Kinetics and thermodynamics of oxidation mediated reaction in L-cysteine and its methyl and ethyl esters in dimethyl sulfoxide-d₆ by NMR spectroscopy.

Ryan J. Dougherty^{1,†}, Jaideep Singh¹ and V.V. Krishnan^{1,2,*}

Abstract

L-Cysteine (L-Cys), L-Cysteine methyl ester (L-CysME) or L-Cysteine ethyl ester (L-CysEE), when dissolved in dimethyl sulfoxide, undergoes an oxidation process. This process is slow enough and leads to nuclear magnetic resonance (NMR) spectral changes that could be monitored in real time. The oxidation mediated transition is modeled as a pseudo-first order kinetics and the thermodynamic parameters are estimated using the Eyring's formulation. L-Cysteine and their esters are often used as biological models due to the remarkable thiol group that can be found in different oxidation states. This oxidation mediated transition is due to the combination of thiol oxidation to a disulfide followed by solvent-induced effects may be relevant in designing cysteine-based molecular models.

Keywords:

NMR, ^1H , ^{13}C , oxidation, kinetics, solvent effect, L-Cysteine. L-Cysteine methyl ester (L-CysME) and L-Cysteine ethyl ester (L-CysEE).

Short title

Oxidation of L-Cys and ester derivatives in DMSO by NMR

Graphical Abstract

- Oxidation induced changes of L-Cysteine and its methyl and ethyl esters in DMSO-d₆
- Real time NMR measurements
- Kinetic and thermodynamic parameters via pseudo-first-order rate process.

Introduction

L-Cysteine (L-Cys) is one of the natural sulfur-containing amino acids. With the capacity to form disulfide bridges, it has an integral role in protein structure. L-Cys and their derivatives are often used as biological models due to the remarkable thiol group that can be found in different oxidation states, and thus dictating the biological function of the proteins. For example, the functional derivative of L-Cys, L-Cysteine ethyl ester (L-CysEE), has many applications in chemistry, biochemistry, food sciences, and biomedical research [1-4]. L-CysEE has been used in the chemical synthesis of the majority of nonsteroidal anti-inflammatory drugs (NSAIDs) due to the presence of the SH functional group (to confer antioxidant properties) and the presence of the natural amino acid scaffold (for reduced toxicity) [1]. Furthermore, the thiol group of cysteine takes part in a variety of biochemical reactions such as the formation of a weak hydrogen bond at receptor sites and thus to initiate or mediate biological responses. L-CysME or L-CysEE is also used as a biomimetic ligand for metal complexes that contain Ni or Zn. These metal complexes serve as models for structural motifs such as zinc fingers [5, 6].

In previous reports, it has been shown that thiols can be directly oxidized by DMSO to a corresponding disulfide [2, 7-13]. When L-Cys and their ester derivatives were dissolved in DMSO, we observed a similar oxidation event. However, to our surprise, we also observed what we believe to be a change in the resulting disulfide, that is predominantly due to the oxidation of thiol along with other solvent-mediated interactions. This observed oxidation mediated change was slow enough to be monitored in real time using NMR spectroscopy. This resulting change is much slower than the characteristic time of the typical NMR experiment and leads to significant changes in the NMR spectrum that could be measured as a function of time. The data fit a *pseudo*-first order process with respect to L-Cys at the temperature range studied. This agrees quite nicely with the rate law proposed by Wallace and Mahon and a detailed derivation of that rate law can be found in their seminal study[13]. The

enthalpic and entropic contributions to the reaction were then estimated via Eyring Plot. Further evidence of an oxidatively induced change was observed from chemical shift changes in the ^{13}C -NMR data. The observed changes and the derived thermodynamic parameters are similar between all the three molecules studied.

Materials and Methods

Materials

Commercially, L-Cys and their ester derivatives are readily available as hydrochloride salts. L-Cysteine, L-Cysteine methyl ester, and L-Cysteine ethyl ester hydrochloride were purchased from Sigma-Aldrich (98% purity) and used with no further modification. Deuterated dimethyl sulfoxide spiked with 0.05% by volume with tetramethylsilane (TMS) was used as a solvent and internal reference (Sigma-Aldrich).

NMR measurements

All the ^1H and ^{13}C NMR experiments were performed in a 400-MHz (^1H resonance frequency) VNMRs spectrometer (Varian-Agilent) and using the one-NMR probe. Measurement of the exact sample temperatures in the NMR probe was calibrated using the chemical shift changes in neat methanol standard (CH_3OH) [14]. The probe temperature was set between 30 to 55 °C in the spectrometer, and the calibrated sample temperature was used in the calculations. Each sample was prepared by freshly dissolving in DMSO-d_6 to a concentration of ~243 mM just before the start of the NMR experiment. The probe was tuned and the pulse width was calibrated at each temperature using the experimental sample. The approximate delay from the time the sample is prepared to start the collection of the first transient was ten minutes. One-dimensional experiments were performed in an arrayed fashion. All the 1D NMR experiments were performed at a pulse angle that corresponds to Ernst angle ($\sim 70^\circ$) [15]. Each one-dimensional experiment was collected over 8 transients, with an acquisition time per FID (free induction decay) of 2.043 s, and a relaxation recycling delay of 4 s. The total time for each experiment in the array was 48.34 s. The array continuously collected until the reaction was almost completed. The reaction completion time ranges from 2 hours at high temperature to >16 hours at low temperatures. All the spectra were processed using Mestrenova (Mestrelab Research, Santiago de Compostela, Spain).

Reaction kinetics:

The oxidation mediated change is modeled as a pseudo-first order reaction process. Ignoring the reverse reaction, the differential rate law can be written as

$$\frac{d[C]}{dt} = k'[C][DMSO] \quad [1]$$

where $[C]$ is considered representative of either one of the three molecules studied; L-Cysteine, L-Cysteine methyl ester (L-CysME) and L-Cysteine ethyl ester (L-CysEE). Upon rearranging Eq. [1], and defining a *pseudo* first order rate constant $k = k' \times [DMSO]$, giving rise to the simplified rate equation,

$$\frac{d[C]}{dt} = k[C] \quad [2]$$

The simplified rate equation (Eq. [2]) can be used to describe the kinetics by virtue of the fact that the total concentration of remains the same, and that are no other any side reactions during the measurement time of the kinetics.

The concentration of reactant and product were calculated according to

$$[C] = \frac{[A]}{[S]} [C]_0 \quad [3]$$

Where $[A]$ is the area of the NMR signal of the reactant, $[S]$ is the total area of the NMR signals from the product, and $[C]_0$ is the initial concentration of the reactant at each temperature. Solution to equation [3] is given by

$$[C] = [C]_0 e^{-kt} \text{ or } [C] = (1 - [C]_0 e^{-kt}) \quad [4]$$

With [C] is defined in Eq. [3]. A linear fit between the estimated transition rates (natural logarithm scale) vs. inverse of temperature in K ($1000/T$) was used to estimate the activation energy of the Eyring equation given by

$$\ln\left(\frac{kh}{k_B T}\right) = \left(\frac{-\Delta H^\ddagger}{R}\right)\left(\frac{1}{T}\right) + \frac{\Delta S^\ddagger}{R} \quad [5]$$

Where h is Planks constant ($6.626\ 97 \times 10^{-34}$ J·s), k_B is Boltzmann constant (1.3807×10^{-23} J/K), R is gas constant (8.314 J/ (K·mol)), and the T is sample temperature in Kelvins. The enthalpy $\Delta H^\ddagger$ was calculated by multiplying the negative slope by R and the entropy $\Delta S^\ddagger$ was calculated by multiplying the intercept value by R .

Results

The oxidation mediated change ensuring the formation of the disulfide adduct is modeled as a pseudo-first order reaction with respect to L-Cys. **Figure 1** highlights the effect of the oxidation mediated changes on L-Cys (Fig.1a), L-CysME (Fig.1b) and L-CysEE (Fig.1c) on the chemical shifts of the $^1\text{H}_\beta$ protons. The spectra plotted in blue are at the beginning of the experiment while the ones plotted in red are after the reaction at 30 °C. The chemical shifts of the ^1H and ^{13}C nuclei at $t \approx 0$ and at the end of reaction are listed in **Table 1**. Supporting information shows a representative ^1H spectrum along with the chemical shift assignments (**Fig. S1**) and the observed chemical shift changes in the ^{13}C spectra (**Fig. S2**) in the case of L-CysEE. The major chemical shift changes occur at the β -position for all the molecules. The $^1\text{H}_\beta$ protons shift downfield by 0.3 ppm while the $^{13}\text{C}_\beta$ resonances shift ~ 13 ppm for all the molecules (Fig. S2). The oxidation mediated chemical shift changes in the $^{13}\text{C}_\alpha$ are in the range of 2.7-2.9 ppm for all the molecules. Rest of the nuclei show no significant changes in the chemical shifts, except the N1 protons, become much narrower and gets slightly shielded with an integration that yields approximately three protons (Fig. S1). This trend continues at all the temperatures studied. The β -protons show the AB part of the ABX spin system with X being the α proton. The chemical shifts of the β -protons are shielded (blue to red spectrum in Fig 1) and the NMR spectra do not show the thiol protons (Fig. S1).

The chemical shifts of the β -protons between the initial and final forms of L-CysEE are well resolved and therefore the area under the curve of each of them is used to quantify the reactants and products. **Figure 2** shows the change in the concentration of the reactants and products with time for each temperature in the case of L-CysEE. The corresponding fit to the *pseudo*-first order process (Eq. [4]) is also shown as continuous curves. The *pseudo*-first order rate constants, k , estimated from the exponential decay of the reactants (Fig. 2b) and that of the increase of the product to maximum (Fig.2a)

estimated, are listed **Table 2** for each molecule. The rate of the reaction follows a typical *pseudo*-first order process. The increase in temperature increased the rate of the reaction: 1.67-2.08 $\mu\text{M}/\text{min}$ at 30 $^{\circ}\text{C}$ to 14-17.5 $\mu\text{M}/\text{min}$ at 55 $^{\circ}\text{C}$ (**Table 2**). The estimated half-life of the reaction ($t_{1/2} = \ln(2)/k$) ranges from 330-415 minutes (30 $^{\circ}\text{C}$) to 39-45 minutes (55 $^{\circ}\text{C}$).

Table 2 also lists Eyring analysis of the *pseudo*-first-order reaction kinetics. Linear fit was used to determine the activated enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) of the reaction process. ($\Delta H^{\ddagger} \text{ kJ/mol} = -\text{slope} \times 1000 / R$, and $\Delta S^{\ddagger} \text{ J/ (mol.K)} = (\text{Y-intercept} - \ln(k_B/h)) \times R$, where R , k_B , and h are gas, Boltzmann and Planks' constants, respectively in SI units). As an example in the case of L-CysEE, the following thermodynamic parameters are estimated (average of rate constants from both the fits): $\Delta H^{\ddagger} = 389.6 \pm 6 \text{ kJ/mol}$ and $\Delta S^{\ddagger} = -40.07 \pm 2 \text{ J/ (mol.K)}$. The average free energy of the reaction of these molecules is $\Delta G^{\ddagger} = 402 \pm 20 \text{ kJ/ (mol)}$. The thermodynamic estimates clearly show a free energy of activation barrier that is large enough to suggest the formation covalent bond formation, such as the S-S bond due to the oxidation process. One of the drawbacks of Eyring plot is that Y-intercept (used to determine $\Delta S^{\ddagger}$) is normally a large extrapolation from the experimental data to high temperatures. In our case, the adjusted R^2 values of the linear fit are 0.99 and lead to reliable estimates $\Delta S^{\ddagger}$.

Discussion

The rate of oxidation mediated change in L-Cys and its ester derivatives DMSO was measured using quantitative NMR spectroscopy. The spectral features of the initial and final forms are clearly distinguishable in the one-dimensional NMR spectra at 400 MHz (Figure 1 and Table 1). The decay of the reactant to form the oxidized product follows a *pseudo*-first-order reaction kinetics (Figure 2). This suggests an oxidation process that takes place following the formation of the disulfide adduct. The rate of conversion increases with temperature with a half-life of the reaction close to five hours at 30 °C to 45 minutes at 55 °C (Table 2). Eyring's activated complex approach is used to determine the enthalpy and entropy of the reaction kinetics (Table 2).

In spite of the importance of L-Cysteine and its ester derivatives, structural and dynamics, studies of these molecules are limited, particularly in solvents such as DMSO. In solvents such as water, the reaction is much faster than in DMSO and NMR-based methods are incapable of measuring the rates of the reaction. Typically, solvents will affect the reaction rates depending on whether the substrate is positively or negatively charged, or electrically neutral [16]. In a study by Walters and Leyden's on molecules L-cysteine , L-cysteine methyl ester and S-methyl-L-cysteine noted significant changes in the chemical shifts of both α and β protons [17]. In the case of L-Cysteine, the chemical shift changes over a pH range measured (pH: 1-13) the α and β protons exhibited a shift of 1.3 and 0.31 ppm, respectively. Such shifts are in the order of 0.85 ppm (α) and 0.38 ppm (β) for L-cysteine methyl ester and 0.98 ppm (α) and 0.43 ppm (β) for S-Methyl-L-cysteine. The chemical shift changes in the present study are in the order of 0.32 ppm (Fig.1 and Table 1).

One of the earliest and detailed study of cysteine and related compounds including L-CysEE was presented by Benesch and Benesch [18]. According to this study, in comparison with cysteine when the carboxyl group is modified, the acidity of the ammonium proton is increased more from the electrostatic

contributions than the inductive effects. As a result, the acidity of the ammonium proton becomes greater than that of the thiol proton. This is an important and differentiable characteristic of L-CysME or L-CysEE in comparison with unmodified L-cysteine. L-CysME and L-CysEE have functional groups with relatively acidic protons, and each proton has a relatively similar pKa value. The large change in the chemical shifts of the proton and carbon nuclei at the C_β position, that is closest to the thiol group (Fig. SX) suggest oxidation. We have noticed in the sample color (sealed tube) changes with time and is related to increase in the absorbance at 310 nm, suggesting the formation of S-S bond (Fig. S3), which further suggests oxidation mediated change [19]. With chemical shifts are highly sensitive to the local environment, the oxidation induced change is the only possible explanation that can accommodate the observed chemical shift differences between the reactant and product all the three molecules studied. The kinetic events measured in this study on fresh samples of L-Cys and its ester derivatives suggest that the reaction process is mediated by the oxidation of the thiol group [20]. More importantly, the estimation of the free energy (~400 kJ/molK) from variable temperature kinetic measurements (Table 2), is large enough such that it establishes the formation of a covalent bond associated with oxidation of the thiol group.

Conclusions

L-Cysteine and its esters are excellent model compounds to study fundamental molecular reactions such as ionization and oxidation. Many of the molecular chemistry involving cysteine and related compounds are well explored in the literature and are routinely discussed in advanced bioorganic and biophysical chemistry texts. In spite of such a vast knowledge in the area, systematic investigation of molecular reactions using NMR spectroscopy allowed the measurement of the rate of the reaction process. The ability to measure the reaction kinetics in real time in such systems further opens up possibilities to explore these reactions and in particular the kinetics. The polarity and the aprotic nature of the solvent slow the reaction kinetics and thus opening up newer possibilities to study fundamental molecular mechanisms related to the oxidation process.

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Figure Captions

Figure 1: Chemical shift changes due to the oxidation mediated changes in f L-Cysteine (a), L-Cysteine Methyl Ester (b) and L-Cysteine Ethyl Ester (c). Sub-spectra of the β -protons of the reactant (blue) and that of the product (red) obtained in dissolving the DMSO- d_6 .

Figure 2: First order kinetics of the reaction process of L-CysEE at various temperatures. The reduction of the reactant ($[CysEE-H]^+$) measured directly by NMR (b) for various sample temperatures and the corresponding increase of the ionized product ($[CysEE]$) (a). The experimental measurements are shown as symbols (symbols are not resolved due to high sampling rate) and the non-linear least square fit to the respective experimental data are shown as dashed lines. Various colors of the data points and dashed lines correspond to the spectrometer set temperature: 30 °C (black), 35 °C (red), 40 °C (green), 45 °C (yellow), 50 °C (blue), and 55 °C (pink).

Table

Table 1. Changes in the NMR parameters in the oxidation process ¹.

Molecule	Nucleus	¹ H (ppm)		¹³ C (ppm)	
		t~0	t >> 1m	t~0	t >> 1m
L-Cys	N1	8.6	8.8		
	α	4.2	4.2	53.9	51.3
	β	3.1	3.3	24.2	37.4
	C1			169.0	169.2
L-CysME	N1	8.9	9.0		
	α	4.3	4.3	54.0	51.1
	β	3.0	3.4	24.1	36.8
	ε1	3.8	3.8	52.9	52.9
	C1			168.1	168.3
L-CysEE	N1	8.8	8.9		
	α	4.3	4.3	53.9	51.1
	β	3.0	3.4	24.2	36.9
	ε1	4.2	4.2	61.9	62.1
	ε2	1.2	1.3	13.9	13.9
	C1			169.6	167.8

¹Chemical shift changes > 0.1 ppm are marked with bold font.

Table 2. Kinetic and thermodynamic parameters of the oxidation¹.

Temperature	L-Cys		L-CysME		L-CysEE	
	$k(\mu\text{M}/\text{min})^2$	$k(\mu\text{M}/\text{min})^3$	$k(\mu\text{M}/\text{min})^2$	$k(\mu\text{M}/\text{min})^3$	$k(\mu\text{M}/\text{min})^2$	$k(\mu\text{M}/\text{min})^3$
30 °C	1.67 ± 0.01	1.69 ± 0.01	1.99 ± 0.02	2.31 ± 0.02	2.08 ± 0.01	2.19 ± 0.01
35 °C	2.74 ± 0.01	3.48 ± 0.01	2.84 ± 0.01	3.75 ± 0.01	3.33 ± 0.01	3.4 ± 0.01
40 °C	3.89 ± 0.01	4.3 ± 0.02	4.73 ± 0.01	5.61 ± 0.01	5.45 ± 0.02	5.73 ± 0.02
45 °C	6.19 ± 0.03	6.95 ± 0.02	7.41 ± 0.02	8.15 ± 0.02	8.13 ± 0.03	8.21 ± 0.03
50 °C	9.68 ± 0.04	11.2 ± 0.11	9.4 ± 0.02	8.5 ± 0.02	11.71 ± 0.05	11.83 ± 0.05
55 °C	14.6 ± 0.04	15.22 ± 0.15	16.89 ± 0.57	17.62 ± 0.6	16.72 ± 0.06	17.22 ± 0.07
Thermodynamic parameters						
$\Delta H^\ddagger$ (kJ/mol)	405.3 ± 30	403.2 ± 25	346.6 ± 36	395.2 ± 20	387.3 ± 10	392.0 ± 10
$\Delta S^\ddagger$ (J/mol.K)	-37.5 ± 1	-37.6 ± 4	-49.5 ± 10	-38.3 ± 4	-40.5 ± 2	-39.6 ± 2
$\Delta G^\ddagger$ (kJ/mol.K)	416.2 ± 55		385.0 ± 57		402.3 ± 20	

¹LCys: L-Cysteine, L-CysME: L-Cysteine methyl ester, L-CysEE: L-Cysteine ethyl ester. ²k measures using the decay of the reactant and ³k measured using the growth of the product.

^1H ppm

Figure 1

Figure 2