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Kinetics and Mechanisms of Chlorine Dioxide and Chlorite Oxidations of Cysteine and Glutathione

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Chlorine dioxide oxidation of cysteine (CSH) is investigated under pseudo-first-order conditions (with excess CSH) in buffered aqueous solutions, p[H$^+$] 2.7–9.5 at 25.0 °C. The rates of chlorine dioxide decay are first order in both ClO_2 and CSH concentrations and increase rapidly as the pH increases. The proposed mechanism is an electron transfer from CS^- to ClO_2 (1.03×10^8 M^{-1} s^{-1}) with a subsequent rapid reaction of the $CS^\bullet$ radical and a second ClO_2 to form a cysteinyl–ClO_2 adduct (CSOClO). This highly reactive adduct decays via two pathways. In acidic solutions, it hydrolyzes to give CSO_2H (sulfinic acid) and HOCl, which in turn rapidly react to form CSO_3H (cysteic acid) and Cl^-. As the pH increases, the (CSOClO) adduct reacts with CS^- by a second pathway to form cystine (CSSC) and chlorite ion (ClO_2^-). The reaction stoichiometry changes from 6 ClO_2:5 CSH at low pH to 2 ClO_2:10 CSH at high pH. The ClO_2 oxidation of glutathione anion (GS^-) is also rapid with a second-order rate constant of 1.40×10^8 M^{-1} s^{-1}. The reaction of ClO_2 with CSSC is 7 orders of magnitude slower than the corresponding reaction with cysteinyl anion (CS^-) at pH 6.7. Chlorite ion reacts with CSH; however, at p[H$^+$] 6.7, the observed rate of this reaction is slower than the ClO_2/CSH reaction by 6 orders of magnitude. Chlorite ion oxidizes CSH while being reduced to HOCl, which in turn reacts rapidly with CSH to form Cl^-. The reaction products are CSSC and CSO_3H with a pH-dependent distribution similar to the ClO_2/CSH system.

Introduction

The ClO_2 radical is a powerful one-electron oxidant ($E°$ = 0.936 V),[1] known for its ability to oxidize inorganic[2−5] and organic species.[6] Chlorine dioxide is also known for its anti-bacterial and anti-viral properties.[7−9] It is used in the pulp industry and in water treatment systems.[10,11] The use of ClO_2 as an aqueous sanitizing agent is growing with applications in vegetable processing,[12−14] citrus packing,[15] and fish,[16,17] meat,[18] and poultry processing.[19,20] Gaseous ClO_2 has been applied in chemosterilization[21] and disinfection of biological warfare agents such as anthrax.[22] The mechanism by which ClO_2 destroys bacteria, viral agents, and spores is of great interest. Disinfection of bacteria may occur as ClO_2 penetrates the membrane and oxidizes the −SH

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group of glucose oxidase to an −S−S− group. This will lead to the disruption of enzyme activity and bacterium death.[23] The primary mechanism of viral inactivation by ClO_2 is still under investigation and is postulated to occur either by damaging the nucleic acid of the virus[24] or by disruption of the viral protein.[7,25,26] Prior evidence of ClO_2 induced damage to protein structures prompted our interest in the reactivity and mechanism of ClO_2 oxidation of amino acids. Previous studies show that ClO_2 reacts more rapidly with cysteine, tyrosine, and tryptophan than with other amino acids.[7,27] A detailed mechanistic study of the ClO_2 oxidation of tyrosine was recently published by the Margerum group.[28]

Biological thiols play a critical role in maintaining cellular redox potentials, as well as protecting the cell from reactive oxygen species.[29] The strongly nucleophilic sulfur group makes cysteine (CSH) the most reactive amino acid to be oxidized by ClO_2.[7] Current literature does not include detailed rate equations or resolved rate constants for the reaction of cysteine with ClO_2 or ClO_2^- at physiologically relevant pH. Darkwa et al.[30] reported the oxidation of cysteine with both ClO_2 and ClO_2^- in acidic solution (pH ≤ 1) to form cysteic acid (CSO_3H). In conditions with excess ClO_2^-, they found oligo-oscillatory formation of ClO_2 and proposed a series of 28 reactions to model the experimental data and estimate rate constants.[30] The formation of the disulfide product (CSSC) has been reported by Lynch et al.[31] for the reaction of cysteine with a mixture of ClO_2 and ClO_2^- at pH 7.

Glutathione (GSH; a tripeptide formed by the amino acids glycine, cysteine, and glutamic acid) is an abundant non-protein intracellular thiol present in animals, plants, and several bacteria.[29,32−34] This tripeptide participates in both chemical and enzymatic reactions to carry out its antitoxic function.[29] Therefore, it is of interest to determine the mechanism of ClO_2 reaction with GSH. Ingram et al.[35] reported the ClO_2^- oxidation of GSH at neutral pH to form glutathione disulfide (GSSG) as the major product. Winterbourne and Brennan[33] reported the HOCl oxidation of GSH to form GSSG, glutathione thiolsulfonate (GSO_2SG), and traces of glutathione sulfonic acid (GSO_3H) as well as a product of an intramolecular reaction of the amine and thiol groups. Other species that have been used to oxidize GSH are H_2O_2[35] and polymeric MnO_2.[32]

In the current work, we determine the kinetics and mechanism of cysteine (CSH) and glutathione (GSH) oxidation by ClO_2. Because chlorite ion is a product of ClO_2 oxidations, we also report the significantly slower oxidation of CSH and GSH by ClO_2^-. The pH dependent formation of two major products, cystine (CSSC) and cysteic acid (CSO_3H), in both the ClO_2 and the ClO_2^- oxidation of cysteine, is shown.

Experimental Section

Reagents. All solutions were made with distilled−deionized water. Commercially available cysteine, cystine, cysteic acid, and glutathione (Sigma) were used without further purification. Chlorine dioxide stock solutions were prepared as described previously,[36] protected from light, and stored in a refrigerator. The chlorine dioxide solution was standardized spectrophotometrically based on the molar absorptivity of ClO_2, $\epsilon = 1230\ M^{-1}\ cm^{-1}$ at 359 nm. Commercially available $NaClO_2$ was recrystallized as previously described,[37] and its purity was determined by ion chromatography. Stock solutions of $NaClO_2$ were standardized spectrophotometrically at 260 nm ($\epsilon = 154\ M^{-1}\ cm^{-1}$). Ionic strength was controlled with recrystallized $NaClO_4$. Phosphate, carbonate, acetate, or formate buffers were used to control the pH.

Methods. All reactions were carried out at 25.0 °C. Ionic strength ($\mu = 1.0$ or 0.10 M) was kept constant by the addition of $NaClO_4$. An Orion model 720A digital pH meter equipped with a Corning combination electrode was used for pH measurements that were corrected to give $p[H^+]$ values (i.e., −log [H^+ concentration] based on electrode calibrations at $\mu = 1.0$ or 0.10 M). A Perkin-Elmer UV−vis spectrophotometer (1.00 cm path length) or an Applied Photophysics Stopped-Flow SX.18 MV (APPSF) spectrometer (0.962 cm path length) with a PD.1 photodiode array or single wavelength detectors was used for the kinetic analysis. Single wavelength stopped-flow reactions on the APPSF instrument with pseudo-first-order rate constants greater than $80\ s^{-1}$ were corrected for mixing by use of a mixing rate constant of $k_{mix} = 4620\ s^{-1}$, where $k_{corr} = (1/k_{obs} - 1/k_{mix})^{-1}$.[38] Under all our conditions, the rate of disproportionation of ClO_2 is much too slow to contribute to the observed reactions.[3] Cysteine (or glutathione) were mixed with buffer prior to the stopped-flow mixing.

A FinniganMAT XL95 (Bremen, Germany) double-focusing mass spectrometer system with electrospray ionization (ESI) was used for product identification. Ion-exchange chromatographic analyses used a Dionex DX 500 Chromatographic System with a Dionex ED40 electrochemical detector and Dionex AD20 absorbance detector. A Dionex Ion-Pac AS9-HC column was used for separations with a 9.0 mM Na_2CO_3 mobile phase at 1.0 mL/min.

Product quantification was obtained by ^1H NMR on a Varian Inova 300 spectrophotometer with D_2O as a solvent. The solvent suppression technique was used to minimize the signal for the water peak and increase the intensity of the product peaks. Because the electronic environment of the observed C−H protons changes with protonation or deprotonation of the compound, large changes in chemical shifts are observed at different pH values of the sample

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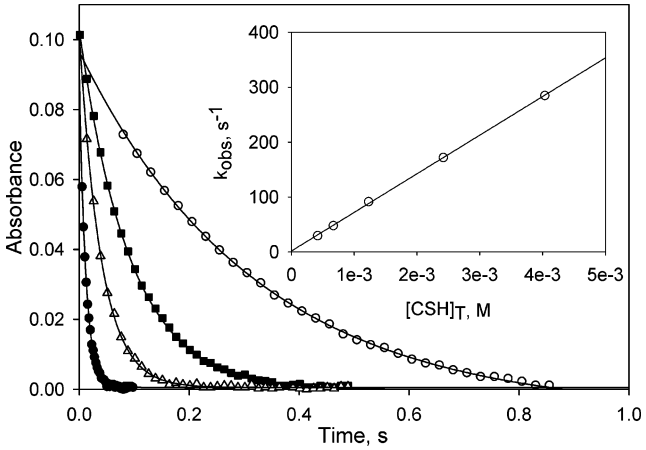
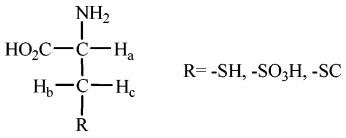


Figure 1. Kinetic traces (symbols) and first-order fits (solid lines) for ClO_2 decay during its oxidation of cysteine. (○) p[H^+] 3.28, $k_{obs} = 3.2 \pm 0.1$ s^{-1}; (■) p[H^+] 3.90, $k_{obs} = 10.9 \pm 0.1$ s^{-1}; (△) p[H^+] 4.24, $k_{obs} = 24.6 \pm 0.3$ s^{-1}; (●) p[H^+] 4.83, $k_{obs} = 85.7 \pm 1.0$ s^{-1}. Conditions: 1.0 mM CSH; 0.10 mM ClO_2; 0.10 M buffer, formate (p[H^+] 3.2−3.9), acetate (p[H^+] 4.2−4.8); μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 359 nm. Inset: Dependence of the first-order rate constant for ClO_2 decay on cysteine concentration. Conditions: 30 μM ClO_2; 50 mM $[OAc]_T$; p[H^+] = 4.9; μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 359 nm. Cell path 0.962 cm.

solution. To avoid this problem, the reaction solutions were adjusted to pH ∼ 11 with NaOH prior to analysis. Sample solutions contained approximately 15% D_2O. Samples were prepared by mixing 0.6 mL of the reaction solution in H_2O with 0.1 mL of an internal standard solution (*t*-BuOH in D_2O). The same internal standard solution was used to obtain calibration curves for CSH, CSSC, and CSO_3H by using C−H peak integrals. Reaction products and remaining cysteine were quantified by using the C−H_a peaks of CSSC and CSO_3H and H_b peaks of cysteine because they have the least amount of interference from the other compounds in solution. Cysteine (CSH), cystine (CSSC), and cysteic acid (CSO_3H) exhibit a doublet of doublets for each of the three C−H protons (H_a, H_b, and H_c shown below) in the carbon backbone.



The protons of the carboxylic group, the S−H proton, and the amine group are not observed in D_2O due to rapid exchange with the solvent.

Results and Discussion

Chlorine Dioxide Oxidation of Cysteine. The oxidation of cysteine (CSH) by chlorine dioxide was studied under pseudo-first-order conditions with excess cysteine from p[H^+] 3 to p[H^+] 6. The reaction kinetics were followed by stopped-flow methods with single-wavelength detection. Chlorine dioxide loss was observed at 359 nm, and first-order fits were obtained (Figure 1) as expressed by eq 1. [Tests of the oxygen level of the solutions (from deairated to O_2 saturated)-showed no dependence of the rate constants on O_2 concentrations.] Variation of cysteine concentration gave a linear plot of k_{obs} vs $[CSH]_T$ (Figure 1 inset). The observed rate constants increase with pH (Figure 2), which indicates that CS^- is the reactive species. Further increase of pH is limited by the time resolution of the method. Equations 2−4 are the proposed initial steps that occur in the reaction between ClO_2 and CSH. The rate-determining step (eq 3) is an electron transfer from CS^- to ClO_2 to form a cysteinyl radical ($CS^{\bullet}$) and chlorite. Thiolate radicals generated from thiolate anions have been observed in photolysis and radiolysis experiments.[39−41] The subsequent step (eq 4) is the rapid coupling of the cysteinyl radical and a second equivalent of ClO_2. The rate expression derived from these steps is given in eq 5, where $[CSH]_T = [CS^-] + [CSH]$ and the pK_{a2} value for the S−H group is 8.18[42] (Table 1). Figure 2 shows an excellent fit of the data with variation of pH dependence due to the ionization of the S−H group. The second-order rate constant for k_1, obtained from the pH dependence plot, is 1.03(0.01) × 10^8 M^{-1} s^{-1} at 25.0 °C, and μ = 1.0 M. An

$$-\frac{d[ClO_2]}{dt} = k_{obs}[ClO_2] \quad (1)$$

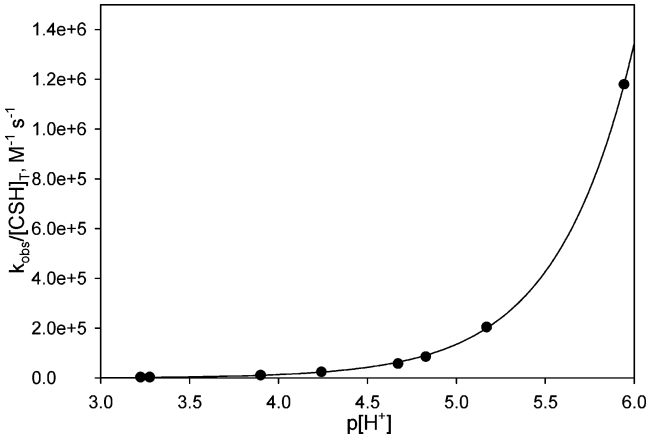


Figure 2. Effect of p[H^+] on the observed ClO_2 decay during cysteine oxidation. Conditions: 1.0 mM CSH and 0.10 mM ClO_2 for p[H^+] 3.2−4.8; 0.20 mM CSH and 0.04 mM ClO_2 for p[H^+] 5.2−5.9; 0.10 M buffer: formate (p[H^+] 3.2−3.9); acetate (p[H^+] 4.2−4.8); phosphate (p[H^+] 5.2−5.9); μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 359 nm. Solid line is a fit of eq 5a using pK_{a2} = 8.18: $k_1 = 1.03\ (0.01) \times 10^8$ M^{-1} s^{-1}.

Table 1. pK_a Values for Cysteine and Related Compounds[a]

compound	pK_{a1}	pK_{a2}	pK_{a3}	pK_{a4}
cysteine[42]	1.89 (OCO−H)	8.18 (S−H)	10.19 $(H_2N-H)^+$	none
cystine[53]	1.65 (OCO−H)	2.35 (OCO−H)	8.26 $(H_2N-H)^+$	8.86 $(H_2N-H)^+$
cysteic acid[54]	1.3 (O_2SO-H)	1.89 (OCO−H)	8.69 $(H_2N-H)^+$	None
glutathione[57]	2.126 (OCO−H)[b]	3.512 (OCO−H)[c]	8.736 (S−H)	9.655 $(H_2N-H)^+$

[a] Cysteine and cystine values are reported for μ = 1.0 M and 25.0 °C. Cysteic acid values are reported for 25.0 °C and unknown ionic strength. Glutathione values are reported for μ = 0.10 M and 25.0 °C. [b,c] pK_a value for the deprotonation of (*b*) OCOH of glutamic acid and (*c*) OCOH of glycine.

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Table 2. pH Dependent Product Distribution for the ClO_2^- Oxidation of CSH[a]

pH	% CSSC[b]	% CSO_3H[b]	other products[c]
2.77	38	51	11
4.0	58	39	3
5.95	92	8	0
6.52	94	0	6

[a] Conditions: initial ClO_2^-/CSH ratio 1:4, 0.25 M buffer (formate, acetate, phosphate), 25.0 °C. [b] Product percentage determined as percent of CSH consumed. [c] Other products taken as the remaining percent of CSH not accounted for by CSSC and CSO_3H.

alternate reaction path between two $CS^\bullet$ radicals to give CSSC provides relatively little product because the concentration of ClO_2 is much larger than the concentration of $CS^\bullet$ under the conditions used.

$$CSH \overset{K_{a2}}{\rightleftharpoons} CS^- + H^+ \tag{2}$$

$$CS^- + ClO_2 \xrightarrow{k_1} CS^\bullet + ClO_2^- \tag{3}$$

$$CS^\bullet + ClO_2 \xrightarrow{\text{fast}} \text{products} \tag{4}$$

$$-\frac{d[ClO_2]}{dt} = 2k_1\left(\frac{K_{a2}}{K_{a2} + [H^+]}\right)[CSH]_T[ClO_2] \tag{5}$$

where

$$k_{obs} = 2k_1\left(\frac{K_{a2}}{K_{a2} + [H^+]}\right)[CSH]_T \tag{5a}$$

Chlorite Ion Oxidation of Cysteine. Cysteine also reacts with ClO_2^-, although the rate is 6 orders of magnitude slower than the ClO_2/CSH reaction at p[H^+] 6.71. Two major products are observed where cysteic acid forms at low pH and cystine forms at higher pH (Table 2). Chlorite (λ_{max} = 260 nm) decay can only be observed at pH < 3.7 due to the interference of increasing amounts of cystine (λ_{max} = 250 nm) formed at higher reaction pH. The formation of cystine is observed above pH 6 where it is a major product (>90%). The formation of CSSC under pseudo-first-order conditions (excess CSH) gives first-order fits as shown in Figure 3. Variation of the CSH concentration at p[H^+] 5.1 shows a first-order dependence in [CSH] (Figure 3, inset). The reactivity decreases above pH 7 as the pK_a (8.18) of the S−H group is approached (Figure 4). In addition, no products are detected by NMR after 12 h for a ClO_2^-/CSH reaction maintained at pH ∼ 12. Therefore, the CS^- anion does not react with ClO_2^-. This observation points to the fundamental difference in mechanism between cysteine oxidation by ClO_2 and by ClO_2^-. The absence of reactivity of the CS^- anion suggests that oxidation occurs through oxygen transfer rather than single electron transfer. In the rate-determining step, chlorite transfers an oxygen atom to CSH and forms HOCl and the highly reactive intermediate CSOH.[43,44] The formation of HOCl was observed in the chlorite ion oxidation of

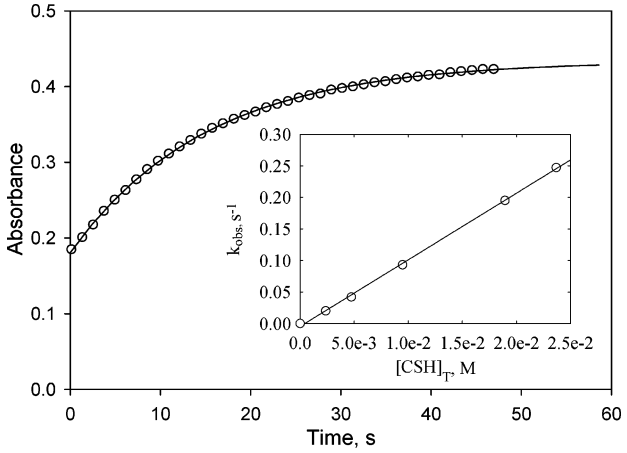

Figure 3. Formation of CSSC during ClO_2^- oxidation of cysteine. Conditions: 10 mM CSH; 1.0 mM ClO_2^-; 0.10 M $[PO_4]_T$; p[H^+] 6.71; μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 260 nm; k_{obs} = 6.6 ± 0.1 × 10^{-2} s^{-1}. Inset: Effect of $[CSH]_T$ on the observed rate constant of CSSC formation during ClO_2^- oxidation of CSH. Conditions: 0.25 mM ClO_2^-; p[H^+] = 4.9−5.3; 0.10 M $[OAc]_T$; μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 250 nm. Cell path 0.962 cm.

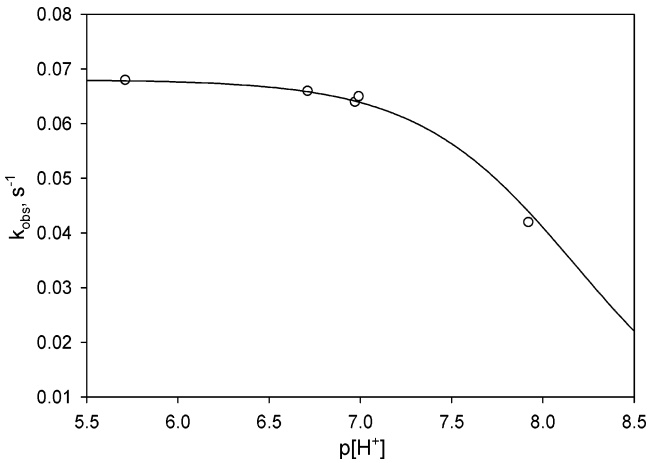

Figure 4. Effect of p[H^+] on the observed CSSC formation during ClO_2^- oxidation of cysteine. Conditions: 10 mM CSH; 1.0 mM ClO_2^-; 0.10 M $[OAc]_T$ or $[PO_4]_T$; μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 260 nm. Solid line is a fit of eq 6 using pK_{a2} = 8.18; k_2 = 3.40 ± 0.03 M^{-1} s^{-1}.

SO_2.[45] Hypochlorous acid as well as OCl^- is known to react rapidly with CSH[46] to form oxidized products of cysteine and chloride ion. Hypochlorus acid oxidation of cysteine has been reported (pH 10−13.5) with a second-order rate constant of 1.2 × 10^9 M^{-1} s^{-1}, while the reaction of OCl^- with CS^- has a rate constant of 1.9 × 10^5 M^{-1} s^{-1}.[46] The short-lived species, sulfenyl chloride, is a proposed reaction intermediate in the Canle et al. work as well as in a number of other reports.[43,47−49] Silverstein and Hager reported the reaction of the thiol TNB, 2-nitro-4-dithiobis(2-nitrobenzoic acid), with HOCl that led to the observed formation of the

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analogous sulfenyl chloride followed by hydrolysis to the sulfonic acid at pH 2.75.[47] Sulfenyl chlorides also can disproportionate by reacting with thiolate anion to form CSSC.[43] Presumably, an increase in the thiolate concentration (i.e., an increase in reaction pH) will lead to the preferential formation of CSSC relative to CSO_3H. Notably, Winterbourn and Brennan reported that increasing amounts of GSSG form with increasing reaction pH during HOCl oxidation of glutathione.[33]

In our reactions ClO_2^- is reduced to chloride ion for an overall four-electron oxidation of cysteine. The rate expression for the pH range 5.7−7.9 is given in eq 6, where $[CSH]_T = [CS^-] + [CSH]$ with a pK_{a2} value of 8.18 for the CSH group.[42] Equation 6 is derived based on the equilibrium concentration of CSH (eq 2) and the overall stoichiometry shown in eqs 7−10. The second-order rate constant (k_2) that is obtained from the pH dependence in Figure 4 equals 3.40 ± 0.03 M^{-1} s^{-1} at 25.0 °C and $\mu = 1.0$ M.

$$\frac{d[CSSC]}{dt} = 2k_2\left(\frac{[H^+]}{[H^+] + K_{a2}}\right)[CSH]_T[ClO_2^-] \qquad (6)$$

$$CSH + ClO_2^- \xrightarrow{k_2} CSOH + OCl^- \qquad (7)$$

$$CSOH + CS^- \xrightarrow{fast} CSSC + OH^- \qquad (8)$$

$$CSH + OCl^- \xrightarrow{fast} CSCl + OH^- \qquad (9)$$

$$CSCl + CS^- \xrightarrow{fast} CSSC + Cl^- \qquad (10)$$

Reaction Products. ESI/MS (positive ion) analysis of an equimolar ClO_2/CSH reaction solution at pH 5 shows both cystine ($[M + H]^+$ 240.85 *m*/*z*) and cysteic acid ($[M + H]^+$ 169.84 *m*/*z*) as products. The cysteic acid product was reported for this system under acidic conditions (pH ≤ 1) by Darkwa et al.[30] The disulfide product was reported for the reaction of cysteine with a mixture of ClO_2 and ClO_2^- at pH 7.[31] Other reports of thiol oxidation in various organic solvents with both ClO_2 and ClO_2^- gave disulfide as the major product.[50−52] The pK_a values for cystine[53] and cysteic acid[54] are given in Table 1.

Ion chromatographic analysis shows that, after ClO_2 oxidation of cysteine at pH 3.2, 93% of all of the chlorine-containing species found is in the form of chloride ion (Table 3). At pH 9.1, the chloride concentration increases with reaction time from 55% at 5 min to 84% at 20 min because of the slow ClO_2^-/CSH reaction at this pH. During cysteine oxidation by ClO_2, the oxidant is initially reduced to ClO_2^-, then to HOCl, and subsequently to Cl^-. The oxidation of cysteine and related biological thiols with HOCl is a well-known rapid reaction.[33,46,47,55] Therefore, ClO_2 behaves as a five-electron oxidant as shown in eq 11.

Table 3. Ion Chromatographic Analysis of Cl-Containing Species after ClO_2 Oxidation of Cysteine[a]

pH	Cl^- (mM)	ClO_2^- (mM)	Cl^-/ClO_2^- ratio
3.2[b]	0.13	0.01	13
9.1[c,d]	0.11	0.09	1.2
9.1[c,e]	0.16	0.02	5.3
10.3[c]	0.04	0.16	0.25

[a] Conditions: [CSH] = 1.0 mM, and 20 mM $[CO_3]_T$. [b] $[ClO_2] = 0.15$ mM. [c] $[ClO_2] = 0.20$ mM. [d] Sample injected at $t \sim 5$ min. [e] Sample injected at $t \sim 20$ min.

Table 4. pH Dependent Product Distribution for the ClO_2 Oxidation of CSH

pH	% CSSC[a]	% CSO_3H[a]	other products[b]
3.6[c]	20	73	7
4.6[c]	60	30	10
6.4[d]	64	11	21
9.1[e]	94	trace	trace

[a] Product percentages determined as percent of CSH consumed. [b] Other products taken as the remaining percent of CSH not accounted for by CSSC and CSO_3H. [c−e]Conditions: initial ClO_2/CSH ratio, (*c*) 1:3, (*d*) 1:2, and (e) 1:5, 0.25 M buffer (formate, acetate, phosphate), 25.0 °C.

$$ClO_2 \xrightarrow{+1e^-} ClO_2^- \xrightarrow{+2e^-} OCl^- \xrightarrow{+2e^-} Cl^- \qquad (11)$$

Use of ^{1}H NMR for product analysis permits the characterization of species after all of the intermediate oxidants (ClO_2^- and HOCl) have reacted. Therefore, product distribution and stoichiometry reflect the oxidation of the substrate by three different oxidants ClO_2, ClO_2^-, and HOCl. In a reaction carried out at pH 6.4 and analyzed at pH ∼ 11, the chemical shifts (ppm) of cysteine protons are H_a (3.5), H_b (2.7), and H_c (2.9). No cysteine peaks are observed after the reaction is complete; however, six new sets of peaks are formed and can be assigned to CSSC (H_a, 3.6; H_b, 2.9; H_c, 3.1) and CSO_3H (H_a, 3.7; H_b, 3.0; H_c, 3.3).

The ClO_2/CSH system reveals a pH-dependent product distribution, where CSO_3H is the major product at low pH and CSSC is the major product at higher pH (Table 4). Under acidic to neutral conditions, the concentrations of CSO_3H and CSSC detected did not account for all of the substrate consumed, although no other products were observed by ^{1}H NMR. The percentages of other products listed in Table 4 are derived solely from the difference between consumed substrate and products formed and were not characterized further. The unknown products may be a mixture of thiolsulfinates, thiolsulfonates, and cleavage products that are present in concentrations too low for detection by ^{1}H NMR.

Reaction stoichiometry also is dependent on pH as shown in Table 5. The ratio of ClO_2:CSH varies from 1:0.9 at pH 3.6 to 1:3.7 at pH 9.1. The pH dependent variation of the ratio is consistent with the balanced equations for two pH extremes by assuming that the low pH product is CSO_3H and the high pH product is CSSC. The balanced overall equations are shown in eqs 12 and 13.

$$6ClO_2 + 5CSH + 3H_2O \xrightarrow{low\ pH} 5CSO_3H + 6Cl^- + 6H^+ \qquad (12)$$

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Table 5. pH Dependent Reaction Stoichiometry for ClO_2 Oxidation of Cysteine

pH	ClO_2/CSH ratio
3.6[a]	1:0.9 (1:0.8 for 100% CSO_3H)[c]
6.5[b]	1:2.4
7.1[a]	1:2
9.5[b]	1:3.7 (1:5 for 100% CSSC)[d]

[a,b]Conditions: initial ClO_2:CSH ratio, (*a*) 1:2 and (*b*) 1:5, 0.25 M buffer (formate, acetate, phosphate), 25.0 °C. [c,d]Theoretical ratios in parentheses are based on reaction stoichiometries in (*c*) eq 12 and (*d*) eq 13.

$$2ClO_2 + 10CSH \xrightarrow{\text{high pH}} 5CSSC + 2Cl^- + 2H^+ + 4H_2O \quad (13)$$

Therefore, at low pH the expected ClO_2/CSH ratio is 6:5 (or 1:0.83) which agrees with the experimental ratio of 1:0.9 at pH 3.6 where CSO_3H is the major product. This stoichiometry is consistent with the proposed reduction of ClO_2 to chloride ion and the resulting oxidation of substrate by three different oxidants, namely, ClO_2, ClO_2^-, and HOCl. The high pH extreme has an expected ClO_2/CSH ratio of 1:5 when CSSC is the major product. Our experiments give a ratio of 1:3.7 at pH 9.1 where CSSC is the major product. The discrepancy between the expected ratio and the experimental value may be due to the incomplete reaction of ClO_2^- with the substrate prior to analysis because this reaction is very slow at high pH.

The product distribution in the ClO_2^- oxidation of cysteine is also found to depend on reaction pH. Analogous to the ClO_2 reaction, the major product at low pH is cysteic acid while cystine is the major product at high pH (Table 2). The reaction stoichiometry is again pH dependent as shown in Table 6. The observed stoichiometry reflects substrate oxidation by both ClO_2^- and the subsequently formed HOCl. The balanced equations for both the low and the high pH pathways are shown in eqs 14 and 15.

$$3ClO_2^- + 2CSH \xrightarrow{\text{low pH}} 2CSO_3H + 3Cl^- \quad (14)$$

$$ClO_2^- + 4CSH \xrightarrow{\text{high pH}} 2CSSC + Cl^- + 2H_2O \quad (15)$$

Experimentally, the low pH reaction shows a ClO_2^-/CSH ratio of 1:0.7 (consistent with the 3:2 ratio in eq 14). The expected ratio for the higher pH is 1:4 when CSSC is the only product. Our experiments yield a ratio of 1:3.5 at pH 6.52, in general agreement with the stoichiometry predicted by eq 15.

The pH dependent product distribution in the ClO_2 and ClO_2^- oxidation of cysteine (Tables 2 and 4) indicates that both oxidants favor the formation of CSO_3H at low pH and the formation of CSSC at higher pH. The HOCl and OCl^- reactions with cysteine and other thiols show a similar dependence of product distribution on pH.[33,43,47] Therefore, all three oxidants react with cysteine through competing pathways to give cysteic acid at low pH and cystine at higher pH.

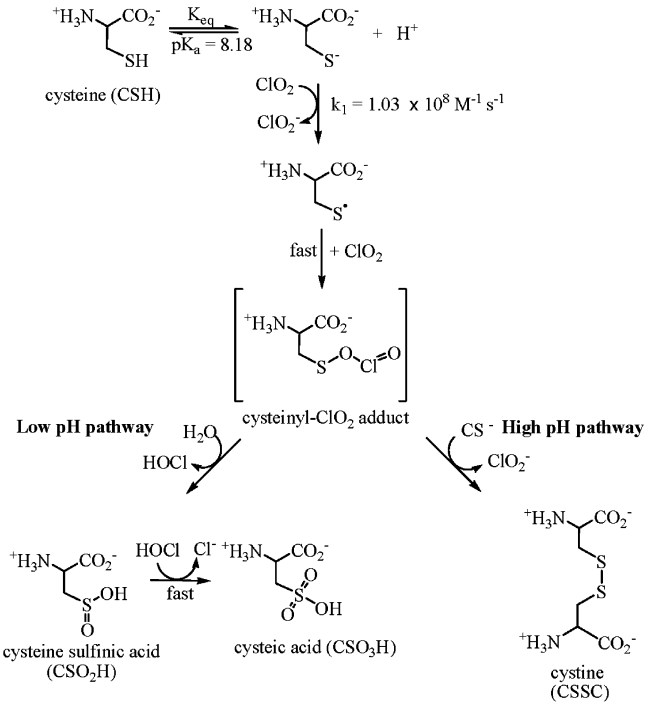
Reaction Mechanism for the ClO_2 Oxidation of Cysteine. The proposed mechanism for the reaction of ClO_2 with cysteine is shown in Scheme 1. Our pH dependence studies

Table 6. pH Dependent Reaction Stoichiometry for ClO_2^- Oxidation of Cysteine[a]

pH	ClO_2^-/CSH ratio
2.77	1:0.7 (1:0.7 for 100% CSO_3H)[b]
4.5	1:1.5
5.95	1:2.3
6.52	1:3.5 (1:4 for 100% CSSC)[c]

[a] Conditions: initial ClO_2^- ratio 1:4, 0.25 M buffer (formate, acetate, phosphate), 25.0 °C. [b,c]Theoretical ratios based on reaction stoichiometries in (*b*) eq 14 and (*c*) eq 15.

Scheme 1. Proposed Mechanism for the Initial Reactions between ClO_2 and CSH and Subsequent Decay of the Cysteinyl−ClO_2 Adduct



show that the reactive species is the thiolate anion and the rate-determining step is an electron abstraction from CS^- by ClO_2 to form the cysteinyl radical. We propose that the cysteinyl radical reacts rapidly with another equivalent of ClO_2 to give a cysteinyl−ClO_2 adduct. The observed kinetics are consistent with the rate expression in eq 5 for the entire pH range at all concentrations of cysteine; however, the ratio of the two major products varies greatly with pH. This indicates that a common intermediate such as the cysteinyl−ClO_2 adduct is present. Although adduct formation cannot be observed directly in this system, we propose its formation based on product distribution and on the observation of analogous ClO_2 adducts with other amino acids such as tyrosine[28] and tryptophan.[56] The cysteinyl−ClO_2 adduct is proposed to disproportionate by two pH-dependent pathways based on the product distribution data. The possibility that CSO_3H is formed by the ClO_2 oxidation of CSSC is eliminated because the reaction was found to be exceedingly slow. The half-life for the ClO_2 oxidation of CSSC at pH 6.7 is approximately 960 s. Under the same conditions, $t_{1/2} = 3 \times 10^{-5}$ s for the ClO_2 oxidation of cysteine (seven orders of magnitude faster than the oxidation of CSSC). According

(56) Stewart, D. J.; Napolitano, M. J.; Margerum, D. W. To be submitted for publication.

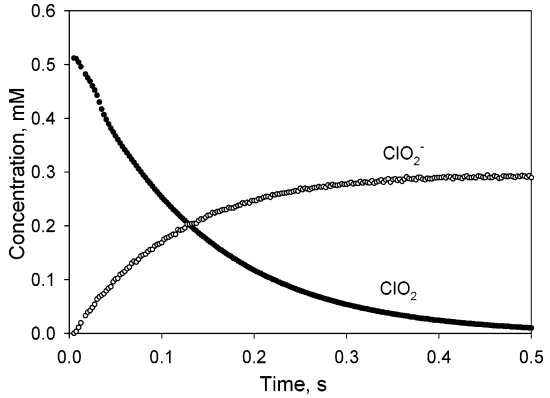
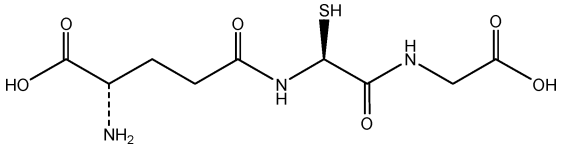


Figure 5. ClO_2 decay and formation of ClO_2^- during the ClO_2 oxidation of CSH. Conditions: 5.0 mM CSH; 0.54 mM ClO_2; 0.10 M formate; p[H^+] 2.73; μ = 1.0 M ($NaClO_4$); 25.0 °C. $k_{obs}(ClO_2)$ = 7.52 ± 0.01 s^{-1}. $k_{obs}(ClO_2^-)$ = 9.42 ± 0.05 s^{-1}.

Scheme 2. Structure of Glutathione (γ-Glutamylcysteinylglycine, GSH)



to the proposed mechanism (Scheme 1), the low pH pathway produces one ClO_2^- and one Cl^- for every 2 equiv of ClO_2 consumed. The stoichiometric ratio of ClO_2 to ClO_2^- at low pH was examined by stopped flow methods. (Slower methods of analysis prevent the quantitative determination of ClO_2^-, because it reacts further with the substrate.) In the reaction of ClO_2 with excess CSH at p[H^+] 2.73, half of the consumed ClO_2 (0.54 mM initial concentration) is converted to ClO_2^- (0.27 mM formed; Figure 5). This observation is consistent with the proposed low pH pathway where 1 equiv of ClO_2 leads to the formation of a cysteinyl radical and 1 equiv of ClO_2^-. This is followed by the reaction of another equivalent ClO_2 to form the adduct. Rapid hydrolysis of the adduct and reaction with HOCl (the HOCl reaction likely involves the CSCl intermediate, but this is not shown explicitly in Scheme 1) form the CSO_3H product and 1 equiv of chloride ion.

The high pH pathway predicts the quantitative conversion of ClO_2 to ClO_2^- (Scheme 1). The subsequent reaction of ClO_2^- with cysteine is very slow at high pH; therefore, the analysis of chlorine-containing species that remains after the reaction was done by ion chromatography. At pH 10.3 (Table 3) the amount of ClO_2^- found is approximately 0.16 mM, where the expected amount is 0.20 mM based on the initial ClO_2 used. The remaining chlorine is accounted for by the formation of the chloride ion (0.04 mM). The slightly lower yield of ClO_2^- than expected for quantitative conversion may be due to some ClO_2^- reaction with the remaining cysteine prior to analysis.

ClO_2 and ClO_2^- Oxidation of Glutathione. The oxidation of glutathione (Scheme 2) by chlorine dioxide is studied under pseudo-first-order conditions with excess tripeptide from p[H^+] 3 to p[H^+] 6 and μ = 1.0 M. Chlorine dioxide loss (359 nm) gives first-order fits (Figure 6). First-order rate constants vary linearly with increasing substrate concentration at constant pH, as expected for a first-order dependence in glutathione. The sharp increase in the observed rate constant with increasing pH (Figure 6, inset) suggests that the reactive portion of the tripeptide is the deprotonated SH group as in the ClO_2/cysteine reaction. Equations 2−4 can be used to describe the ClO_2 oxidation of GSH. The fit of the data in Figure 6 to eq 5a with a pK_{a3} value of 8.74[57] for the SH group of glutathione gives a second-order rate constant k_3 = 1.40 (0.01) × 10^8 M^{-1} s^{-1}. The similarity between the second-order rate constants in the ClO_2 oxidation of gluathione and cysteine provides further evidence that the primary attack of ClO_2 is at the SH portion of the tripeptide.

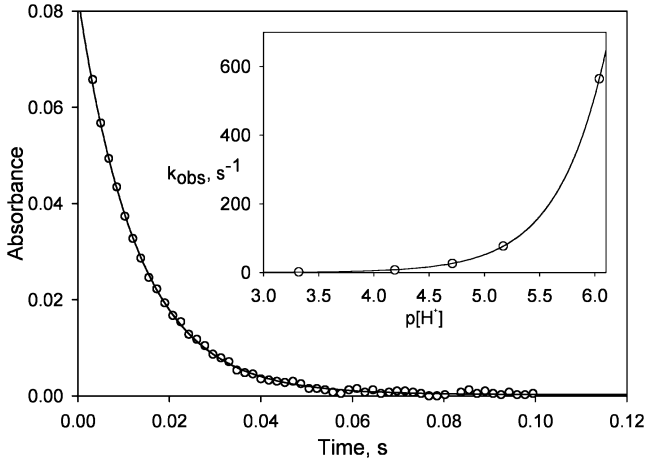


Figure 6. Kinetic trace and first-order fit for ClO_2 decay during oxidation of glutathione. Conditions: 1.0 mM GSH; 0.10 mM ClO_2; 0.10 M buffer; p[H^+] = 5.17; μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 359 nm; k_{obs} = 76.8 s^{-1}. Inset: p[H^+] dependence on the observed ClO_2 decay during glutathione oxidation. Conditions: 1.0 mM GSH; 0.10 mM ClO_2; p[H^+] 3.32−6.04; 0.10 M buffer; formate (p[H^+] 3.32); acetate (p[H^+] 4.19−5.17); phosphate (p[H^+] 6.04); μ = 1.0 M ($NaClO_4$); 25.0 °C; λ = 359 nm; Solid line is a fit of eq 5a using pK_{a2} = 8.74: k_1 = 1.40 (0.01) × 10^8 M^{-1} s^{-1}; λ = 359 nm, cell path = 0.962 cm.

Chlorite oxidation of glutathione shows similar behavior as the chlorite oxidation of cysteine. The ClO_2^-/GSH reaction is approximately six orders of magnitude slower than in the ClO_2/GSH reaction. The relative reactivity of ClO_2 and ClO_2^- with glutathione is similar to their reactivities with cysteine. The kinetics are followed by monitoring the formation of product at 260 nm and give first-order fits under pseudo-first-order conditions with glutathione in excess. The reactivity decreases above pH 7 as the pK_{a3} (8.74) of the S−H group is approached. The decrease in the first-order rate constant with increasing pH is due to the absence of reactivity of the deprotonated SH group and is analogous to the results seen in the ClO_2^-/cysteine reaction. The kinetic behavior of the glutathione reaction is almost identical to the cysteine system and indicates that the mechanistic details are similar for both substrates.

Conclusions

A mechanism for the oxidation of cysteine by chlorine dioxide is proposed. The rate-determining step is the single electron transfer from CS^- to ClO_2 to form a cysteinyl radical and chlorite ion. The subsequent step is the rapid coupling

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of the cysteinyl radical and a second equivalent of ClO_2 to form a cysteinyl−OClO intermediate. This common intermediate reacts further by two pH dependent pathways that lead to the observed products. The low pH pathway favors the formation of CSO_3H, and the higher pH pathway favors the formation of CSSC. Stoichiometric studies reveal that ClO_2 behaves as a five-electron oxidant as the subsequently formed ClO_2^- and HOCl also react with cysteine to give the observed products. Independent studies of the ClO_2^-/cysteine reaction show the oxidation to be 6 orders of magnitude slower than the ClO_2 reaction. Studies of pH effects show that the most likely mechanism of chlorite oxidation of cysteine is oxygen transfer to CSH instead of electron transfer from the CS^- anion as found for ClO_2. Although the mechanism of cysteine oxidation by ClO_2 is very different from oxidation by ClO_2^-, the products of both reactions are the same with a similar pH dependent product distribution.

The ClO_2 oxidation of glutathione was also investigated because of its abundance in cells and its function in maintaining cellular redox potentials. The kinetic behavior of GSH oxidation by both ClO_2 and ClO_2^- follows closely to that found in the cysteine system. Therefore, we conclude that the primary reactive site of GSH is the thiol group and that the mechanistic details proposed in the cysteine system apply to glutathione. Furthermore, the second-order rate constants for electron transfer to ClO_2 from both anions of cysteine and anions of glutathione are very close in value.

The Margerum group has been interested in the reactivity of ClO_2 with amino acids and nucleic acids. Three of the most reactive amino acids (cysteine, tyrosine, and tryptophan) and the nucleic acid 5′-guanosine monophosphate (5′GMP)[58] have been investigated. The relative reactivities within this group place cysteine as the most reactive substrate. The following order was calculated at pH 7.0 based on the experimental data; cysteine 1.0×10^7 M $s^{-1} \gg$ tyrosine 1.8×10^5 M s^{-1} > tryptophan 3.4×10^4 M s^{-1} > 5′GMP 1.5×10^3 M s^{-1}. The thiol group of cysteine is the most nucleophilic of the group and is the most reactive. Therefore, if the anti-viral and anti-bacterial power of ClO_2 stems from its ability to destroy protein function, the primary target could be the exposed SH group of biological thiols.

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Supporting Information Available: Molar absorptivity values, ^1H NMR spectrum, p[H^+] dependence, and effect of glutathione concentration on the observed rate constant of ClO_2 decay at constant pH. This material is available free of charge via the Internet at http://pubs.acs.org.

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