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Reactive Sulfur Species: Kinetics and Mechanism of the Equilibrium between Cysteine Sulfenyl Thiocyanate and Cysteine Thiosulfinate Ester in Acidic Aqueous Solution

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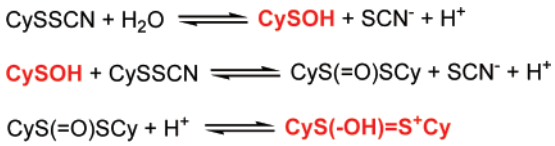

The kinetics and mechanism of the hydrolysis of cysteine sulfenyl thiocyanate (CySSCN) to give cysteine thiosulfinate ester (CyS(=O)SCy) have been investigated between pH 0 and 4. The reaction is reversible. The hydrolysis of CySSCN is second-order in [CySSCN] and inverse first-order in [H^+] and [SCN^-]. The following mechanism is proposed for the hydrolysis of CySSCN (where the charge depends upon the pH): $CySSCN^{0/+} + H_2O \rightleftharpoons CySOH^{0/+} + SCN^- + H^+$, $CySOH^{0/+} + CySSCN^{0/+} \rightarrow CyS(=O)SCy^{0/+/2+} + SCN^- + H^+$; $k_1 = 3.36 \pm 0.01 \times 10^{-3}\ s^{-1}$, $K_1k_2 = 0.13 \pm 0.05\ Ms^{-1}$ (which yields $k_2/k_{-1} = 39$ M). The observed rate law rules out alternative mechanisms for $1 \leq pH \leq 4$, including the condensation of two molecules of cysteine sulfenic acid (CySOH) to give CyS(=O)SCy. The equilibrium can be approached from the opposite direction. The reaction of CyS(=O)SCy with SCN^- to give CySSCN is first-order in [CyS(=O)SCy], [SCN^-], and [H^+] (for [H^+] > 0.4 M). The following mechanism is proposed: $CyS(=O)SCy^{2+} + H^+ \rightleftharpoons CyS(OH)=SCy^{3+}$, K_a; $CyS(OH)SCy^{3+} + SCN^- \rightarrow CySOH^+ + CySSCN^+$, $k_{-2} = 0.239 \pm 0.007\ M^{-2}s^{-1}/K_a\ M^{-1}$. Since cysteine sulfenic acids are known to play an important function in many enzymes, and SCN^- exists in abundance in physiologic fluids, we discuss the possible role of sulfenyl thiocyanates in vivo.

Introduction

Under conditions of oxidative stress, cysteine (CySH) residues in peptides are among the first moieties to undergo oxidative modification. In fact, the thiol−disulfide equilibrium plays an important role as a redox buffer in eukaryotes, and many prokaryotes, vis-à-vis the cysteine-containing tripeptide glutathione and enzymes that keep it principally in a reduced state.[1,2] However, under conditions of severe oxidative stress, CySH residues can be over-oxidized to give higher oxidation states, including the sulfenic acid CySOH (which rapidly dimerizes to give the corresponding thiosulfinate ester, CyS(=O)SCy),[3,4] the sulfinic acid $CySO_2H$, and eventually the sulfonic acid $CySO_3H$. Being perhaps the most powerful nucleophile in vivo, cysteinate (CyS^-) is particularly susceptible to attack by electrophiles. Not surprisingly, therefore, CySH residues are particularly prone to oxidation by the hypohalous acids (HOX).[4] Hypochlorous (X = Cl) and hypobromous acids (X = Br) are generated in vivo by the oxidation of the corresponding halide by hydrogen peroxide in reactions that are catalyzed by defensive peroxidases (including myeloperoxidase and eosinophil peroxidase). Pattison and Davies have investigated the rates of reaction of HOCl with the organic components of proteins and lipids.[5−7] For the organic moieties that have been studied thus far, the sulfur-containing amino acids CySH and methionine (Met) have been found to be the most reactive, although both hypohalous acids exhibit somewhat promiscuous reaction chemistry. We have shown that thiocyanate (SCN^-), a ubiquitous inorganic anion in vivo, is a competitive reductant

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of HOCl and HOBr.[8,9] The product of the reaction of HOX with SCN^- is hypothiocyanite ($OSCN^-$),[10] which, like the hypohalites, is part of the defensive stratagem of mammals. In addition to the aforementioned nonenzymic production of $OSCN^-$ by oxidation of SCN^- by HOX, $OSCN^-$ is also generated by the same defensive peroxidases that generate HOX as well as enzymes that specialize in its production (e.g., lactoperoxidase and salivary peroxidase). In contrast to HOX, $OSCN^-$ appears to exclusively target CySH groups in vivo, apparently to produce cysteine sulfenyl thiocyanates (CySSCN).[11] The fate of CySSCN groups in vivo has not been previously investigated, but it appears likely that they hydrolyze to produce generally unstable CySOH groups. In the present study, we report the kinetics and mechanisms of the hydrolysis of CySSCN in acidic aqueous media. Under the conditions of our study, the first product that is observed upon hydrolysis of CySSCN is the ester CyS(=O)SCy. Surprisingly, we have observed that the reaction is reversible, and CyS(=O)SCy reacts with SCN^-, under favorable conditions, to regenerate CySSCN. Thus, we report herein the kinetics and mechanism of the interconversion of CySSCN and CyS(=O)SCy and briefly discuss the possible physiological relevance of the reaction.

Results

Products of the Reaction of the Hydrolysis of CySSCN. The only product of the hydrolysis of CySSCN that is observed by ^{1}H NMR under the conditions of all of the kinetic experiments that are discussed herein is CyS(=O)SCy. Thus, hydrolysis of CyS(=O)SCy and its subsequent reactions (which would result in the formation of cysteine sulfinic acid, $CySO_2H$)[3,4] are not competitive with the other reactions that we consider here. Conversely, the reaction of CyS(=O)SCy with SCN^- under suitably acidic conditions (as observed by ^{1}H NMR) is CySSCN, or in some cases an equilibrium mixture of CySSCN and CyS(=O)SCy.

Kinetics of Hydrolysis of CySSCN for pH < 1. At low pH and high [SCN^-] (e.g., [CySSCN] = 0.5 mM, [H^+] = 0.5 M and [SCN^-] = 0.1 M), CySSCN is stable. In the absence of added SCN^- at pH < 1, hydrolysis of CySSCN is a single-exponential first-order process (Figure 1, inset) that is essentially independent of $[CySSCN]_0$ and [H^+] (data not shown).

Kinetics of CySSCN Hydrolysis for 1 ≤ pH ≤ 4. Biphasic kinetic traces were observed when the approach to equilibrium of the hydrolysis of CySSCN was monitored to completion. We attributed these complex kinetics to the fact that the approach to equilibrium is not an elementary reaction (vide infra). Using the initial rate method, second-order dependence on $[CySSCN]_o$ was observed at pH 2.60 in the presence of 0.1 M SCN^- (Figure 2). The initial rates for hydrolysis of CySSCN exhibit an inverse first-order dependence on [SCN^-] at pH 1.82 (Figure 3). An inverse first-order dependence on [H^+] was also observed for 1.87 ≤ pH ≤ 2.37 in the presence of 0.1 M SCN^- (Figure 4). An inverse first-order dependence on [SCN^-] was also observed for the first phase at pH 2.89 (data not shown).

Kinetics of the Reaction of CySSCN with 2-Mercaptoethanol. To determine the pK_a of the carboxylic acid moiety of CySSCN, the reaction of CySSCN with 2-mercaptoethanol was investigated as a function of pH in the presence of 0.1 M SCN^-. Thiocyanate was added to eliminate the contribution of hydrolysis of CySSCN to the overall rate of the reaction in question. The absence of an intercept in Figure 5 indicates that CySSCN reacts with 2-mercaptoethanol much faster than

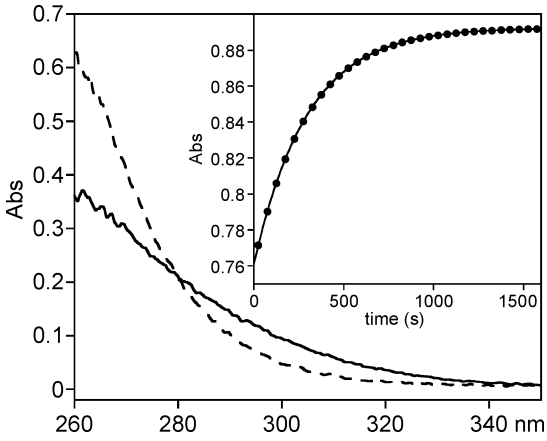

FIGURE 1. Solid line: Spectrum of CySSCN (0.95 mM) at pH 0. Dashed line: Spectrum of CyS(=O)SCy following the hydrolysis of CySSCN (0.95 mM) at pH 2.89. Spectrum recorded 50 s after a pH-jump from 0 to 2.89. No SCN^- was added. Inset: Absorbance increase at 264 nm observed for hydrolysis of CySSCN (1.5 mM) at [H^+] = 0.3 M in the absence of added SCN^-. Ten percent of the data are shown together with a first-order fit ($k_1 = 3.36 \pm 0.01 \times 10^{-3}$ s^{-1}).

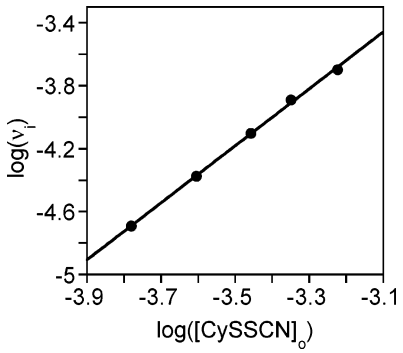

FIGURE 2. Log−log plot of the initial rates (ν_I, M s^{-1}) versus $[CySSCN]_0$ (M) for the hydrolysis of CySSCN at pH 2.60 in the presence of 0.1 M SCN^-. The slope of the linear fit is 1.81 ± 0.03.

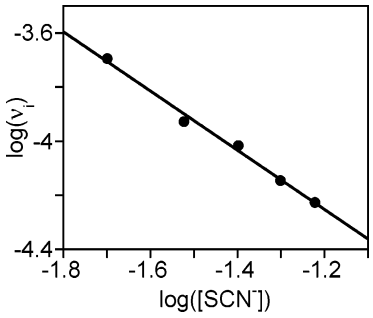

FIGURE 3. Log−log plot of the initial rates (ν_I, M s^{-1}) versus [SCN^-] (M) for the hydrolysis of CySSCN (0. 5 mM) at pH 1.82. The slope of the linear fit is −1.09 ± 0.06.

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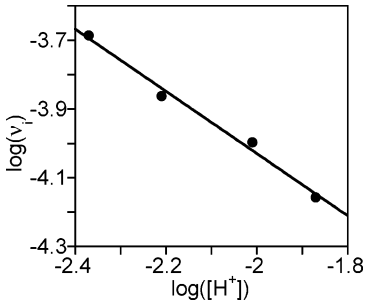

FIGURE 4. Log−log plot of the initial rates (v_I, M s^{-1}) versus [H^+] (M) for the hydrolysis of CySSCN (0. 5 mM) in the presence of 0.1 M SCN^-. The slope of the linear fit is −0.90 ± 0.06.

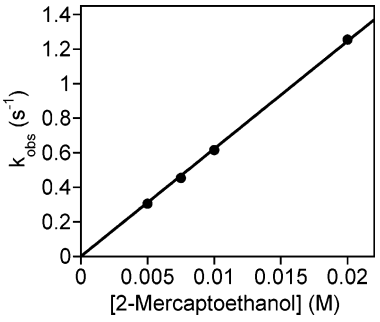

FIGURE 5. Plot of k_{obs} vs [2-mercaptoethanol] for the reaction of CySSCN (0.5 mM) with 2-mercaptoethanol at pH 2.17 in the presence of 0.1 M SCN^- and I = 1.0 M.

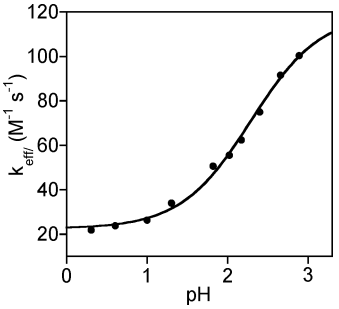

FIGURE 6. pH profile obtained for the reaction of CySSCN (0.5 mM) with 2-mercapoethanol (10 mM) in the presence of 0.1 M SCN^-. The pK_a of the carboxylic group in CySSCN is obtained from the curve fitting to be pK_a = 2.29 ± 0.09.

CySSCN hydrolyzes at pH 2.17. A plot of k_{eff} versus pH (Figure 6) was fit to the following equation

$$k_{eff} = \frac{k_x[H^+] + k_y K_a}{K_a + [H^+]}$$

to give a pK_a of 2.29 ± 0.09. The latter equation assumes that CySSCN exhibits a rapid proton equilibrium (2-mercaptoethanol does not have a pK_a in this pH region) and that there are parallel pathways for the reactions of the protonated (k_x = 22.4 ± 1.3 M^{-1} s^{-1}) and unprotonated (k_y = 119(9) M^{-1} s^{-1}) forms of CySSCN.

Kinetics of the Reaction of CyS(═O)SCy with SCN^-. In the absence of SCN^-, CyS(═O)SCy is stable at pH 0. As demonstrated by ^{1}H NMR and authentic samples,[12,13] the reaction of 20 mM CyS(═O)SCy with 0.1 M SCN^- at pH 0 produces 40 mM CySSCN. The molar absorptivity of CySSCN is less than twice that of CyS(═O)SCy at 260 nm (Figure 1), so a decrease in absorbance indicates the production of CySSCN, whereas an increase indicates that CyS(═O)SCy is produced (Figure 1, inset). Beginning with CyS(═O)SCy, we have observed that the approach to equilibrium is a single-exponential process in the presence of excess SCN^-. The pseudo-first-order rate constants exhibit a first-order dependency on [SCN^-] (Figure 7). A first-order dependency on [H^+] is observed when the pH > 0.3, but the reaction becomes pH-independent at higher pH (Figure 8, note the log scales).

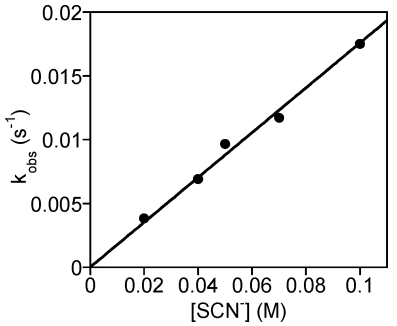

FIGURE 7. Plot of k_{obs} vs [SCN^-] for the reaction of SCN^- with CyS(═O)SCy at [H^+] = 0.9 M and I = 1.0 M.

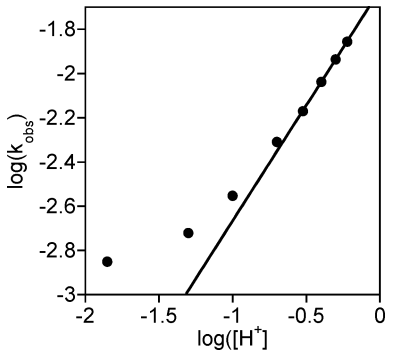

FIGURE 8. Plot of log(k_{obs}/s^{-1}) vs log([H^+]/M) for the reaction of CyS(═O)SCy (0.5 mM) with SCN^- (0.1 M). The linear portion of the plot has a slope of 1.005 ± 0.006, which yields the reaction order with respect to [H^+] for the acid-catalyzed pathway. The reaction tends to be zero-order in [H^+] at low acid concentration corresponding to an acid-independent pathway.

Discussion

Mechanism of the Hydrolysis of CySSCN. Hydrolysis of CySSCN could be expected to initially produce either CySOH (cysteine sulfenic acid) and SCN^- or CySH and HOSCN (hypothiocyanous acid). If HOSCN were produced from the hydrolysis of CySSCN, we would expect it to react rapidly with SCN^- at low pH to give $(SCN)_2$ (thiocyanogen), which would in turn react with excess SCN^- to give $(SCN)_3^-$ (trithiocyanate).[14] The latter reactions are much faster than the reaction of CySH with $(SCN)_3^-$ (unpublished results). Since $(SCN)_3^-$ exhibits a very large molar absorptivity,[14,15] and we do not observe an increase in absorption that might be attributed to its

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formation as a transient species,[16,17] we conclude that CySSCN hydrolyzes initially to give CySOH and SCN^-. Although we do not have an electronic spectrum for CySOH in the pH range of this study, at higher pH its molar absorptivity is comparable to CySSCN and CyS(=O)SCy.[3,4] The sulfenic acid is not expected to be stable, and (under certain conditions) it should condense with itself to give CyS(=O)SCy,[3,4] the product that is eventually observed when CySSCN hydrolyzes:[18]

$$\mathrm{CySSCN} + \mathrm{H_2O} \rightleftharpoons \mathrm{CySOH} + \mathrm{SCN^-} + \mathrm{H^+} \quad (1)$$

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

However, the condensation of two molecules of CySOH is not the only possible pathway to produce CyS(=O)SCy.[18] It is conceivable that the CySOH that is produced from the hydrolysis of CySSCN (eq 1) reacts with a second molecule of CySSCN:

$$\mathrm{CySSCN} + \mathrm{CySOH} \rightleftharpoons \mathrm{CyS(=O)SCy} + \mathrm{SCN^-} + \mathrm{H^+} \quad (2')$$

The net reaction is of course the same for both mechanisms:

$$2\mathrm{CySSCN} + \mathrm{H_2O} \rightleftharpoons \mathrm{CyS(=O)SCy} + 2\mathrm{SCN^-} + 2\mathrm{H} \quad (3)$$

However, the rate laws that describe these reactions are not the same. Thus, beginning with CySSCN, the initial rate for the approach to equilibrium for the first mechanism (eq 1 → eq 2) is given by (see the Supporting Information)

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{dt}} = 2k_2\left\{\frac{2k_1[\mathrm{CySSCN}]}{k_{-1}[\mathrm{H^+}][\mathrm{SCN^-}](\sqrt{1+\alpha[\mathrm{CySSCN}]}+1)}\right\}^2 \quad (4)$$

where k_1 and k_{-1} are the rate constants for the forward and reverse reactions of eq 1, and k_2 is the rate constant for the forward reaction of eq 2. The value of α is given by

$$\alpha = \frac{8k_1k_2}{k_{-1}^2[\mathrm{H^+}]^2[\mathrm{SCN^-}]^2} \quad (5)$$

Before deriving the rate law for the alternative mechanism, it is worthwhile to delineate two limiting equations. If $1 \ll \sqrt{\alpha[\mathrm{CySSCN}]}$, eq 4 simplifies to

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{d}t} = k_1[\mathrm{CySSCN}] \quad (6)$$

Thus, at very high pH and/or very low $[SCN^-]$, pseudo-first-order kinetics might be expected. If $1 \gg \alpha[\mathrm{CySSCN}]$, eq 4 simplifies to

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{d}t} = 2K_1^2k_2\frac{[\mathrm{CySSCN}]^2}{[\mathrm{H^+}]^2[\mathrm{SCN^-}]^2} = k_{\mathrm{eff}}[\mathrm{CySSCN}]^2 \quad (7)$$

where the equilibrium constant is given by $K_1 = k_1/k_{-1}$. Accordingly, provided $[H^+]$ and $[SCN^-]$ are held constant, pseudo-second-order kinetics might be expected at very low pH and/or very high $[SCN^-]$.

For the alternative mechanism (eq 1 → eq 2′), a different rate law can be derived for the initial rate of hydrolysis of CySSCN (see the Supporting Information)

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{d}t} = \frac{2k_1k_2[\mathrm{CySSCN}]^2}{k_{-1}[\mathrm{H^+}][\mathrm{SCN^-}] + k_2[\mathrm{CySSCN}]} \quad (8)$$

where k_1 and k_{-1} are the rate constants for the forward and reverse reactions of eq 1 and k_2 for this mechanism is the rate constant for the forward reaction of eq 2′. If $k_{-1}[H^+][SCN^-] \ll 2k_2[\mathrm{CySSCN}]$, eq 8 simplifies to

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{d}t} = 2k_1[\mathrm{CySSCN}] \quad (9)$$

Thus, at very high pH and/or very low $[SCN^-]$, once more, pseudo-first-order kinetics might be expected. If $k_{-1}[H^+][SCN^-] \gg 2k_2[\mathrm{CySSCN}]$, eq 8 simplifies to

$$\text{rate} = \frac{-\mathrm{d[CySSCN]}}{\mathrm{d}t} = 2K_1k_2\frac{[\mathrm{CySSCN}]^2}{[\mathrm{H^+}][\mathrm{SCN^-}]} = k_{\mathrm{eff}}[\mathrm{CySSCN}]^2 \quad (10)$$

Provided $[H^+]$ and $[SCN^-]$ are held constant, pseudo-second-order kinetics might be expected at very low pH and/or very high $[SCN^-]$.

Equations 6 and 9 predict that at very high pH and low $[SCN^-]$, pseudo-first-order kinetics can be expected. However, such conditions do not differentiate between the alternative mechanisms that are described above. In contrast, while the pseudo-first-order limits of eq 6 and eq 9 cannot distinguish between the two mechanisms, the $[H^+]$ and $[SCN^-]$ dependencies of eqs 7 and 10 can indeed distinguish between the two mechanisms. For $1 \leq \mathrm{pH} \leq 4$, the kinetics of hydrolysis of CySSCN exhibit second-order dependency on $[\mathrm{CySSCN}]_0$ (Figure 2) and inverse first-order dependencies on $[SCN^-]$ (Figure 3) and $[H^+]$ (Figure 4). These observations suggest that under the conditions of our study, the CySOH that is produced from the hydrolysis of CySSCN (eq 1) reacts with CySSCN (eq 2′) rather than with itself (eq 2).

The rate constant for the hydrolysis of $CySSCN^+$ (the fully protonated form) can be obtained from Figure 1 (inset, $k_1 = 3.36 \pm 0.01 \times 10^{-3}\ \mathrm{s^{-1}}$). In principle, the pseudo-second-order rate constants of Figures 2−4 give K_1k_2. However, there is a potential complication because all of the CySH-derived species of eqs 1 and 2′ are expected to exhibit Brønsted acid/base chemistry between pH 1 and 4. Determination of the acid dissociation constants of CySSCN by spectrophotometric titration is problematic because of its propensity to undergo hydrolysis, so we determined $\mathrm{p}K_{a1} = 2.29 \pm 0.09$ using a kinetic method (vide supra). For CyS(=O)SCy, $\mathrm{p}K_{a1}$−$\mathrm{p}K_{a4}$ occur at 1.4, 2.0, 7.3, and 7.9, respectively.[18] We have previously established that the acid dissociation constant of the sulfenic moiety of CySOH ($\mathrm{p}K_{a2}$) is between pH 6 and 10.[4] The first acid dissociation constant of CySOH ($\mathrm{p}K_{a1}$) presumably occurs at about pH 2. In general, the protonation states of CySH derivatives have a small but discernible effect on electrophilic/nucleophilic reactions. For example, we have observed that the

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relative second-order rate constants for the nucleophilic attack of OH^- on $CyS(=O)SCy^n$ for $n = -2$, -1, and 0 are $1:1.7 \times 10^2:1.4 \times 10^4$.[18] Thus, a two-order-of-magnitude increase in the rate of reaction is observed for each additional positive charge. Similarly, the rate of nucleophilic attack of CyS^- on $CyS(=O)SCy^n$ for $n = -2$, -1, and 0 are 1:16:131 (1 order of magnitude of increase for each proton).[3] However, the data of Figure 4 suggest that the nucleophilic attack of H_2O on $CySSCN^0$ vs $CySSCN^+$ occurs at about the same rate. It would appear that protonation of the carboxylic group of CySSCN does not have a marked effect on the electrophilicity of CySSCN toward neutrally charged nucleophiles. Accordingly, we calculate K_1k_2 from Figures 2−4 to be 0.082 ± 0.008, 0.112 ± 0.007, and 0.18 ± 0.01 M s^{-1}, respectively. Using an average value of 0.13 ± 0.05 M s^{-1}, we compute $k_2/k_{-1} = 39$ M.

Mechanism of the Reaction of CyS(=O)SCy with SCN^-. Under reaction conditions that are analogous to those that were employed to study the hydrolysis of CySSCN, the reaction of CyS(=O)SCy with SCN^- should be the microscopic reverse of eq 2′. As discussed previously, the hydrolysis of CySSCN at pH < 1 in the absence of added SCN^- produces CyS(=O)SCy in a first-order process. However, the addition of SCN^- drives the equilibrium in the other direction, and with sufficient [SCN^-], CySSCN is produced exclusively. As predicted from the reverse of eq 2′, the reaction of CyS(=O)SCy with SCN^- is first-order in [SCN^-] (Figure 7, vide supra). The reaction also exhibits a first-order dependency on [H^+] at low pH, but a deviation from first-order behavior is observed at higher pH. The pH limits of the data of Figure 8 ($1.9 \leq pH \leq 0.2$) encompass the first pK_a of CyS(=O)SCy ($pK_{a1} = 1.4$). Accordingly, $CyS(OH)SCy^{3+}$ is the reactive species for the data of Figure 8 that exhibit a first-order dependency on [H^+], but $CyS(=O)SCy^{2+}$ is the reactive species for the data that deviate from that first-order behavior. In contrast to the insensitivity of the charge state of CySSCN toward the neutral nucleophile H_2O (vide supra), the reaction of $CyS(=O)SCy^{n+}$ toward SCN^- appears to be sensitive to the charge, as expected from Debye−Hückel Theory.[19] Also, we note that Kice et al. have previously observed acid catalysis of nucleophilic reactions of aromatic thiosulfinate esters at low pH.[20] Using the four data points that fall on the line of Figure 8, we compute the rate constant for the reaction of $CyS(OH)SCy^{3+}$ with SCN^- to be $k_{-2} = 0.239 \pm 0.007$ M^{-2} s^{-1}.

Kinetics and Thermodynamics of the Equilibrium. We have independently measured three kinetic parameters, k_1, K_1k_2, and k_{-2}. While there is insufficient data to deconvolute the four rate constants that define $K_{12} = k_1k_2/k_{-1}k_{-2}$, we can compute the equilibrium constant itself:

$$K_{12} = K_1K_2 = \frac{[CyS(=O)SCy][SCN^-]^2[H^+]^2}{[CySSCN]^2} = \frac{k_1k_2}{k_{-1}k_{-2}} = \frac{0.13\ M\ s^{-1}}{0.24\ M^{-2}\ s^{-1}} = 0.54\ M^3 \quad (11)$$

Since k_1k_2/k_{-1} was determined in the pH range of 1.9−2.4 (Figure 4), and k_{-2} was determined for [H^+] 0.4−0.9 M (the linear data of Figure 8), there is an assumption that the protonation states of CySSCN and CyS(=O)SCy do not affect K_{12}, and this assumption is probably not valid. While the data of Figure 4 suggest that the $CySSCN^0 \rightleftharpoons CySSCN^+$ equilibrium does not have a marked effect on K_1k_2, the data of Figure 8 suggests that the $CyS(=O)SCy^+ \rightleftharpoons CyS(=O)SCy^{2+}$ equilibrium ($pK_{a2} = 1.3$) does impact the value of k_{-2}. The $CyS(=O)SCy^0 \rightleftharpoons CyS(=O)SCy^+$ equilibrium ($pK_{a2} = 2.0$) presumably also impacts the value of k_{-2}. Thus, there are at least three values of K_{12} below pH 4. The data we have collected are insufficient to resolve the effects of these equilibria. However, eq 11 predicts that CySSCN should be relatively stable at low pH and high [SCN^-]. Accordingly, we find for [H^+] = 0.5 M and [SCN^-] = 0.1 M, CySSCN is stable for several days.

Relevance of Sulfenyl Thiocyanates in a Biological Setting. There are very few examples of thiosulfinate esters in nature. The most widely cited example is allicin, one of the components of garlic.[21−23] Accordingly, the equilibrium that is defined by eq 3 (K_{12}) does not have much importance in a biological context. However, there are many examples of cysteine sulfenic acid residues in proteins: e.g., NADH[1] peroxidase,[24] NADH oxidase,[25] nitrile hydratase,[26] both classes of methionine sulfoxide reductases,[27] and certain peroxiredoxins.[28−32] Therefore, since SCN^- is found in abundance in many physiologic fluids, the equilibrium of eq 1 (K_1) could be of relevance. Importantly, we have not measured K_1 in the present study, so it is not possible to predict whether such an equilibrium would favor the formation of a sulfenyl thiocyanate at physiologic pH and [SCN^-]. Also, it is important to point out that most sulfenic moieties in proteins are not solvent accessible, and therefore their dielectric environments are significantly perturbed relative to an aqueous solution. Nonetheless, our observation of the equilibrium interconversion of CySSCN and CyS(=O)SCy, presumably vis-à-vis CySOH, raises the intriguing possibility that sulfenyl thiocyanates may play a role in the biological chemistry of sulfenic acids.

Conclusions

We have reported the kinetics and mechanism of the reversible interconversion of CySSCN and CyS(=O)SCy for $0 \leq pH \leq 4$. Using initial rate data, the rate law favors a mechanism for which hydrolysis of CySSCN yields a transient molecule of CySOH that subsequently reacts with CySSCN to give CyS(=O)SCy. While the observed rate law rules out the alternative mechanism of condensation of two molecules of

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cysteine sulfenic acid (CySOH) to give CyS(═O)SCy for the conditions of our measurements, we are confident that situations exist that favor the condensation of CySOH. When the equilibrium is approached from the opposite direction, the reaction of CyS(═O)SCy with SCN^- yields CySSCN via an acid-catalyzed mechanism. Uncatalyzed reaction pathways appear to exist. Since cysteine sulfenic acids are known to play an important function in many enzymes, and SCN^- exists in abundance in physiologic fluids, we are particularly intrigued by the possibility that sulfenic moieties in biological macromolecules may exist under some circumstances as sulfenyl thiocyanates. We are currently exploring this possibility.

Experimental Section

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, $HClO_4$ 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-Cysteine, DCl (35 wt % solution in D_2O), NaOD (40 wt % solution in D_2O), $NaClO_4$, K_3PO_4, $NaH_2PO_4 \cdot H_2O$, Na_2HPO_4, and $Na_3PO_4 \cdot 12H_2O$, deuterium oxide (99.9%) were used as received. The synthesis of CyS(═O)SCy was accomplished using a published procedure.[13]

Synthesis of CySSCN. CySSCN was prepared using a modification of a published procedure.[11,12] Solutions of $(SCN)_2$ (30−40 mM) were prepared by reacting excess $Pb(SCN)_2$(s) with Br_2 in CCl_4. The reaction was allowed to proceed until a colorless solution was obtained. The concentration of $(SCN)_2$ was determined spectrophotometrically ($\epsilon_{296} = 140\ M^{-1}\ s^{-1}$). Stock solutions of CySSCN (20−40 mM) were prepared in 1.0 M $HClO_4$ by reacting CySH with $(SCN)_2$ (using 10% excess). The aqueous phase was carefully separated from the organic phase using a glass Pasteur pipet. The concentrations of stock solutions of CySSCN were determined based on $[CySH]_T$ used for the synthesis by measuring the ^1H NMR spectra in 1.0 M HCl containing 50% D_2O.

pH/pD Measurements. The $[OH^-]$ for the unbuffered solutions were determined by acid−base titration against standardized HCl or standardized $HClO_4$ solutions. The $[H^+]$ of the buffered solutions were determined with a pH meter using an Ag/AgCl combination pH electrode. Unless stated otherwise, the ionic strength was kept constant at 0.76 M for all solutions ($NaClO_4 + HClO_4 + NaH_2PO_4$). To obtain the $[H^+]$ or $[OH^-]$ of the buffered solutions from the measured pH values, all pH measurements were corrected for the "Irving factor"[33] and the ionic product of water (pK_w) that were measured by titration of a 1.0 M $NaClO_4$ solution by a standardized solution of 0.1 M NaOH (in 1.0 M $NaClO_4$). pD measurements in D_2O were made using the same pH electrode by adding 0.4 units to the measurement.[34]

NMR Studies. ^1H NMR spectra were recorded with a 300 MHz 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/vis Spectroscopy. Electronic spectra were measured using a conventional diode-array spectrophotometer using quartz cells with calibrated 1 mm, 2 mm, and 1 cm path lengths at 20 °C or the monochromator of a stopped-flow instrument with a Xe arc lamp at 18 °C.

General Description of the Kinetic Measurements. Kinetic measurements were made with a double-mixing stopped-flow spectrophotometer using a Xe arc lamp and a PMT detector or a conventional diode-array spectrophotometer, depending upon the speed of the reaction. All of the kinetic measurements were made at a temperature of 18 °C maintained in the observation cell with a refrigerated circulator. The kinetics of the hydrolysis of CySSCN were investigated by doing a pH-jump from 0 to higher values using NaOH solutions. The adiabatic temperature increases that were associated with the pH-jump experiments were determined experimentally to be less than 1 °C.

Hydrolysis of CySSCN. The $[H^+]$ dependency was investigated by doing a pH jump from 0 to a higher pH in a single-mixing mode. CySSCN (1.0 mM) in 1.0 M $HClO_4$ was mixed with 0.2 M PO_4^{3-} containing OH^-, ClO_4^-, and SCN^-. The ionic strength was adjusted to 0.76 M after mixing. Initial rates were measured in the pH region 1.87 − 2.37 by monitoring the absorbance increase at 260 nm. SCN^- (0.1 M) was present in all measurements. The $[SCN^-]$-dependency was investigated using a single-mixing method at pH 1.82 by measuring the initial rates and at pH 2.89 by monitoring the reaction until completion. For the initial rate measurements, CySSCN (1.0 mM) in 1.0 M $HClO_4$ was mixed with 0.2 M PO_4^{3-} containing OH^-, ClO_4^-, and SCN^-. The $[SCN^-]$ were varied from 20 to 60 mM at a constant pH of 1.82 and an ionic strength 0.76 M. The initial absorbance increase at 260 was monitored. At pH 2.89, the $[SCN^-]$ dependency was investigated by monitoring the reaction at 300 nm until completion. CySSCN (1.9 mM) in 1.0 M $HClO_4$ was mixed with 0.2 M PO_4^{3-} containing OH^-, ClO_4^- and SCN^-. Biphasic absorbance decay was monitored at 300 nm. After mixing, the concentration of SCN^- was varied from 7 − 72 mM at pH 2.89 and ionic strength 0.76 M.

Reaction of CyS(═O)SCy with SCN^-. The $[H^+]$ and [SCN] dependencies of the reaction rates were investigated under pseudo-first-order conditions using excess SCN^- over CyS(═O)SCy in single-mixing stopped-flow and diode-array experiments. The $[H^+]$ in the CyS(═O)SCy solutions before mixing was kept at 0.6−1.0 M to suppress the rate of hydrolysis of CyS(═O)SCy. CyS(═O)-SCy (1 mM) in 1.0 M $HClO_4$ was mixed with SCN^- solutions containing H^+ and ClO_4^-. After mixing, the $[H^+]$ was 0.9 M, while $[SCN^-]$ was varied from 0.02−0.10 M. The total ionic strength was 1.0 M. Single-exponential absorbance increases at 300 nm were observed in all cases. The $[H^+]$-dependency was investigated using the diode-array spectrophotometer. CyS(═O)SCy (0.5 mM) in 0.6 M $HClO_4$ ($\mu = 1.0$ M) was mixed with 0.2 M SCN^- containing H^+ and ClO_4^- ($\mu = 1.0$ M) using a vortexer, and the kinetics were measured immediately thereafter. $[H^+]$ was varied from 0.014 − 0.60 M at constant $[SCN^-] = 0.1$ M and an ionic strength of 1.0 M. The reaction was monitored at 260 nm, which exhibited single-exponential absorbance decrease in all cases.

Control Experiments. Use of 10% excess or more of $(SCN)_2$ over CySH in the preparation of CySSCN has been shown to have no effect on its hydrolysis kinetics. Thus, $(SCN)_2$ (35.6 mM) was extracted into 1.0 M $HClO_4$ in the absence of CySH, this solution was allowed to decompose for 24 h, a pH-jump to $[H^+] = 0.5$ M was made on a 2 mM sample, and time-resolved spectra were collected for 3000 s. No absorbance change was observed in the UV region where hydrolysis of CySSCN at $[H^+] = 0.5$ M shows absorbance increase over the same period of time. In another control experiment, a pH-jump to $[H^+] = 0.1$ M was performed in the presence of 0.1 M SCN^-. Again, there was no absorbance change in the time-resolved spectra collected over a period of 1500 s. Under similar conditions, CySSCN displayed an absorbance increase in the UV region over the same period of time. These control experiments suggest that the decomposition products of $(SCN)_2$ do not contribute to the spectral changes that are observed in connection with the hydrolysis of CySSCN. Also, we have demonstrated that

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cystine (CySSCy), which is present in stock solutions of CySSCN in variable concentrations, has no effect on the hydrolysis process. Thiocyanate, when present at high concentrations, was observed to be light-sensitive, giving slow absorbance increase in the UV region. Fresh solutions of SCN^- were employed in our studies to avoid contribution of the decomposition of SCN^- to the time-resolved spectra.

Kinetic Data Analysis. The monochromatic kinetic traces were fit with HI-TECH KinetAsyst 3.14 software (Hi-Tech, UK). 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) or Mathematica (Wolfram Research, Version 5.2).

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Supporting Information Available: The rate laws of eqs 4 and 8 are derived. This material is available free of charge via the Internet at http://pubs.acs.org.

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