{1}H NMR measurements, the conditions after mixing were as follows: $[CyS(=O)SCy]_0 = 20$ mM, [CySSCy] = 3.2 mM (an impurity), [iP] = 0.05 M, [borate] = 0.05 M, $I = 1.0$ M, and pH = 5.99–9.74. The acid dissociation constants were evaluated by fitting the ^{1}H NMR chemical shifts (of a doublet of doublets that corresponds to one of the β protons of CyS(=O)SCy) vs pH data set using the program PSEQUAD (*48*).

Kinetic Measurements of the Hydrolysis of CyS(=O)SCy. For fast reactions, kinetic measurements were made with a HI-TECH SF-61 DX2 stopped-flow spectrophotometer using a Xe arc lamp and a photomultiplier tube (PMT) detector. During all of the stopped-flow measurements, a temperature of 18 °C was maintained in the observation cell with a Lauda RC-20 circulator. The adiabatic temperature increases that were associated with the pH jump experiments were determined experimentally to be less than 1 °C. The pH dependencies of the reaction rates at pH > 12 were investigated in single-mixing stopped-flow experiments. The $[H^+]$ of the CyS(=O)SCy stock solutions were maintained at 0.5 M to reduce the rate of hydrolysis. The pH at time zero was achieved by mixing with the appropriate amount of NaOH. The ionic strength of the reaction mixtures was kept constant by adding the same amount of $NaClO_4$ to the NaOH and CyS(=O)SCy solutions. When the hydrolysis reaction was sufficiently slow (below pH 12), kinetic data were measured with a HP 8452A diode array UV–vis spectrophotometer using buffered solutions. Turbulent mixing was achieved using a hand mixer that was comprised of two Hamilton syringes that were connected to a T-mixer or by addition of the reagents while vortexing the solution. The monochromatic kinetic traces were fit with HI-TECH KinetAsyst 3.14 software (Hi-Tech, United Kingdom). Polychromatic data were analyzed using SPECFIT/32 (Spectrum Software Associates), a multivariate data analysis program. The concentration dependencies of the pseudo-first-order rate constants were obtained by linear least-squares fits of the data with KaleidaGraph 3.6 (Synergy Software).

Results

Kinetics of the Hydrolysis of CyS(=O)SCy. The rate of hydrolysis of CyS(=O)SCy was studied in the 6–14 pH range. For fast reactions, the kinetic traces were measured using stopped-flow methods by following the depletion of CyS(=O)SCy monochromatically. Polychromatic UV spectra were assembled from individual time traces that were measured at different wavelengths. For sufficiently slow reactions, time-resolved spectra were collected with a HP 8452A diode array UV–vis spectrophotometer. The pseudo-first-order rate constants were obtained by fitting the polychromatic spectral data using singular-value decomposition (SVD) analysis (Figure 1). All kinetic traces fit well to single exponential equations, which indicates that the rate of hydrolysis exhibits a first-order dependency on $[CyS(=O)SCy]_0$. The rate of hydrolysis also exhibits a first-order dependency on $[OH^-]$ between pH 10 and pH 14 (Figure 2). The reaction begins to deviate from its first-order dependency on $[OH^-]$ under more acidic conditions (Figure 2, inset, and Figure 5), and hydrolysis is no longer rate limiting at pH > 13 (vide infra).

Measurement of the Acid Dissociation Constants of CySSCy and CyS(=O)SCy. The two ammonium acid dissociation constants of CyS(=O)SCy were measured by UV (Figure 3) and ^{1}H NMR spectroscopy (Figure 4) at 20 °C and $I = 1.0$ M ($NaClO_4$). Because of the instability of the species (relatively rapid hydrolysis for the pH range that was investigated), a pH jump method was used to carry out these

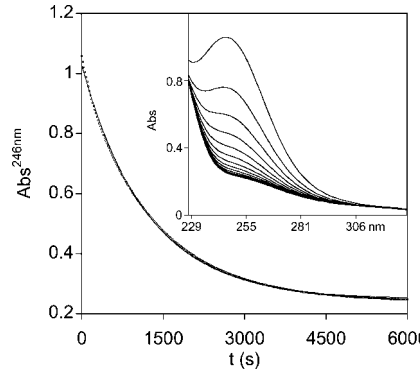


Figure 1. Change in absorbance that is observed at a representative wavelength (246 nm) upon the pH jump of 0.6 mM CyS(=O)SCy from $[H^+] = 0.5$ M to pH 7.44 together with the corresponding fit. The UV spectra were recorded at time intervals of 20 s, but for clarity, the transient spectra are illustrated at intervals of 480 s in the inset. Fit of these data to a first-order rate law with SVD analysis of the entire UV spectrum (inset) yields a k_{obs} of $(8.10 \pm 0.01) \times 10^{-4}$ s^{-1}. Conditions: $[CyS(=O)SCy]_0 = 0.6$ mM, [CySSCy] = 0.096 mM (from the impurity of the authentic ester sample), [iP] = 0.1 M, $I = 1.0$ M ($NaClO_4$ + iP), pH = 7.44, and $T = 20$ °C.

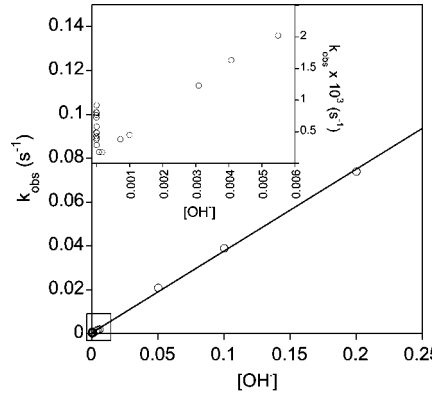


Figure 2. Effect of $[OH^-]$ on the hydrolysis of CyS(=O)SCy. Kinetic traces were collected at $\lambda = 260$ nm. The data in the box of the main figure are expanded in the inset. Conditions: combination of two sets of data (i) at $[OH^-] \geq 0.05$ M: $[CyS(=O)SCy^{2-}]_0 = 0.25$ mM, [CySSCy] = 0.04 mM (from the impurity of the authentic ester sample), $[OH^-] = 0.05$–0.2 M, $I = 1.0$ M ($NaClO_4$ + NaOH), and $T = 18$ °C and (ii) $[CyS(=O)SCy^{2-}]_0 = 0.6$ mM, [CySSCy] = 0.096 mM (from the impurity of the authentic ester sample), pH = 6.5–12 ([iP] or [TRIS] = 0.1 M), $I = 1.0$ M (iP/TRIS + $NaClO_4$), and $T = 20$ °C.

experiments (see Materials and Methods). The data fit well to an equation using only one floating parameter by the nonlinear least squares method in both cases resulting in $pK_a = 7.62 \pm 0.01$ for the NMR data and 7.62 ± 0.05 for UV data.

Products of the Hydrolysis of CyS(=O)SCy. We have previously reported that the hydrolysis of CyS(=O)SCy ultimately produces a 1:1 molar ratio of $CySO_2H$ and CySSCy at pH 11.3 (*49*). The same products in the same ratio are observed between pH 10 and pH 14. At pH < 10, the formation of an unsymmetrical dimer (as indicated by the ^{1}H NMR spectra of the reaction mixture) was also observed as an intermediate. An authentic sample of this unsymmetrical dimer was obtained by precipitating it (together with CySSCy) from a 0.2 M DCl solution by adding NH_4OH. The spectroscopic signatures of the species (^{1}H NMR, IR (*45, 50*), EI MS, and TOF MS ES+) are consistent with the formulation $CyS(=O)_2SCy$. To further evidence the formation of the unsymmetrical dimer, the stoichiometry of its hydrolysis was investigated by ^{1}H NMR spectroscopy. A pH jump of the dimer to pH 13 resulted in

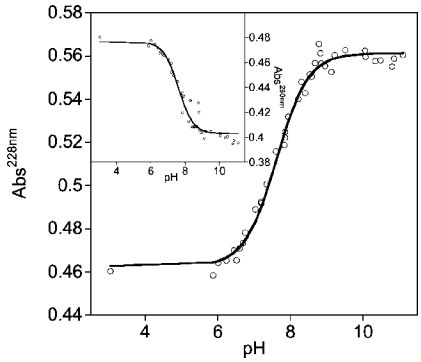


Figure 3. Change in the absorbance of the spectrum of CyS(=O)SCy at 228 and 260 nm (inset) as a function of pH (circles) and the fit that is obtained by floating only one parameter. The calculated pK_a is 7.62 ± 0.05. Conditions: [CyS(=O)SCy]$_0$ = 0.3 mM, [CySSCy] = 0.048 mM (from the impurity of the authentic ester sample), [iP] = 0.05 M, [borate] = 0.05 M, I = 1.0 M ($NaClO_4$ + iP + borate), pH = 3.04–11.10, and T = 20 °C.

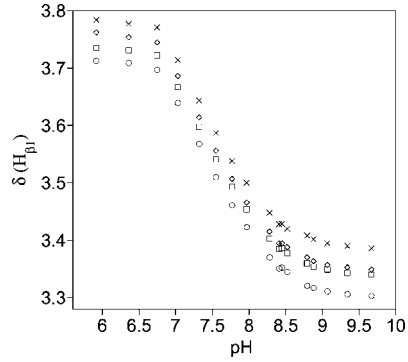


Figure 4. ^{1}H NMR titration curves of CyS(=O)SCy showing the change in the chemical shifts of a doublet of doublets that corresponds to one of the β protons as a function of pH. The calculated pK_a is 7.62 ± 0.01. Conditions: [CyS(=O)SCy]$_0$ = 20 mM, [CySSCy] = 3.2 mM (from the impurity of the authentic ester sample), [iP] = 0.05 M, [borate] = 0.05 M, I = 1.0 M ($NaClO_4$ + iP + borate), pH = 5.99–9.74, and T = 20 °C.

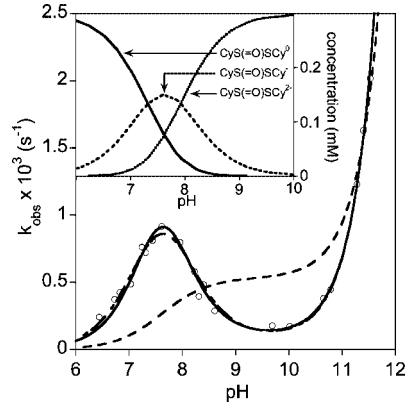


Figure 5. Observed pseudo-first-order rate constants for the hydrolysis of CyS(=O)SCy (circles) and the calculated distribution of the different protonated forms of CyS(=O)SCy for pK_{a1} = 7.32 and pK_{a2} = 7.92 (inset) as a function of pH. Polychromatic data were collected in the 218–400 nm range. The data were fit using SVD analysis of the entire spectrum. Fits using the equation for the two K_a model in Scheme 3 where pK_{a1} = pK_{a2} = 7.62 (solid) and pK_{a1} = 7.32 and pK_{a2} = 7.92 (dot-dashed) are illustrated. A fit for the one K_a model of Scheme 3 is illustrated as a dashed line. Conditions: [CyS(=O)SCy]$_0$ = 0.6 mM, [CySSCy] = 0.096 mM (from the impurity of the authentic ester sample), [iP] or [TRIS] = 0.1 M, I = 1 M ($NaClO_4$ + iP/TRIS), pH = 6.44–11.53, and T = 20 °C.

rapid formation of a 1:4 mixture of CySSCy and $CySO_2H$. Hence, the stoichiometry of the hydrolysis reaction is

$$3CyS(=O)_2SCy^{2-} + 2OH^- \rightarrow 4CySO_2^{2-} + CySSCy^{2-} + 2H^+ \quad (2)$$

The formulation of $CyS(=O)_2SCy$ was further confirmed by determining the product of its reaction with cysteine acid (CySH). A solution of the unsymmetrical dimer was titrated with 1–6 molar equivalent of CySH at [DCl] = 1.15 M in the presence of a known amount of the internal standard DSS. The stoichiometry of the net reaction was found to be 1:1 CyS(=O)SCy:CySH (to produce 1:1 $CySO_2H$:CySSCy) as expected for the following redox balance:

$$CyS(=O)_2SCy + CySH \rightarrow CySO_2H + CySSCy \quad (3)$$

Thus, we conclude the unsymmetrical dimer is indeed $CyS(=O)_2SCy$.

As noted earlier, the only products of the hydrolysis of CyS(=O)SCy above pH 10 are $CySO_2H$ and CySSCy. However, $CyS(=O)_2SCy$ is observed as a transient species when the hydrolysis reaction of CyS(=O)SCy is carried out below pH 10. While the final products of the hydrolysis reactions are always a stoichiometric amount of $CySO_2H$ and CySSCy, the amount of intermediate $CyS(=O)_2SCy$ increases with respect to $CySO_2H$ until it becomes the dominant species when the pH is less than 6 (after five half-lives of the hydrolysis reaction of CyS(=O)SCy and before the subsequent hydrolysis of $CyS(=O)_2SCy$ occurs) To address the issue of whether $CySO_2H$ forms directly from the hydrolysis of CyS(=O)SCy or vis-à-vis the intermediate $CyS(=O)_2SCy$, we carried out the following experiment: Solutions of CyS(=O)SCy (5 mM) and $CyS(=O)_2SCy$ (8.8 mM) in 0.2 M DCl were stored at 20 °C in the dark, and the ^{1}H NMR spectra of the reaction mixtures were recorded periodically. The thiosulfinate ester CyS(=O)SCy was observed to hydrolyze more rapidly than the thiosulfonate ester $CyS(=O)_2SCy$ to produce a mixture of $CySO_2H$, CySSCy, and $CyS(=O)_2SCy$ (vide supra). In contrast, $CyS(=O)_2SCy$ hydrolyzed more slowly to produce $CySO_2H$ and CySSCy. Importantly, no measurable $CySO_2H$ was produced by the hydrolysis of $CyS(=O)_2SCy$ in the timeframe when a significant amount of $CySO_2H$ was produced by the hydrolysis of CyS(=O)SCy. Hence, this side-by-side comparison demonstrates that (i) the $CySO_2H$ was produced by the hydrolysis of CyS(=O)SCy (and also subsequently by the more slow hydrolysis of $CyS(=O)_2SCy$) and (ii) $CyS(=O)_2SCy$ is a relatively stable intermediate of the hydrolysis of CyS(=O)SCy at pH < 7. This result suggests the operation of parallel reaction pathways (vide infra).

Discussion

Observed and Theoretical Products of the Hydrolysis Reaction of CyS(=O)SCy. If it is assumed that the elementary reactions that cause the interconversions of the species in Scheme 1 are bimolecular (i.e., termolecular reactions are unlikely), it is possible to compute the number of pairwise combinations for n = 7 objects, where the order is unimportant and allowing each object to pair with itself, using the formula:

$$\frac{n!}{2\times(n-2)!} + n = \frac{7!}{2\times 5!} + 7 = 28 \quad (4)$$

In addition, we must add three hydrolysis reactions (for CySSCy, CyS(=O)SCy, and $CyS(=O)_2SCy$) for a total of 31 pairs of reactants. The products that are obtained from these bimolecular reactions must conserve the mass and the number of electrons (i.e., there must be redox balance). An analysis of these possible

combinations produces 34 reactions that meet these requirements. Thus, there is a bewildering collection of possible reaction pathways. These pathways include two different elementary hydrolysis steps for CyS(=O)SCy. The first is the reverse of eq 1, a reaction that produces two molar equivalents of CySOH:

$$\mathrm{CyS(=O)SCy + H_2O \rightarrow 2CySOH} \quad (5)$$

Alternatively, the first products of the hydrolysis could be:

$$\mathrm{CyS(=O)SCy + H_2O \rightarrow CySO_2H + CySH} \quad (6)$$

With respect to the relatively slow hydrolysis reaction, CySH and CySOH are both reactive species that can eventually produce $CySO_2H$ and CySSCy, the products that are observed above pH 10. Below pH 10, $CyS(=O)_2SCy$ is observed as an intermediate. Like CyS(=O)SCy, $CyS(=O)_2SCy$ is an electrophilic species that is subject to hydrolysis and other reactions that involve nucleophiles (e.g., CySH). The products of the decomposition reactions of $CyS(=O)_2SCy$ are identical to those of CyS(=O)SCy (i.e., $CySO_2H$ and CySSCy are eventually formed, albeit in different molar ratios than when CyS(=O)SCy hydrolyzes). Because our observations are consistent with multiple reaction pathways for the hydrolysis reaction of CyS(=O)SCy, we focus our attention initially on the information that is provided by equilibrium and kinetics measurements (i.e., the reactions that lead up to rate-limiting hydrolysis of CyS(=O)SCy). Later, we will discuss the possible reactions that follow the rate-limiting step.

Acid Dissociation Constants of CySSCy and CyS(=O)SCy. Fitting the data in Figures 3 and 4 with only one floating parameter resulted in $pK_{a3} = pK_{a4} = 7.62$. The ^1H NMR data cannot be fit using two parameters. Fitting the UV data using two parameters results in a larger uncertainty as compared to when only one parameter is floated. The fact that only one inflection point is observed during the titration of CyS(=O)SCy between pH 3 and pH 11, which is a range that should encompass both of the acid dissociation constant values for the ammonium groups (i.e., $CyS(=O)SCy^0 \rightleftharpoons CyS(=O)SCy^{-1} \rightleftharpoons CyS{=}OSCy^{2-}$), suggests that the acid dissociation constants are essentially independent of one another. For a diprotic acid that has two identical (microscopic) dissociation sites, there is a statistical factor of 4 between the macroscopic ionization constants that corresponds to $pK_{a4} - pK_{a3} = \log(4) \approx 0.6$ (which is a difference that we are not likely to be able to resolve in our titration curves) (*51*). Accordingly, we assign the measured pK_a to the mean value:

$$\overline{pK_a} = \frac{pK_{a3} + pK_{a4}}{2} = \frac{7.32 + 7.92}{2} = 7.62(1) \quad (7)$$

For the sake of comparison, we have measured the corresponding acid dissociation constants of CySSCy by potentiometric titration in 1.0 M $NaClO_4$ and 20 °C. The two acid dissociation constants that were obtained (8.30 ± 0.01 and 8.88 ± 0.02) are comparable with the previously reported values at 1.0 M NaCl ionic strength and 25 °C (8.29 ± 0.01 and 8.88 ± 0.01) (*52*).

Kinetics and Mechanisms of the Hydrolysis of CyS(=O)SCy. The hydrolysis of CyS(=O)SCy is rate determining below ca. pH 13, as indicated by the observed first-order kinetic traces that are observed when the $[OH^-]$ is buffered (Figure 1). The pseudo-first-order rate constants that were measured between pH 10 and pH 13 exhibit a first-order dependence on $[OH^-]$ (Figure 2). In this pH range, CyS(=O)SCy is fully deprotonated. Accordingly, our observations establish the following rate law between pH 10 and pH 13:

$$\mathrm{rate} = \frac{\mathrm{d}[CyS(=O)SCy^{2-}]}{\mathrm{d}t} = P_2 k_2 [CyS(=O)SCy^{2-}][OH^-] \quad (8)$$

where k_2 is the rate constant for the reaction of $CyS(=O)SCy^{2-}$ with OH^- and P_2 is a proportionality constant ($1 \leq P_x \leq 3$, vide infra). The slope of the linear fit of the data between pH 10 and pH 13 in Figure 2 yields $P_2k_2 = 0.376 \pm 0.003$ $M^{-1}\ s^{-1}$.

At pH > 13, the observed kinetic traces begin to deviate from single exponential behavior. For example, at low $[CyS(=O)SCy]_0$ (<~0.1 mM), double exponential curves were observed at pH 14, where the first (faster) reaction fits to a single exponential equation and, after correcting for the $[OH^-]$, results in a second-order rate constant that is similar to P_2k_2. At relatively high $[CyS(=O)SCy]_0$ (>~1 mM), the observed kinetic traces at 260 nm are best-described with a single second-order equation. However, polychromatic data suggest that the observed spectral changes are due to more than one reaction that serendipitously exhibit similar rates. We will focus the remainder of our discussion on the kinetics that are observed below pH 13.

The observed pseudo-first-order rate constants (k_{obs}) for the hydrolysis of CyS(=O)SCy begin to deviate from their first-order dependency on $[OH^-]$ below pH 10, and a bell-shaped profile is observed for plots of k_{obs} vs pH between pH 6 and pH 10 (Figure 5). The rate starts to increase below 10. The reaction kinetics exhibit an inflection point with a maximum rate at about pH 7.7, which is indicative of a competitive acid–base equilibrium. The rate decreases below pH 7.7, suggesting that OH^-, and not H_2O, is the reactive species. The observed increase in the rate below pH 10 suggests that the protonation of the amines of CyS(=O)SCy results in an increase in reactivity. The maximum of the pH profile is approximately located at the macroscopic $pK_a = 7.62$ that we have determined for CyS(=O)SCy. Because CyS(=O)SCy reacts as an electrophile, we interpret the increased reactivity of the protonated species as a charge effect. Furthermore, because the observed rate constants continue to decrease below pH 6, OH^- is still the reaction partner of CyS(=O)SCy under very acidic conditions. Thus, the half-life of the reaction at pH 0 is on the order of days under the experimental conditions that are given in the caption of Figure 5 (data not shown). The data for pH 6 < pH < 10 could not be fit using only one acid dissociation constant (which describes two reaction pathways as illustrated in the "One K_a Model" of Scheme 3) as shown by the dashed line in Figure 5. However, the data fit well to an equation that employs the two acid dissociation constants $pK_{a3} = 7.32$ and $pK_{a4} = 7.92$ (which produces three possible pathways as illustrated in the "Two K_a Model" of Scheme 3 and the dot-dashed line of Figure 5). It is noteworthy that the quality of the fit is relatively insensitive to the difference between pK_{a3} and pK_{a4} (for $0 \leq \Delta pK_a \leq 1$) as the use of a single value for the two acid dissociation constants (i.e., $pK_{a3} = pK_{a4} = 7.62$) produced an equally acceptable fit of the data (Figure 5, solid line). This observation further supports our conclusion that the two acid dissociation constants of CyS(=O)SCy are very close to one another. Because the rate has to be defined in terms of the change in the concentration of CyS(=O)SCy (the observable in the rate equations of Scheme 3) and the stoichiometry of the net reaction will depend upon the mechanism (including the rate steps that follow the rate-determining hydrolysis step

Scheme 3. Definitions of the Rate Laws for the Hydrolysis of CyS(=O)SCy

One K_a Model:

$$CyS(=O)SCy^0 = CyS(=O)SCy^- + H^+ \qquad K_{a3} = \frac{[CyS(=O)SCy^-][H^+]}{[CyS(=O)SCy^0]}$$

$$CyS(=O)SCy^0 + OH^- \rightarrow \text{products} \qquad k_0$$

$$CyS(=O)SCy^- + OH^- \rightarrow \text{products} \qquad k_1$$

$$\text{Rate} = \frac{-d[CyS(=O)SCy]_{tot}}{dt} = (P_0 \cdot k_0[H^+] + P_1 \cdot k_1 K_{a3}) \frac{[OH^-]}{[H^+] + K_{a3}}$$

Two K_a Model:

$$CyS(=O)SCy^0 = CyS(=O)SCy^- + H^+ \qquad K_{a3} = \frac{[CyS(=O)SCy^-][H^+]}{[CyS(=O)SCy^0]}$$

$$CyS(=O)SCy^- = CyS(=O)SCy^{-2} + H^+ \qquad K_{a4} = \frac{[CyS(=O)SCy^{2-}][H^+]}{[CyS(=O)SCy^-]}$$

$$CyS(=O)SCy^0 + OH^- \rightarrow \text{products} \qquad k_0 = (5.0 \pm 0.01)\times 10^3\ M^{-1}s^{-1}/P_o$$

$$CyS(=O)SCy^- + OH^- \rightarrow \text{products} \qquad k_1 = 60 \pm 18\ M^{-1}s^{-1}/P_1$$

$$CyS(=O)SCy^{2-} + OH^- \rightarrow \text{products} \qquad k_2 = 0.36 \pm 0.01\ M^{-1}s^{-1}/P_2$$

$$\text{Rate} = \frac{-d[CyS(=O)SCy]_{tot}}{dt} =$$

$$(P_0 \cdot k_0[H^+]^2 + P_1 \cdot k_1 K_{a3}[H^+] + P_2 \cdot k_2 K_{a3} K_{a4}) \frac{[OH^-]}{[H^+]^2 + K_{a3}[H^+] + K_{a3}K_{a4}}$$

that may consume additional molar equivalents of CyS(═O)SCy), a variable proportionality constant (P_x) has to be introduced to the rate equations: $P_0k_0 = (5.0 \pm 0.01) \times 10^3\ M^{-1}\ s^{-1}$, $P_1k_1 = 60 \pm 18\ M^{-1}\ s^{-1}$, and $P_2k_2 = 0.36 \pm 0.01\ M^{-1}\ s^{-1}$ (Scheme 3).

Proportionality Constant P_x. Although the rate law only describes the rate-limiting step (and the reversible steps that precede it), the overall mechanism should include the subsequent steps that produce the observed products. There are in fact a multitude of alternative pathways that could succeed the hydrolysis step (vide infra), and the rate law (Scheme 3) does not distinguish between them. Nonetheless, it is important to discuss the limiting mechanisms since their stoichiometries affect the definition of the rate constants we report herein (vide infra). From the perspective of the value of P_x, we need only concern ourselves here with those elementary reactions that change the [CyS(═O)SCy] (since this is the observable on which the rate laws of Scheme 3 are based). As a consequence of the fact that only nonradical pathways are considered here (i.e., all of the species in Scheme 1 are closed-shell compounds and we are dealing with group transfer reactions), P_x will take on only integer values for each discrete mechanism (although fractional values for P_x are possible by taking admixtures of multiple mechanisms). We have elected to use separate proportionality constants for the reactions that involve different states of protonation of CyS(═O)SCy because we do not know how protonation will affect the partitioning between the operative mechanisms.

The minimum value of P_x will be 1, which is the situation where no additional molar equivalents of CyS(═O)SCy are consumed after the rate-limiting step (P_x cannot be less than 1 since any mechanism that produces CyS(═O)SCy must eventually consume the same number of molar equivalents in the rate-limiting step). Only reactions that consume additional molar equivalents of CyS(═O)SCy after the rate-limiting step will increase the value of P_x. Two representative mechanisms that produce the observed products above pH 10 are illustrated in Scheme 4. The mechanism at the top of Scheme 4 does not consume any CyS(═O)SCy during the fast steps that follow

Scheme 4. Definition of the Proportionality Constant

$CyS(=O)SCy^{x-} + OH^- \rightarrow 2\ CySO^{x-} + (x-1)\ H^+$ rds

$2/3\{2\ CySO^{x-} \rightarrow CySO_2^{x-} + CyS^x\}$ fast

$2/3\{CySO^{x-} + CyS^{x-} + (x-1)\ H^+ \rightarrow CySSCy^{x-} + OH^-\}$ fast

---

$CyS(=O)SCy^{x-} + 1/3\ OH^- \rightarrow 2/3\ CySO_2^{x-} + 2/3\ CySSCy^{x-} + 1/3(x-1)\ H^+$

$$\text{Rate} = \frac{-d[CyS(=O)SCy]_{total}}{dt} \Rightarrow P_x = 1$$

$CyS(=O)SCy^{x-} + OH^- \rightarrow 2\ CySO^{x-} + (x-1)\ H^+$ rds

$2\{2\ CySO^{x-} \rightarrow CySO_2^{x-} + CyS^x\}$ fast

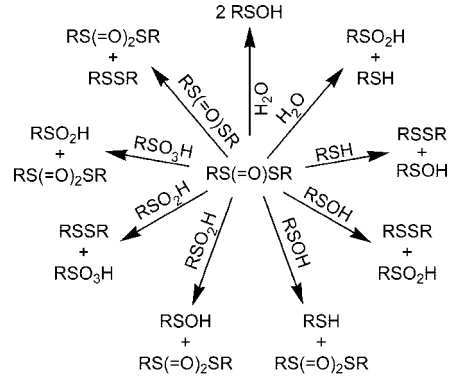
$2\{CyS(=O)SCy^{x-} + CyS^{x-} \rightarrow CySO^{x-} + CySSCy^{x-}\}$ fast

---

$3\ CyS(=O)SCy^{x-} + OH^- \rightarrow 2\ CySO_2^{x-} + 2\ CySSCy^{x-} + (x-1)\ H^+$

$$\text{Rate} = \frac{1}{3} \times \frac{-d[CyS(=O)SCy]_{total}}{dt} \Rightarrow P_x = 3$$

Scheme 5. Elementary Bimolecular Redox Reactions



rate-limiting hydrolysis; therefore, $P_x = 1$ for this pathway. In contrast, the lower mechanism consumes twice as much CyS(═O)SCy in the fast steps as is consumed in the rate-limiting step, so $P_x = 3$ for this pathway. Note that other mechanisms can be devised for the alternative intermediate products of eq 6 as well as for mechanisms that involve $CyS(=O)_2Cy$, but these mechanisms also produce proportionality constants that are in the range ($1 \leq P_x \leq 3$). We note that there is insufficient information available at this time to distinguish between the possible mechanisms that follow the rate-limiting hydrolysis of CyS(═O)SCy (Scheme 6); hence, the absolute values of P_x remain unknown. However, when such information becomes available (i.e., after the study of some of the other reactions that comprise the 34 possible reactions, vide supra), it will be possible to rule out pathways that are not kinetically competent with respect to the rate-limiting hydrolysis reaction.

Comparison of the Hydrolysis Mechanisms of Thiosulfinate Esters. The mechanisms of reactions of thiosulfinate esters are characterized by their reactions with nucleophiles (*53*). In general, "hard" bases such as OH^- tend to attack the sulfinyl sulfur center, whereas "soft" nucleophiles such as thiolates target the sulfenyl center. We are aware of only two previous mechanistic studies of the hydrolysis reactions of thiosulfinate esters. One of these investigations was by Kice et al. for the hydrolysis of PhS(═O)SPh in mixed aqueous/dioxane solvent systems containing 0.005–0.05 M OH^-. Remarkably, Kice et al. observed that the corresponding thiosulfonate ester $PhS(=O)_2SPh$ hydrolyzes more rapidly than PhS(═O)SPh under these conditions, and PhS(═O)SPh is in fact an intermediate in the hydrolysis of $PhS(=O)_2SPh$. Furthermore, it was concluded

Scheme 6. Possible Two- and Three-Step Reaction Mechanisms for the Hydrolysis of RS(═O)SR That Yields a 1:1 Ratio of RSO_2H and RSSR (R = Cy)

1	RS(=O)SR → 2 RSOH 2 {RSOH + RS(=O)SR → RSSR + RSO_2H}	9	RS(=O)SR → RSH + RSO_2H 2 RSOH → RSH + RSO_2H} 2 {RSH + RS(=O)SR → RSOH + RSSR}
2	RS(=O)SR → 2 RSOH 2 {RSH + RSOH → RSSR} 2 {RS(=O)SR → RSH + RSO_2H}	10	RS(=O)SR → RSH + RSO_2H RSH + RSO_2H → 2 RSOH 2 {RSOH + RS(=O)SR → RSO_2H + RSSR}
3	RS(=O)SR → 2 RSOH 2 {2 RSOH → RSH + RSO_2H} 2 {RSH + RS(=O)SR → RSOH + RSSR}	11	RS(=O)SR → RSH + RSO_2H RSH + RS(=O)SR → RSOH + RSSR RSOH + RS(=O)SR → RSO_2H + RSSR
4	RS(=O)SR → 2 RSOH 2 {RSO_3H + RSOH → 2 RSO_2H} 2 {RSO_2H + RS(=O)SR → RSSR + RSO_3H}	12	RS(=O)SR → RSH + RSO_2H RSH + $RS(=O)_2SR$ → RSO_2H + RSSR 2 RS(=O)SR → $RS(=O)_2SR$ + RSSR}
5	RS(=O)SR → 2 RSOH 2 {RSOH + RS(=O)SR → RSH + $RS(=O)_2SR$} 2 {RSH + $RS(=O)_2SR$ → RSO_2H + RSSR}	13	2 {RS(=O)SR -> RSH + RSO_2H} 2 {RSH + RSOH → RSSR} RS(=O)SR →2 RSOH
6	RS(=O)SR → 2 RSOH 2{$RS(=O)_2SR$+RSOH→RSO_2H+RS(=O)SR} 2 {2 RS(=O)SR → RSSR + $RS(=O)_2SR$}	14	2 {RS(=O)SR → RSH + RSO_2H} RSH + RSOH → RSSR RSH + RS(=O)SR → RSOH + RSSR
7	RS(=O)SR → 2 RSOH 2 {$RS(=O)_2SR$ + RSOH → RSO_3H + RSSR} 2 {RSO_3H+RS(=O)SR→RSO_2H+$RS(=O)_2S$}	15	2 {RS(=O)SR → RSH + RSO_2H} 2 {RSOH → RS(=O)SR} 2 {RSH + RS(=O)SR → RSOH + RSSR}
8	3 {RS(=O)SR → 2 RSOH} 2 {RSH + RSOH → RSSR} 2 {2 RSOH → RSH + RSO_2H}	16	3 {RS(=O)SR → RSH + RSO_2H} 2 {RSH + RSOH → RSSR} RSH + RSO_2H → 2 RSOH

that OH^- attacks the sulfinyl center to produce the corresponding thiol and sulfinic acid:

$$PhS(=O)SPh + OH^- \rightarrow PhSO_2^- + PhSH \qquad (9)$$

Under these conditions, the PhSH that is liberated is more reactive than OH^- toward PhS(═O)SPh, (*55*) and the following subsequent reaction was proposed

$$PhS(=O)SPh + PhS^- \rightarrow PhSSPh + PhSO^- \qquad (10)$$

Under the alkaline conditions that were employed by Kice, the $PhSO^-$ that is released in the latter reaction disappears slowly to give the following net reaction:

$$3PhSO^- + H_2O \rightarrow PhSO_2^- + PhSSPh + 2OH^- \qquad (11)$$

However, the mechanism of the latter reaction remains unexplored. It is clear that PhS(═O)SPh undergoes hydrolysis via a mechanism that is very different than that of CyS(═O)SCy. We suspect that the aromatic groups play an important role in orchestrating this difference. A second relevant mechanistic study has been described by Oae et al. who reported that OH^- attacks the sulfinyl center of several thiosulfinate esters (as evidenced by ^{18}O tracer studies in mixed aqueous/dioxane solvent systems) (*56*). Thus, it is possible that the initial reaction of OH^- with CyS(═O)SCy is at the sulfinyl center to produce $CySO_2H$ and CySH (viz. eq 6) rather that two molar equivalents of CySOH (reverse of eq 1 and eq 5). We note that there have been a handful of additional kinetic measurements for the hydrolysis of thiosulfinate esters in previous studies (*57, 58*), but detailed mechanistic studies did not accompany these data.

Redox Cascades in Biological Settings. The issue of whether allicin is an "active principle" is often a matter of heated debate (*59–61*). However, the concepts of drug precursor and causative agent become ill-defined when dealing with chemically reactive agents. Redox-active compounds, such as allicin, can often times transfer their redox equivalents to reaction partners in facile redox cascades. For example, there exist nine nondegenerate bimolecular reactions of CyS(═O)SCy that interconvert the derivatives of Scheme 1 (Scheme 5). As discussed in the Results section (and as illustrated in Scheme 5), the 1:1 ratio of CySSCy and $CySO_2H$ that is observed after hydrolysis of CyS(═O)SCy cannot be formed directly from the hydrolysis of CyS(═O)SCy via an elementary reaction. We have written a computer program to show that there are 16 unique reaction pathways of two ($n = 1$) or three ($n = 15$) steps that can account for the observed reaction products (Scheme 6; for the sake of simplicity, water

was omitted from the otherwise balanced equations). Interestingly, CySOH is involved in all but one of the reaction pathways (reaction 12 of Scheme 6), but the later mechanism can be ruled out due to the fact that the bimolecular reaction of CyS(═O)SCy with itself to produce $CyS(=O)_2SCy$ and CySSCy is not kinetically competent {the kinetic traces under the applied experimental conditions fit well to a single exponential equation and lowering the $[CyS(=O)SCy]_0$ did not result in the change of k_{obs}}. Thus, CySOH is implicated as an intermediate in the redox cascade that begins with the hydrolysis of CyS(═O)SCy.

Scheme 6 illustrates the complexity of the mechanistic problem for the seemingly simple net reaction of Scheme 4 (where the initial reaction must be one of only two possibilities, the stoichiometry of the reactants and products are known, and the basis set consists of only seven redox derivatives). The situation in vivo is expected to be exceedingly complex, wherein cysteine derivatives are incorporated into small molecules, like glutathione, as well as a multitude of proteins. Also, we note that all thiosulfinate esters do not exhibit the same propensity for reaction. Thus, the half-life for hydrolysis of allicin at neutral pH is on the order of days (*61*), whereas the half-life for hydrolysis CyS(═O)SCy under similar conditions is about 15 min (Figure 1). However, allicin reacts with components of whole blood at rates that far exceed its rate of hydrolysis (*61*). Thus, hydrolysis of allicin (and by analogy other thiosulfinate esters) is not expected to be the only pathway of importance in vivo. Some of these reactions are likely to have deleterious effects. For example, the cytotoxic antimicrobial leinamycin bears a thiosulfinate moiety that is believed to be responsible for triggering redox cascades that eventually induce DNA damage vis-à-vis reactive oxygen species ($O_2^{\bullet-}$, H_2O_2, and $HO^{\bullet}$) (*40*). Similar thiol-triggered Fenton reactions are known for allicin (*30*), as well as for cysteine derivatives (*62*). Of course, in excess, all oxidants are expected to eventually overwhelm host defense mechanisms, thereby ultimately leading to host tissue damage and inflammatory disease.

Conclusions. We have an interest in studying the kinetics and mechanisms of CySOH, the only common oxidation derivative of cysteine that has never been isolated or characterized. In principal, the hydrolysis of CyS(═O)SCy offers one possible approach to producing CySOH. Unfortunately, regardless of whether the hydroxide attacks the sulfenic center to yield sulfenic acid (eq 5) or the sulfinic center to yield thiol and sulfinic acid, the reaction is too slow to be of utility in studying the intermediates that are produced (except perhaps above pH 13). Therefore, we are currently investigating alternative approaches to produce CySOH in situ.

Acknowledgment. We appreciate the financial support that we have received from the National Science Foundation (CHE-0503984), the American Heart Association (0555677Z), the Petroleum Research Fund (42850-AC4), and the National Institutes of Health (1 R21 DE016889-01A2). We are also grateful to Dr. Gyula Tircso for his assistance with the computer program PSEQUAD.

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