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Author manuscript

Free Radic Res. Author manuscript; available in PMC 2017 January 01.

Published in final edited form as:
Free Radic Res. 2016 ; 50(2): 143–149. doi:10.3109/10715762.2015.1077385.

Thiyl radicals and induction of protein degradation

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Abstract

Thiyl radicals are important intermediates in the redox biology and chemistry of thiols. These radicals can react via hydrogen transfer with various C-H bonds in peptides and proteins, leading to the generation of carbon-centered radicals, and, potentially, to irreversible protein damage. This review summarizes quantitative information on reaction kinetics and product formation, and discusses the significance of these reactions for protein degradation induced by thiyl radical formation.

Keywords

Thiyl radicals; hydrogen atom transfer; carbon-centered radicals; D-amino acids; glutathione

Introduction

An increased level of protein oxidation is an important hallmark of biological oxidative stress [1–7]. Protein oxidation is the result of increased levels of reactive oxygen and nitrogen species (ROS and RNS), generated via various enzymatic and non-enzymatic pathways, and a manifold of different reactions of these ROS and RNS with proteins. Usually, aromatic and sulfur-containing amino acids are more susceptible to attack by ROS and RNS [8]. However, depending on the nature of ROS and RNS, also aliphatic amino acids may be targeted. With few exceptions, the reactions of ROS and RNS with amino acids generate reactive intermediates, which can subsequently react with other, secondary targets [9]. Such secondary reactions can “move” the final reaction products away from the sites of initial attack. In addition, such secondary reactions can trigger processes, which would possibly not have been initiated by the primary reactions of ROS and RNS. The current review focuses specifically on this area, summarizing recent results on hydrogen abstraction reactions of thiyl radicals within proteins and model peptides. These results suggest that secondary, hydrogen abstraction, reactions of thiyl radicals may have the potential for extensive, irreversible protein damage. In the following, we will briefly introduce reactions which can lead to the formation of thiyl radicals in physiologic and pathologic environment, summarize our current knowledge on hydrogen transfer reactions of thiyl radicals, and conclude with a discussion of the relevance of these reactions for protein degradation.

Formation of thiyl radicals

The redox chemistry of thiols has been detailed in many articles [10–19] and shall only be reviewed here with regard to thiyl radical formation. Chemically, protein thiyl radicals are

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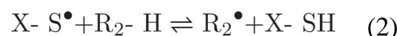
generated via many pathways. While physiologically only a few of them may be significant [11, 19, 20] additional pathways will operate under pathologic conditions, or during the exposure of organisms to exogenous stresses such as, e.g., ionizing radiation. Protein thiyl radicals are generated during the reaction of hydrogen peroxide with heme proteins, including hemoglobin [21–23], and similar pathways are expected for non-heme iron complexes and other protein-associated redox-active transition metals. Significant levels of redox-active transition metals can be present under conditions of iron overload [24], neurodegenerative diseases [25], and as a result of over exposure to other metals, e.g. in the case of manganese [26, 27]. Physiologically, peptide and protein thiyl radicals can form through electron/hydrogen transfer between Cys and tyrosyl radicals [28, 29] or carbon-centered radicals [30]. Chemically, thiyl radicals can be generated by reaction of Cys with tryptophan radicals/radical cations [31], peroxyl radicals [32] (including superoxide [33, 34], where superoxide-induced protein thiyl radical formation has been implicated in S-glutathionylation of mitochondrial complex I [35, 36] and endothelial nitric oxide synthase [37, 38]), carbon-centered radicals [30], nitrogen dioxide ($^{\bullet}NO_2$) [39, 40], carbonate radical ($CO_3^{\bullet-}$) [40] and the hydroxyl radical ($HO^{\bullet}$). Protein thiyl radicals have been involved in mechanisms leading to S-nitrosation, and specifically in mechanisms of nucleotide exchange of various GTPases [41–47].

Hydrogen transfer reactions of thiyl radicals

Hydrogen abstraction by thiyl radicals from organic substrates had been documented decades ago [48, 49], and thiols had been added to synthetic processes to facilitate hydrogen transfer via "polarity reversal catalysis" [50, 51]. Here, a primary organic radical ($R_1^{\bullet}$) abstracts a hydrogen from a thiol, yielding a thiyl radical, which, in turn, reacts via hydrogen abstraction with a second organic substrate, R_2-H. The net reaction is hydrogen transfer between $R_1^{\bullet}$ and R_2-H, catalyzed by the thiol.

$$R_1^{\bullet} + X\text{-}SH \rightleftharpoons R_1\text{-}H + X\text{-}S^{\bullet} \quad (1)$$



$$X\text{-}S^{\bullet} + R_2\text{-}H \rightleftharpoons R_2^{\bullet} + X\text{-}SH \quad (2)$$

It is this concept of "polarity reversal catalysis", which, when extended and applied to proteins, suggests that thiyl radicals could be efficient promotors of protein damage, even by radicals or oxidants, which would not react rapidly with most amino acids. A simplified reaction sequence for such protein damage is displayed in reaction sequence 3, 4 and 7. Cys thiyl radicals (Cys-$S^{\bullet}$) are generated through reaction 3, and a measurable fraction of these thiyl radicals reacts with amino acid (AA) C-H bonds (reaction 4) rather than with one of the biologically available and abundant antioxidants, glutathione (GSH) and ascorbate (Asc^-) (reactions 5 and 6, where $Asc^{\bullet}$ represents an ascorbyl radical). In competition to the reverse reaction (–4), the amino acid radical, (AA)$C^{\bullet}$, must convert into another intermediate or product (reaction 7; see below), which effectively removes (AA)$C^{\bullet}$ from equilibrium 4. For

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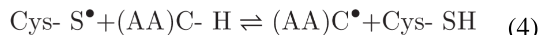
simplicity, the reaction of Cys-S$^\bullet$ with molecular oxygen [17] was omitted in this reaction scheme, as this reaction is reversible; however, it should be noted that the radical anion complex [Cys-S-S-G]$^{-\bullet}$ will efficiently react with oxygen.

$$R_1^\bullet + Cys\text{-}SH \rightleftharpoons R_1\text{-}H + Cys\text{-}S^\bullet \quad (3)$$



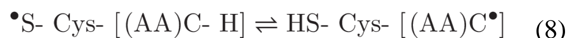
$$Cys\text{-}S^\bullet + (AA)C\text{-}H \rightleftharpoons (AA)C^\bullet + Cys\text{-}SH \quad (4)$$

$$Cys\text{-}S^\bullet + GSH \rightleftharpoons [Cys\text{-}S\text{-}SG]^{-\bullet} + H^+ \quad (5)$$

$$Cys\text{-}S^\bullet + Asc^- \rightarrow Asc^\bullet + Cys\text{-}S^- \quad (6)$$

$$(AA)C^\bullet \rightarrow \text{intermediate/product} \quad (7)$$

Reaction sequence 3, 4 and 7 can proceed *intra*- and *inter*molecularly: rate constants $k_4 = 10^3$–10^5 $M^{-1}s^{-1}$ have been measured for *inter*molecular hydrogen transfer reactions [52, 53] while *intra*molecular reactions (such as reaction 8, measured for several model peptides) proceed with $k_8 \approx 10^5$ s^{-1} (and $k_{-8} \approx 10^6$ s^{-1}) [54].



$$^\bullet S\text{-}Cys\text{-}[(AA)C\text{-}H] \rightleftharpoons HS\text{-}Cys\text{-}[(AA)C^\bullet] \quad (8)$$

The actual extent to which reaction sequence 3, 4 and 7 may lead to protein damage will be defined by the respective rate constants and the availability of glutathione and ascorbate. Physiologic concentrations of glutathione and ascorbate will favor reactions 5 and 6 if these antioxidants have access to Cys-S$^\bullet$. However, this may not always be the case with proteins where Cys residues are frequently buried in the interior [55] (see also below). A role for thiols [56, 57] in the protection of cells against ROS (for example during exposure to ionizing radiation) has been established. However, it has been realized that secondary radicals from thiol or ascorbate oxidation may induce damage as well, for example during γ-irradiation of solutions containing deoxyguanosine [58]. Radiation chemistry, ESR, NMR and mass spectrometry experiments have provided complimentary evidence for *intra*molecular hydrogen atom transfer reactions between thiyl radicals and C-H bonds in model peptides [54, 59, 60] and glutathione (GSH) [30, 61–65]. The equilibria between thiyl and carbon-centered radicals were directly monitored by ESR spectrometry, while NMR and mass spectrometry monitored the loss of proton signal or covalent incorporation of

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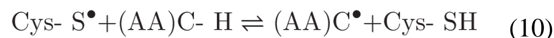
deuterium into reaction products, respectively, when reactions were carried out in D_2O. In these experiments, covalent deuterium incorporation is the result of reactions (9)-(12).



Importantly, thiyl radicals of glutathione react with the C-H bonds of all three amino acids present in glutathione: γ-Glu, Cys and Gly [62, 63]. Here, the *intra*molecular reactions of thiyl radicals with C-H bonds of the Cys residue itself suggests 1,2 – and 1,3-hydrogen transfer processes (Scheme 1, equilibrium 14, and Scheme 3, equilibrium 24, respectively), reactions, which were confirmed recently by pulse radiolysis studies of a series of model compounds.

In equilibrium 14, a thiyl radical exists in equilibrium with an α-mercaptoalkyl radical through a formal 1,2-hydrogen transfer. Rate constants for this equilibrium are on the order of $k_{14} \approx 10^5\ s^{-1}$ and $k_{-14} \approx 1.5\times10^5\ s^{-1}$ at acidic pH [66]. When thiyl radicals were generated from glutathione in D_2O, we observed covalent H/D-exchange for a total of two C-H bonds within Cys, consistent with at least one deuterium incorporated into the original $^{\beta}$C-H bond (and the other deuterium either into the second $^{\beta}$C-H bond or the $^{\alpha}$C-H bond) [62]. Complimentary evidence for such hydrogen transfer processes comes from recent ESR spectroscopy studies on *E. coli* class III ribonucleotide reductase, where covalent deuterium incorporation into the $^{\beta}$C-H bond of Cys-175 was observed during experiments carried out in D_2O [67]. Theoretical calculations suggest that equilibrium 19 should be located predominantly on the left hand side [68], and analogous calculations were performed for the equilibrium between HO-$CH_2CH_2S^{\bullet}$ and HO-CH_2-$^{\bullet}$CH-SH [69]. However, more recent data by Morris et al. indicate that deprotonation of the mercapto group (equilibrium 20) lowers the C-H bond energy (of $CH_3S^{\bullet}$) by ca. 49.4 kJ/mol compared to that of CH_3SH [70]. By analogy to carbon-centered radicals from aliphatic alcohols [71, 72], α-mercaptoalkyl radicals may have significantly lower pK_a values of the mercapto group compared to alkyl mercaptanes, i.e. the deprotonation reaction 21 is expected to shift equilibrium 19 towards the right hand side.

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$$CH_3S^\bullet \rightleftharpoons {}^\bullet CH_2SH \quad (19)$$

$$CH_3SH \rightleftharpoons CH_3S^- + H^+ \quad (20)$$

$$^\bullet CH_2SH \rightleftharpoons {}^\bullet CH_2S^- + H^+ \quad (21)$$

In fact, the covalent H/D-exchange at the $^{\beta}$C-H bond of Cys-175 of ribonucleotide reductase was rationalized by the intermediary formation of a deprotonated α-mercaptoalkyl radical [67].

For the covalent modifications of proteins, the potential formation of α-mercaptoalkyl radicals is significant. Addition of oxygen (reaction 15) leads to a peroxyl radical, which may react via hydrogen abstraction or electron transfer with other amino acids, or via elimination of superoxide (Scheme 2, reaction 22). Hydrogen abstraction and electron transfer reactions will lead to additional protein radicals, while reaction 22 yields thioaldehyde, a tautomeric form of dehydrocysteine (equilibrium 23).

Such products were, in fact, observed when thiyl radicals were generated in several model peptides [59, 60, 73, 74] and proteins [75], but, interestingly, also in iron regulatory protein 2 (IRP2), potentially as a result of iron-dependent degradation [76, 77]. Importantly, both the reactions of thiyl radicals of model peptides and Cys oxidation in IRP2 also reveal the conversion of Cys to Ala. The mechanism for Ala formation likely involves β-elimination, which may proceed via another radical intermediate, $^{\alpha}C^{\bullet}$ radicals (see below).

Noteworthy, the reaction of α-mercaptoalkyl radicals with oxygen proceeds in competition with other pathways. The reaction of thiyl radicals with molecular oxygen [17] proceeds with $k_{18} = 2.2\times10^{9}\ M^{-1}s^{-1}$ (rate constant measured for the addition of oxygen to the thiyl radical from β-mercaptoethanol) but the efficient reverse reaction, $k_{-18} = 6.2\times10^{5}\ s^{-1}$, likely precludes significant product formation via this pathway. Reaction 16, with the deprotonated form of glutathione, GS–, proceeds with k_{16} ca. $4.5\times10^{8}\ M^{-1}s^{-1}$ [65], and generates a disulfide radical anion. Analogous reactions will occur with other protein Cys residues, i.e. can proceed *intra*molecularly when the protein structure permits. While the reverse reaction proceeds with k_{-16} ca. $2\times10^{5}\ s^{-1}$ [65], the efficient reaction of the disulfide radical anion with molecular oxygen (reaction 17) will generate significant yields of disulfide. Schemes 1 and 3 do not display any reaction of thiyl radicals with ascorbate. While this reaction is efficient, it will only occur with thiyl radicals of protein Cys residues which can be accessed by ascorbate. As frequently Cys residues are buried [55], accessibility by ascorbate cannot be necessarily taken for granted.

Scheme 3, equilibrium 24, displays a 1,3-hydrogen transfer reaction of the Cys thiyl radical, where by analogy to reactions of penicillamine thiyl radicals, $k_{24} \approx 8\times10^{4}\ s^{-1}$ and $k_{-24} \approx$

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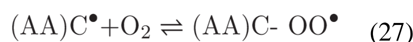
1.4×10^6 s^{-1} [66]. This reaction leads to $^{\alpha}C^{\bullet}$ radicals, which can react with molecular oxygen (reaction 25). The generation of peroxyl radicals at the $^{\alpha}C$ position will lead to additional hydrogen and electron transfer processes, as well as fragmentation reactions [78]. Moreover, the $^{\alpha}C^{\bullet}$ radicals can eliminate $^{\bullet}SH$ (Scheme 4, reaction 26), which proceeds with $k_{26} \approx 5 \times 10^3$ s^{-1} [66]. The latter reaction generates dehydroalanine, an electrophile which can cross-link with nucleophilic amino acids such as Cys and Lys.

Beyond 1,2- and 1,3-hydrogen transfer reactions, thiyl radicals will be able to react with C-H bonds of other amino acids when the protein structure permits, i.e. via "long range hydrogen transfer" (based on the position of amino acids within the protein sequence) (reaction 8). Theoretical calculations by Rauk and co-workers show that thiyl radicals should react with $^{\alpha}C$-H bonds of protein amino acids when located in flexible or β-sheet structures but not within α-helices [79–82].

Our data on thiyl radical reactions within insulin are consistent with this prediction [83]. Importantly, thiyl radicals can react with both $^{\alpha}C$-H and side chain C-H bonds [52, 53]. As a consequence, these *inter*- and *intra*molecular hydrogen transfer reactions equilibria generate significant fractions of intermediary carbon-centered radicals. As shown for $^{\alpha}C^{\bullet}$ and $^{\beta}C^{\bullet}$ radicals in Schemes 3 and 1, respectively, but generally applicable to any carbon-centered radical, these are precursors for peroxyl radicals (reaction 27) and the various routes of peroxyl radical chemistry[78].



$$(AA)C^{\bullet} + O_2 \rightleftharpoons (AA)C\text{-}\ OO^{\bullet} \quad (27)$$

The extent to which peroxyl radical formation will compete against alternative pathways will depend on the oxygen concentration, which, in tissue is in the range of ca. 3–70 μM [84–86], and oxygen diffusion across the three-dimensional structure of the proteins [87].

The reversibility of the 1,3-hydrogen transfer (reaction 24) and of any long-range hydrogen transfer between thiyl radicals and $^{\alpha}C$-H bonds bear the potential for epimerization. In fact, D-alanine formation was detected in model peptides [60], and during light-induced thiyl radical generation in IgG1[88]. These observations are consistent with synthetic applications, where thiyl radicals where used for the racemization of amines [89].

Significance for protein degradation

Based on the reactions summarized above, any reactive species capable of forming a protein thiyl radical is theoretically able to induce protein degradation via the general reaction sequence 3, 4 and 7 (where reaction 27 represents one potential pathway for the irreversible conversion of an amino acid radical $(AA)C^{\bullet}$ according to the general reaction 7). An interesting case can be made for superoxide: superoxide would not efficiently react with any of the essential amino acids except Cys (for which rate constants have been measured on the order of 10^2–10^3 $M^{-1}s^{-1}$ [33, 34]). Part of the reaction of superoxide with Cys generates thiyl radicals, and, therefore, via the mechanisms summarized above, superoxide is theoretically able to induce damage of aliphatic amino acids, promoted by thiyl radicals. To

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what extent superoxide would practically react with protein thiols certainly depends on the environment and especially the availability of superoxide dismutases.

However, in this context it is important to note that a role of superoxide was discussed with respect to thiyl radical formation in endothelial NOS [37, 38] and mitochondrial NADH dehydrogenase [90], where protein thiyl radicals appear to play a role in self-inactivation [90]. In the latter case the authors utilized immunospin-trapping for the localization of radicals on Cys and Tyr, suggesting a hydrogen transfer equilibrium between radicals from Cys and Tyr. Additional examples for thiyl radical-dependent protein degradation are forthcoming for 3-glyceraldehyde phosphate dehydrogenase (GAPDH) and the sarco/endoplasmic reticulum Ca-ATPase (SERCA) (Mozziconacci and Schöneich, unpublished results). Importantly, hydrogen transfer reactions to protein thiyl radicals are not restricted to proteins but may proceed between Cys-$S^{\bullet}$ and lipids, carbohydrates and DNA, provided close contact of Cys-$S^{\bullet}$ with the respective C-H bonds such as potentially present in protein complexes with these molecules.

Acknowledgments

I would like to acknowledge the contributions of various co-workers and collaborators to our research on thiyl radicals, including Drs. D. Pogocki, V. Sharov, O. Mozziconacci, S. Zhou, B.A. Kerwin, Y. J. Wang, T. Nauser and W.H. Koppenol. Financial support by the NIH (P01AG12993), Amgen and Genentech is gratefully acknowledged.

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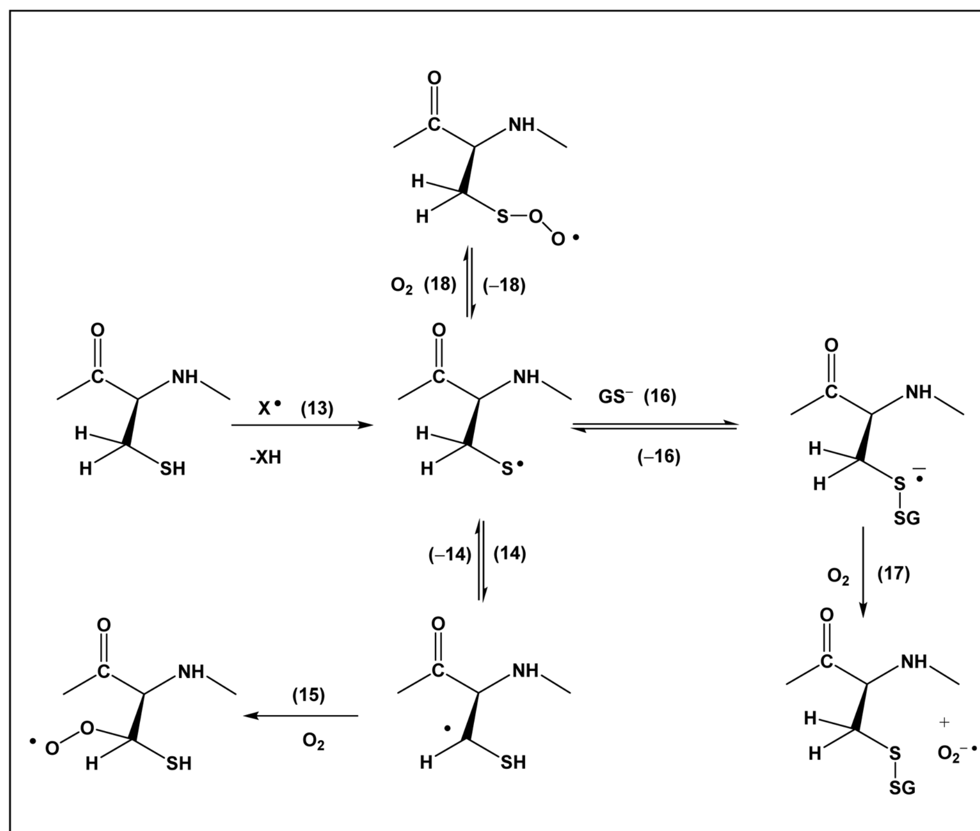

Scheme 1.

1,2-Hydrogen transfer of Cys thiyl radicals and competitive reactions

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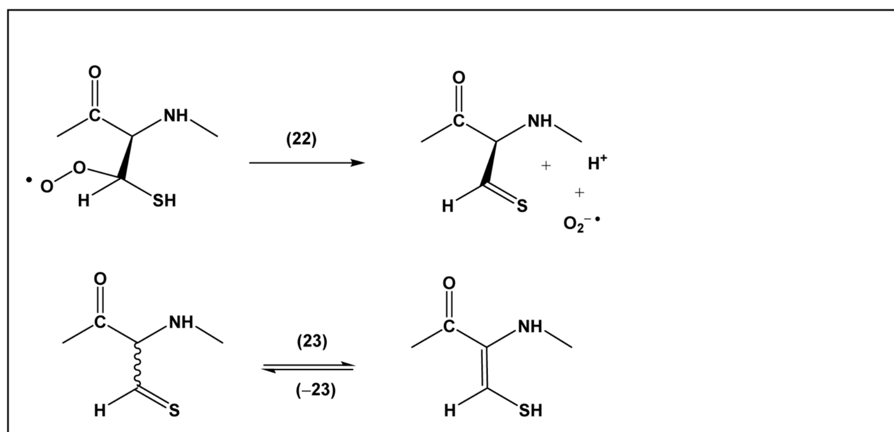

Scheme 2.
Formation and tautomers of Cys thioaldehyde

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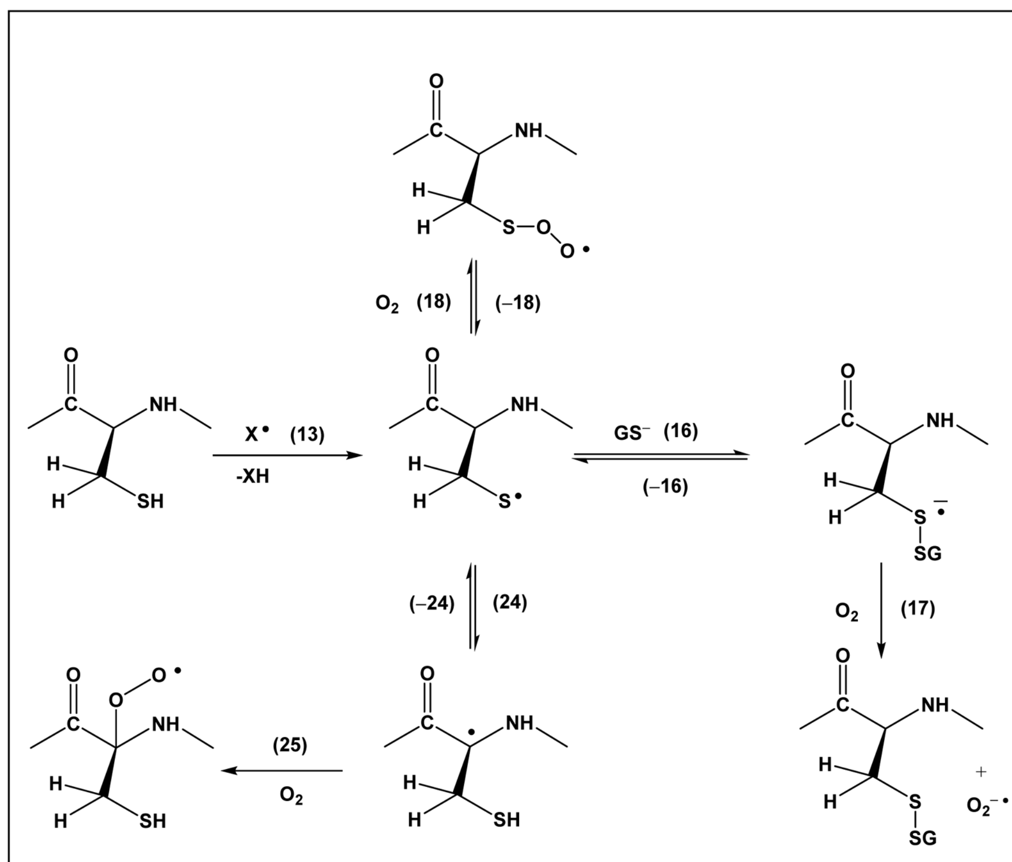

Scheme 3.

1,3-Hydrogen transfer of Cys thiyl radicals and competitive reactions

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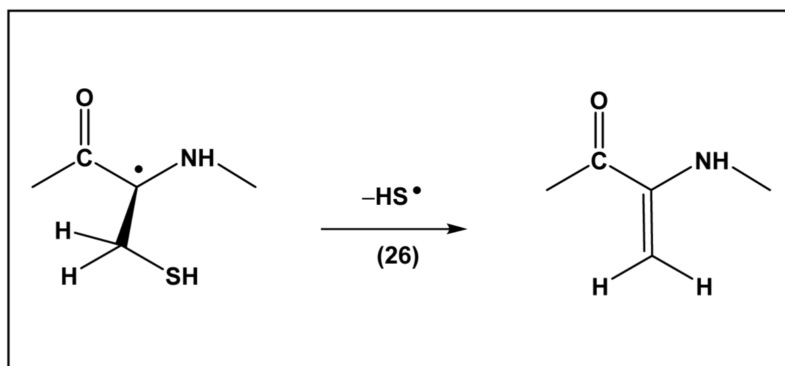

Scheme 4.

Elimination of $HS^{\bullet}$ from Cys $^{\alpha}C^{\bullet}$ radical