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NMR study of thiosulfate-assisted oxidation of L-cysteine

Tatyana V. Berestova,*[a] Liana A. Khamitova,[a] Olga V. Lusina,[a] Ludmila G. Kuzina,[a] Alexander N. Lobov[b] and Akhat G. Mustafin[a,b]

[a] *Department of Chemistry, Bashkir State University, 450074 Ufa, Russian Federation. E-mail: berestovatv@gmail.com*

[b] *Ufa Institute of Chemistry, Ufa Federal Research Center of the Russian Academy of Sciences, 450054 Ufa, Russian Federation*

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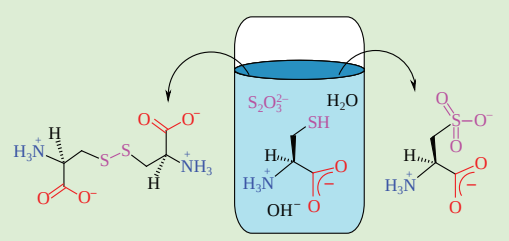
The reaction of L-cysteine with sodium thiosulfate in aqueous solution at pH 9 affords mainly L-cystine with noticeable amounts of L-cysteine sulfonic anion $^{-}O_2CCH(NH_3^+)CH_2SO_3^-$. NMR study revealed the formation of intermediate L-cysteine sulfenic acid and L-cysteine *S*-sulfite, the latter existing in two active forms $^{-}O_2CCH(NH_2)CH_2S(=S)O_2^-$ and $^{-}O_2CCH(NH_2)CH_2SSO_2^-$.



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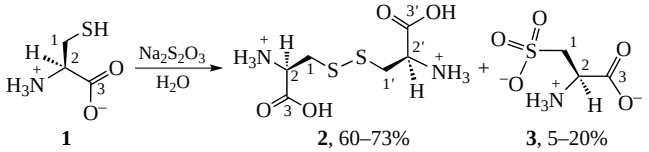
Studies on the biological role of sulfur-containing amino acids and the mechanism of their oxidation depending on the reaction conditions are important and relevant tasks in the modern biochemistry. The interest in the oxidation of L-cysteine[1–3] and other thiols[4,5] with potential biological activity is caused by its biological role in the organism, which involves their participation in post-translational modifications of proteins, intercellular signal transmission, redox homeostasis, regulation of gene expression or binding of heavy metals. It has recently been shown that the oxidation of cysteine residues of SARS-CoV-2 S-glycoprotein reinforces the binding of viral particles to the cellular receptors of the cell.[7]

Oxidation of thiols[1,7] can proceed in several directions depending on the oxidant nature and the reaction conditions.[8] The oxidative processes of biologically active thiols are best carried out in the presence of H_2O_2,[9–12] $NaClO_3$ or ClO_2.[13] The reactions of L-cysteine with sulfur-containing compounds giving L-cysteine derivatives with various states of sulfur oxidation are documented.[14] Similar reactions of L-cysteine oxidation can take place in biological systems with participation or formation of sulfur-containing anions, including thiosulfate.[15–17] The oxidation of L-cysteine can occur at different rates depending on the reaction conditions, the molecular structure of thiol[9–11] and the oxidant nature.[12,13] The oxidation of thiols most often results in L-cysteine sulfenic, sulfinic and sulfonic acids, as well as sulfenamide, disulfide and other sulfur-containing compounds.[1,9,10,12,18] The reactions of L-cysteine with H_2O_2 and other sources of reactive oxygen species occurs quite quickly. Currently, the mechanism of this process is being studied intensely by NMR[13,19] or UV spectroscopy,[20] chromatographic methods[10,12,21] and some others techniques.

The oxidation rate of thiols in the presence of Fe^{III} (refs. 12, 21–23) and Cu^{III} (refs. 19, 20) compounds is rather high and, as a rule, the reaction results in disulfides. This reaction has been studied most thoroughly in the presence of Cu^{II} salts.[19,20] In this case, the metal ions can act as the oxidizing agents in this reaction: $\varphi°(Fe^{III}/Fe^{II}) = 0.77$ V; $\varphi°(Cu^{II}/Cu^{I}) = 0.34$ V, while L-cysteine acts as the reducing agent: $\varphi°$(L-cysH/(L-cystH) = 0.025 V.[24] L-Cystine is usually the final product in the reaction of L-cysteine with Cu^{II} salts, and the reaction proceeds through the formation of metal complexes Cu^{I}–L-cysteine.[19,20] This is a distinguishing feature of L-cysteine compared to other aliphatic α-amino acids that generally form chelate N,O-complexes in reactions with Cu^{II}.[25–29] Moreover, the formation of biologically active disulfides from thiols is an important in vital activity, and similar reactions attract the attention of many researchers.[9–13,19–23]

In this communication, we report on the reaction of sodium thiosulfate with L-cysteine **1** that is of interest as a counter synthesis of oxidation products, depending on the nature of the oxidizing agent and pH value (Scheme 1). It has been found that the oxidation of L-cysteine solution in the presence of $S_2O_3^{2-}$ is actually S-thiolation as it occurs through the formation of L-cysteine *S*-sulfite **3**. In fact, the oxidation of L-cysteine **1** in the presence of $Na_2S_2O_3$ to give L-cystine **2** occurs slowly in 3 h. The yield of disulfide **2** is about 73% that allows intermediate adducts to be identified in the reaction solution. The formation of L-cystine **2** (60–73%) and L-cysteine *S*-sulfite anion **3** (5–20%) is achieved with the use of equivalent amounts of the reactants within 3–5 h (see Scheme 1).

This reaction represents self-oxidation/self-healing process. The reaction products were analyzed by NMR and IR spectroscopy and by the ESI-MS method. Determination of L-cystine **2**, the major product, can be performed quite correctly by IR spectroscopy, because IR spectra of L-cysteine **1** and L-cystine **2** differ significantly.[30] These differences are primarily



1 **2**, 60–73% **3**, 5–20%

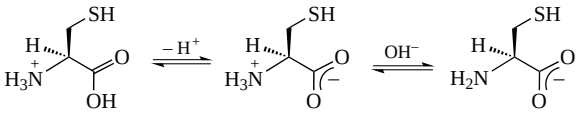
Scheme 1

due to the properties of crystal packing of various forms of molecules of poorly soluble L-cystine.[31,32]

It is known that appearance of ^{1}H NMR spectra of amino acids having a zwitterionic form at the isoelectric point depend on the pH value. Therefore, the ^{1}H NMR spectra of cysteine **1** were initially studied at various pH values in order to compare the relative signal shifts. An aqueous solution of L-cysteine was studied directly in an NMR tube, and we have found that zwitterion form of L-cysteine **1** in solution was generated at pH ~ 6 which corresponded to its isoelectric point. The addition of an HCl or NaOH solution to zwitterion **1** resulted in protonated L-cysH_2^+ **4** or deprotonated L-cys$^-$ **5** forms of L-cysteine, respectively (Scheme 2). The corresponding changes in chemical shifts for **4** and **5** relative to zwitterion **1** were observed in the ^{1}H NMR spectra (Table 1). With pH growth, there is a tendency of rather large upfield shift for the proton at the asymmetric center of L-cysteine in the ^{1}H NMR spectra.[33] The protons for the methylene moiety are less variable, however, there is also a tendency for the signals to undergo some upfield shift at pH 9 compared to pH 3. At the same time, the ^{13}C NMR spectra show no significant shift of carbon atoms with pH variation. Thus, the ^{13}C NMR method makes it possible to determine cysteine-containing compounds with sufficient accuracy in solutions having various pH values.

A study of the oxidation of L-cysteine with $Na_2S_2O_3$ was carried out in D_2O by NMR spectroscopy using two-dimensional methods. It was found that, along with the major oxidation products, L-cystine **2** and L-cysteine sulfonic anion (L-cysO_3^-) **3**,[13,34] the reaction mixture also contained the L-cysteine *S*-sulfite anion (L-cysSO_2^-) **6** and L-cysteine sulfenic acid (L-cysOH) **7** as intermediate products.[14,35] Identification of compounds **3**, **6**, **7** was made by the ESI-MS method and was based on literature data.[13,34–36] The most probable form of ions **3**, **6**, **7** in solution at pH 3 and pH 9 was determined using the Marvin program.† Some ^{1}H NMR spectra of compounds **3** and **7** at pH 3 were taken from literature.[13,14,30] Also, for a correct assessment and analysis of the NMR spectra of compounds **3**, **6**, **7**, theoretical ^{1}H and ^{13}C spectra were simulated for the compounds under study using the Marvin program,† ChemDrawUltra program‡ and quantum chemical calculations (for details, see Online Supplementary Materials). The theoretical calculations in ChemDrawUltra correlated well with experimental data compared to other methods.

According to ^{1}H NMR spectroscopy data, in the beginning of the reaction after mixing the L-cysteine **1** and $Na_2S_2O_3$ solutions in the ratio of 1 : 1 at room temperature (pH ~ 8–9), we observed a gradual transition from L-cysteine **1** to L-cysteine *S*-sulfite



4 (L-cysH_2^+) **1** (L-cysH) **5** (L-cys$^-$)

Scheme 2

Table 1 ^{1}H, ^{13}C and ^{15}N NMR of different forms of L-cysteine in solution depending on pH value.

Cysteine form	pH	δ ^{1}H NMR		δ ^{13}C NMR			δ ^{15}N NMR
		CH_AH_B	CH_C	C_1	C_2	C_3	NH_2/NH_3^+
L-cysH_2^+ **4**	3	3.07 dd, 3.14 dd	4.16 dd	24.61	55.33	171.49	36.36
L-cysH **1**	6	3.02 dd, 3.10 dd	3.98 dd	24.98	55.95	172.42	37.25
L-cys$^-$ **5**	9	3.00 dd, 3.04 dd	3.89 dd	25.28	56.23	173.13	37.54

† *Marvin. ChemAxon*. https://chemaxon.com/products/marvin (accessed 29 March 2022).

‡ https://perkinelmerinformatics.com/products/research/chemdraw/.

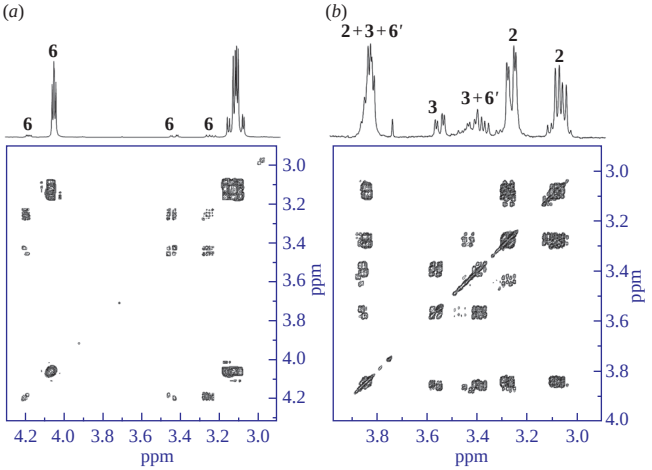


Figure 1 {^{1}H, ^{1}H} COSY spectra after combining the stock solutions of L-cysteine and $Na_2S_2O_3$ in the ratio of 1 : 1 (*a*) in 60 min after the reaction start, (*b*) in 10 days at the end of the reaction.

anion **6** [Figure 1(*a*)]. Moreover, a white precipitate of sparingly soluble L-cystine **2** appeared in the tube during the reaction. The spectra contained three double doublets at 3.24, 3.44 ppm for H_A and H_B protons, respectively, and at 4.18 ppm for H_C proton. They were assigned to L-cysteine *S*-sulfite **6** [Figures 1(*a*) and 2(*a*)]. In the ^{13}C NMR spectra, the signal for the carbon atom C^1 in compound **6** is located at 37.84 ppm, and that for C^2 at 53.36 ppm. The molecular mass of **6** corresponds to the L-cysteine *S*-sulfite anion, *m/z* 185.1154 (for details, see Online Supplementary Materials). Moreover, the ^{1}H NMR spectra of the reaction mixture showed the presence of trace amounts of L-cysOH **7**, as proven by ESI-MS. For compound **7** at pH 9 in D_2O, a set of doubled signals was observed in the ^{1}H NMR spectrum: dd 2.89 for H_A and dd 3.05 for H_B, along with 4.20 ppm for the H_C proton.

Additional NMR experiment was carried out when the main product, L-cystine **2**, was removed from the reaction mixture by filtration. An equilibrium system with a considerable amount of product **7** was established in the resulting solution [see Figure 2(*b*)]. At the same time, chemical shifts for product **2** were almost not detected. Thus, in this case, an equilibrium system was observed when compounds **1**, **6** and **7** were present simultaneously. In addition, an analogous product **6′** was detected along with product **6**. Apparently, intermediate **6** can exist as two tautomers **6** and **6′** (Scheme 3). Compound **6** can be converted into L-cysteine sulfenic acid **7** with subsequent formation of L-cystine **2**, while the adduct **6′** promotes the formation of L-cysteine sulfonic anion **3** (at pH 9), and possibly other sulfur-containing products of L-cysteine oxidation (see Scheme 3).

Based on the results obtained, we suggested a scheme for the oxidation of L-cysteine with participation of $Na_2S_2O_3$ to give L-cystine and additional product **3** (see Scheme 3). In the first stage of the reaction of L-cysteine **1** with $Na_2S_2O_3$, L-cysteine *S*-sulfite anion **6** may be initially formed almost immediately after mixing the reagents [see Figures 1(*a*) and 2(*a*)]. At the same time, the pH of the solution would grow significantly due to the release of hydroxide ions OH^-. In addition, hydrogen sulfide is formed as a reaction side product which is released in the first stage of the process (step *A*, see Scheme 3). Thus, in the NMR spectra recorded during the reaction we observed a gradual disappearance of characteristic signals for L-cysteine **1** and the appearance of L-cysSO_2^- **6** peaks, and then the appearance of traces of L-cysOH **7** and a slow accumulation of L-cystine **2**. In addition, at the end of the reaction (after 10 days), the appearance of characteristic peaks for L-cysO_3^- **3** was observed (see Figure 2).

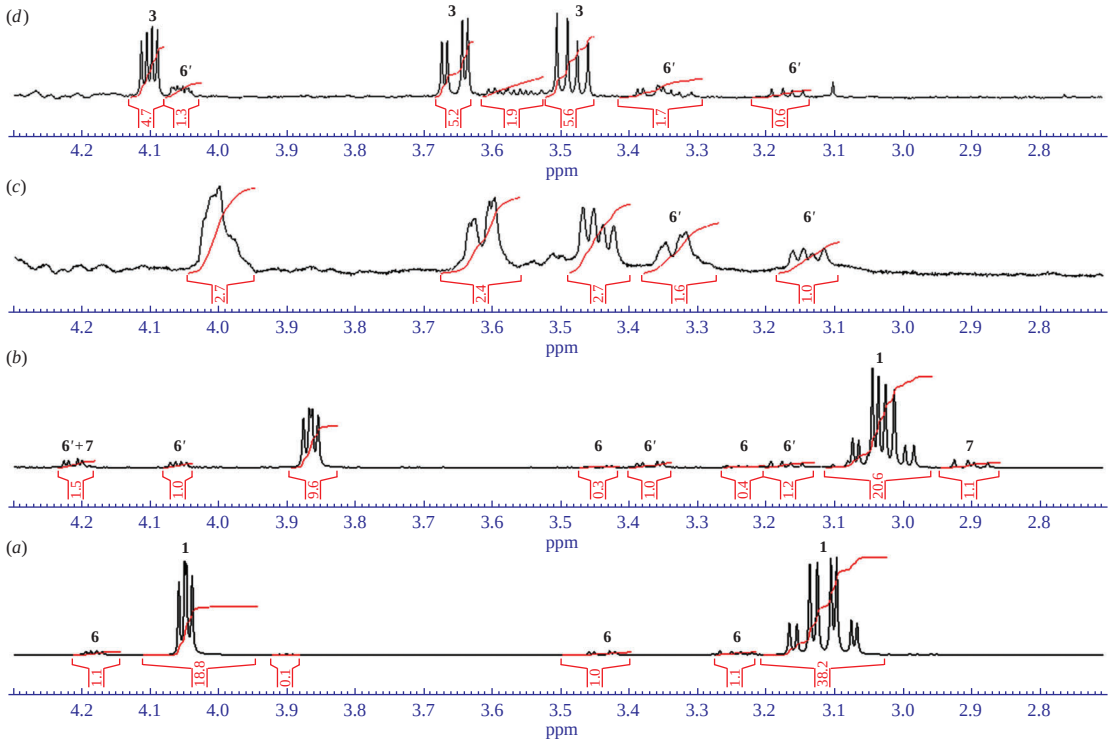
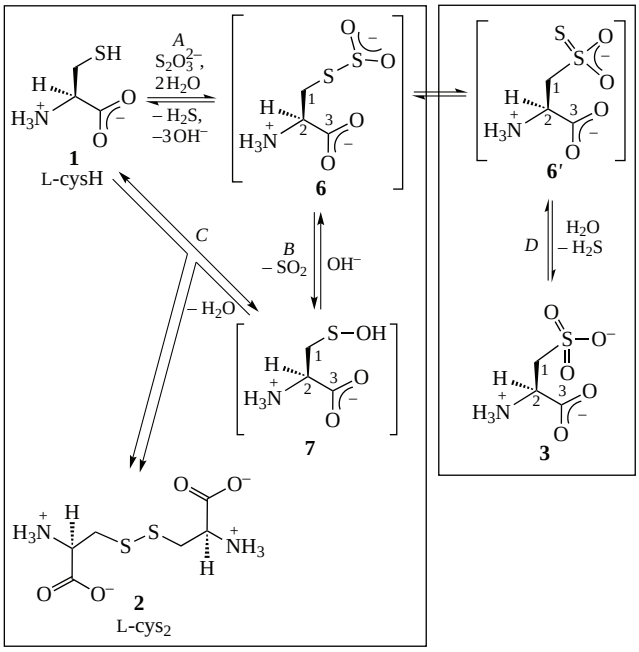


Figure 2 ^{1}H NMR spectra after mixing the solutions of L-cysteine **1** and of $Na_2S_2O_3$ in the ratio of 1 : 1 (after the resulting precipitate of **2** was filtered off): (*a*) in 60 min after the reaction start; (*b*) in 3 days in a closed tube; (*c*) in 10 days in a closed tube; (*d*) in 30 days in a closed tube.



Scheme 3

According to the suggested scheme, in step *A*, $S_2O_3^{2-}$ is attached to L-cysteine **1** to form L-cysSO_2^- **6** with evolution of H_2S. Compound L-cysSO_2^- **6** may convert into L-cysOH **7** with evolution of SO_2[37] (step *B*). At the same time, the pH value increases even more through the release of OH^- ions. Apparently, L-cysteine sulfenic acid **7** is a very reactive compound[38] and it is capable of reacting with starting L-cysteine **1** (step *C*) to produce major product L-cystine **2**.[39] In this case, disulfide **2** would precipitate due to poor solubility.[31,32] The formation of product **3** that is observed in the end of the reaction in an amount of 6–20% is apparently due to formation of compound **6′** in the reaction medium. In this case, in an aqueous reaction mixture containing product **6′** the rearrangement of the S–O bond can occur with formation of **3** and release of H_2S (step *D*).

To conclude, detailed NMR study of the oxidation of L-cysteine with sulfur-containing salts with formation of L-cystine gave new insight into the reaction mechanism. This process is essentially S-thiolation comprising formation of L-cysteine *S*-sulfite anion (L-cysSO_2^-) having a S–S bond. Also, L-cysteine sulfenic acid **7** that formed was detected as an intermediate in the oxidation of the L-cysteine. The reaction of cysteine with thiosulfate is an equilibrium process involving L-cysteine *S*-sulfite anion and L-cysteine sulfenic acid as the key intermediates. The reaction can occur in two directions because of the possible transition **6**↔**6′**. It is assumed that compound **7** eventually leads to L-cystine **2**, whereas **6′** gives L-cysteine sulfonic anion. Thus, the shift of the **6**↔**6′** equilibrium in a particular direction determines the formation of L-cystine and L-cysteine sulfonic anion in this reaction.

This study was performed in accordance with state assignments UIC UFRC RAS no. 1021062311386-8-1.4.1 ‘Methods of chromatography, mass spectrometry, IR, UV, EPR and NMR spectroscopy for establishing the structure and identification of organic, bioorganic molecules and polymers’.

NMR spectra were recorded using the equipment at the Center for Collective Use ‘Chemistry’ of the Ufa Institute of Chemistry of the UFRC RAS and RCCU ‘Agidel’ of the UFRC RAS.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2023.01.031.

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