Published in final edited form as:
Biochem Soc Trans. 2011 October ; 39(5): 1254–1259. doi:10.1042/BST0391254.

Cysteine residues as catalysts for covalent peptide and protein modification: a role for thiyl radicals?

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Abstract

Cysteine thiyl radicals engage in reversible *intra*molecular hydrogen transfer reactions with amino acid residues in peptides and proteins. These reactions can be experimentally demonstrated through covalent H/D exchange when experiments are carried out in D_2O. To this end, hydrogen transfer reactions have been observed between Cys thiyl radicals and Gly, Ala, Ser, Val and Leu in both model peptides and a protein, insulin. The relevance of such reactions for protein oxidation under conditions of oxidative stress is discussed.

Introduction

Protein cysteine (Cys) residues are prominent targets for chemical modification [1], and such modifications can have significant functional and conformational consequences [2, 3]. For example, the reversible modification of Cys through S-nitrosation, S-glutathiolation or sulfenic acid formation plays an important role in signaling processes [2, 3]. Thiyl radicals ($RS^{\bullet}$) have been implicated in at least one possible mechanism of S-nitrosation, i.e. the radical-radical reaction 1 [4-6]. In theory, thiyl radicals can also serve as origin for S-glutathiolation (reactions 2 and 3, where GS^- denotes the thiolate form of glutathione) and sulfenic acid formation (reactions 4 and 5).

$$RS^{\bullet}+NO \rightarrow RSNO \quad (1)$$

$$RS^{\bullet}+GS^- \rightarrow [RSSG]^{\bullet-} \quad (2)$$

$$[RSSG]^{\bullet-}+O_2 \rightarrow RSSG+O_2^{\bullet-} \quad (3)$$

$$RS^{\bullet}+O_2 \rightleftharpoons RSOO^{\bullet} \quad (4)$$

$$RSOO^{\bullet}+R'SH \rightarrow RSO^{\bullet}+R'SOH \quad (5)$$

Thiyl radicals easily form via hydrogen transfer from Cys to a variety of carbon- and oxygen-centered radicals, or via electron transfer from the thiolate form of Cys to any suitable one-electron acceptor [7]. In radiation biology, and the biology of oxidative stress, in general, hydrogen transfer processes of thiols had been referred to as “repair reactions”, for example “repairing” radicals located on DNA strands [8]. Only later was the reverse reaction, hydrogen abstraction by thiyl radicals, recognized when thiyl radicals were utilized for the inversion at chiral centers in organic molecules [9, 10]. Time-resolved pulse radiolysis experiments provided rate constants for hydrogen abstraction by thiyl radicals (reaction 7) from aliphatic alcohols and ethers, which are on the order of 10^3-10^4 $M^{-1}s^{-1}$ [11,

12]. These are several orders of magnitude slower compared to hydrogen transfer processes *from* thiols to carbon-centered radicals from alcohols and ethers (reaction -7), which generally proceed with rate constants on the order of 10^6-10^8 $M^{-1}s^{-1}$ [7]. Hence, for aliphatic alcohols and ethers, equilibrium 7 is located far on the left hand side, well accounted for by the differences in the homolytic bond dissociation energies (BDE) between the S-H bond of Cys, BDE(S-H, Cys) = 367 kJ/mol [13], and the C-H bonds of aliphatic alcohols and ethers, e.g., BDE(C-H) = 393 kJ/mol for CH_3OH [14]. Nevertheless, several enzymes utilize thiyl radicals for turnover: in ribonucleotide reductase [15-17], benzylsuccinate synthase [18, 19], and glycerol dehydratase [20], thiyl radicals engage in hydrogen transfer reactions with substrates (while in pyruvate formate lyase, thiyl radicals add to the carbonyl group of pyruvate [17, 21]).

$$RS^{\bullet} + -O-CH(R')- \rightleftharpoons RSH + -O-C^{\bullet}(R')- \quad (7\text{-}7)$$

A more efficient hydrogen abstraction by thiyl radicals would be expected from substrates containing C-H bonds of lower homolytic bond dissociation energies. The $^{\alpha}$C-H bonds of amino acid residues in peptides and proteins can have significantly lower homolytic bond dissociation energies, depending on peptide and protein conformation. For example, for fully optimized structures of model peptides, $BDE(C\text{-}H, Gly)_{opt}$ = 348 kJ/mol [22], while for an α-helix, $BDE(C\text{-}H, Gly)_{helix} \approx$ 361 kJ/mol [22], i.e.values which are smaller than BDE(S-H, Cys) = 367 kJ/mol [13]. Moreover, peptides and proteins offer the opportunity for *intra*molecular hydrogen transfer reactions. Therefore, the formation of thiyl radicals in peptides and proteins may lead to hydrogen abstraction from various amino acid sites, which ultimately may result in irreversible damage to the peptide or protein. Our laboratory has recently provided evidence for quite efficient hydrogen abstraction reactions by thiyl radicals within peptides and proteins in solution. These experiments are summarized below, followed by a discussion of the potential consequences of these reactions.

Hydrogen abstraction by thiyl radicals from C-H bonds in peptides

The *inter*molecular reactions of thiyl radicals with amino acids within model peptide structures, N-acetyl amino acid amides and diketopiperazines, proceed with rate constants on the order of k = 10^3-10^5 $M^{-1}s^{-1}$ [23]. Considering the significantly lower BDEs of the $^{\alpha}$C-H bonds of amino acid residues compared to the C-H bonds of alcohols and ethers, the similar rate constants suggest that hydrogen transfer reactions by thiyl radicals are not controlled by thermodynamics alone. In fact, polar effects promote the reactions of thiyl radicals with alcohols and ethers [10, 24], but may be of lower significance for the reactions of thiyl radicals with amino acid residues in peptides. It has been concluded that the $^{\alpha}$C-H bonds of amino acid residues are deactivated by inductive effects, especially in reactions with highly oxidizing radicals such as the hydroxyl radical ($HO^{\bullet}$) and chlorine radical ($Cl^{\bullet}$), but such inductive effects are less likely to control the reactions of less oxidizing radicals (such as thiyl radicals) [25].

Experimental evidence for *intra*molecular hydrogen transfer reactions between thiyl radicals and nearby amino acid residues in peptides and proteins was obtained through covalent H/D exchange [26-29], as outlined in Scheme 1 for a model peptide containing the –Cys-Ala- subsequence. Generally, thiyl radicals were generated through the photolytic cleavage of disulfide bonds and the ensuing incorporation of deuterium quantified by mass spectrometry.

Reaction 8 represents the initial *intra*molecular hydrogen transfer from the $^{\alpha}$C-H bond of Ala in position n+1 relative to the Cys thiyl radical, generating a carbon-centered radical and Cys. In D_2O, the S-H bond rapidly converts to an S-D bond (reaction 9), which reacts with

the carbon-centered radical (reaction 10). Pulse radiolysis experiments have provided rate-constants for the reversible hydrogen transfer between Cys thiyl radicals and Ala for the model peptide N-Ac-Cys-Ala-Ala-Asp-Ala-Ala-Ala (reactions 10/-10), where $k_{10} \approx 10^4$ $M^{-1}s^{-1}$ and $k_{-10} \approx 10^5$ $M^{-1}s^{-1}$ [30]. Importantly, equilibrium 10/-10 bears the opportunity for the inversion of the chiral center at the αC-position of Ala, and L-Ala-to-D-Ala conversion was experimentally detected after photolysis of the disulfide bond-containing dipeptide $(Leu\text{-}Gly\text{-}Ala\text{-}Cys\text{-}Ala\text{-}Gly\text{-}Leu)_2$ [29]. Substitution of Ala by Gly in the model peptide N-Ac-Cys-Gly-Gly-Asp-Gly-Gly-Gly resulted in a 10-fold enhancement of both the forward (reaction 11) and reverse (reaction -11) hydrogen transfer compared to that with Ala, i.e. $k_{11} \approx 10^5$ $M^{-1}s^{-1}$ and $k_{-11} \approx 10^6$ $M^{-1}s^{-1}$ [30]. Hence, K_{10} ($= k_{10}/k_{-10}$) ≈ K_{11} ($=k_{11}/k_{-11}$), which may be expected considering the rather similar $^{\alpha}$C-H BDEs of Gly (348-350 kJ/mol [13, 31] and Ala (345 kJ/mol [31]), while the absolute rate constants differ significantly.

$$\text{N-Ac-Cys}(S^{\bullet})[Ala_5Asp] \rightleftharpoons \text{N-Ac-Cys}(SH)[Ala[{}^{\alpha}C^{\bullet})Ala_4Asp] \quad (10\text{-}10)$$

$$\text{N-Ac-Cys}(S^{\bullet})[Gly_5Asp] \rightleftharpoons \text{N-Ac-Cys}(SH)[Gly({}^{\alpha}C^{\bullet})Gly_4Asp] \quad (11\text{-}11)$$

Additional evidence for the intermediary formation of $^{\alpha}C^{\bullet}$ radicals was derived from complementary experiments, where thiyl radicals were generated via the photolysis of acetone in the presence of Cys-containing peptides [29]. The photolysis of acetone (CH_3COCH_3) yields $^{\bullet}CH_3$ and $CH_3CO^{\bullet}$; when $^{\bullet}CH_3$ and $CH_3CO^{\bullet}$ were reacted with Leu-Gly-Ala-Cys-Ala-Gly-Leu in D_2O, significant incorporation of deuterium was detected in all amino acids of the subsequence Cys^4-Ala^5-Gly^6. The photolysis of deuterated acetone (CD_3COCD_3) leads to $^{\bullet}CD_3$ and $CD_3CO^{\bullet}$, which were reacted with Gly-Gly-Cys-Gly-Gly-Leu. Both carbon-centered radicals are expected to react primarily with the Cys thiol group, yielding thiyl radicals, which equilibrate with $^{\alpha}C^{\bullet}$ radicals (Scheme 2; reactions 12/-12). Mass spectrometry analysis revealed a series of radical-radical combination products consistent with equilibrium 12/-12. For example, the fragmentation pattern of a reaction product with m/z 508.2 is rationalized by the reaction of $CD_3CO^{\bullet}$ with (i) the thiyl radical of Gly-Gly-Cys-Gly-Gly-Leu (Scheme 2; reaction 13) and (ii) the $^{\alpha}C^{\bullet}$ radical at the Gly residue at position n+1 from Cys (Scheme 2; reaction 14).

From the relative intensity of characteristic mass spectrometric fragments of both reaction products we estimate that the ratio of thiyl radical to $^{\alpha}C^{\bullet}$ radical in equilibrium 12 is on the order of ca. (5-10):1, which is good agreement with the equilibrium constant $K_{11} \approx 0.1$, determined for the *intra*molecular reaction of Cys thiyl radical and Gly in the peptide N-Ac-Cys-Gly-Gly-Asp-Gly-Gly-Gly.

Hydrogen abstraction by thiyl radicals from C-H bonds in proteins

The *intra*molecular hydrogen abstraction by thiyl radicals in a protein was experimentally studied through the photolysis of insulin [27]. Insulin possesses three disulfide bonds, of which two connect the A- and B-chains, and one is an *intra*chain disulfide bond located on the A-chain. It should be noted that the photolysis of insulin can generate thiyl radicals not only via direct homolysis of one or more of the disulfide bonds, but also via photo-induced electron transfer from a Tyr residue to the disulfide bond, resulting in cleavage of the disulfide bond into thiyl radical and thiolate. Importantly, when insulin was subjected to photoirradiation in D_2O, only six amino acids incorporated significant amounts of deuterium (where the letter and number in parenthesis indicate the location on A- or B-chain, respectively): Leu(B6), Gly(B8), Ser(B9), Val(B18), Gly(B20), and Cys(A20). This rather selective H/D exchange is best rationalized by the secondary structure of insulin, which may (i) limit the access of thiyl radicals to amino acid residues, and (ii) prevent hydrogen transfer

reactions through control of C-H BDEs. We note, that the BDE of a Gly $^{\alpha}$C-H bond is significantly higher when Gly is located in an α-helix (BDE = 361 kJ/mol [22]) or in a β-sheet (BDE = 402 kJ/mol [22]), compared to a Gly residue within a fully optimized structure (BDE = 348 kJ/mol [22]).

More recently, we have extended our studies to radical reactions with Cys residues on (i) glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and (ii) the sarco/endoplasmic reticulum Ca-ATPase (SERCA). In preliminary experiments, both proteins were exposed in D_2O to radicals generated through (1) the thermal decomposition of 2,2′-azobis-2-methylpropanimidamide, dihydrochloride (AAPH), and (2) the decomposition of peroxynitrite ($ONOO^-$) in the presence of bicarbonate (HCO_3^-). These reactions resulted in significant incorporation of deuterium into specific peptide sequences of both GAPDH and SERCA (unpublished data), analogous to our results with model peptides delineated in reactions 8-10 in Scheme 1.

Relevance to the chemistry and biology of protein oxidation

During conditions of oxidative stress, tissue is exposed to a variety of reactive oxygen and nitrogen species, which display significantly different reactivities towards biomolecules [32]. For example, the hydroxyl radical (generated through exposure to ionizing radiation, the reduction of hydrogen peroxide, or the homolysis of peroxynitrous acid) is a strong oxidant which reacts very fast to diffusion-controlled with most amino acids [33]. In fact, the reactivity of amino acids in a protein towards hydroxyl radicals is controlled largely by their surface exposure. On the other hand, superoxide shows very low reactivity towards proteins (except for protein-bound redox-active transition metals). Other radicals of biological significance are NO, NO_2, carbon-centered radicals (-$^{\bullet}$CH-), peroxyl radicals ($ROO^{\bullet}$), alcoxyl radicals ($RO^{\bullet}$), phenoxyl radicals (e.g., tyrosyl radicals, $TyrO^{\bullet}$), and the carbonate radical ($CO_3^{\bullet-}$) [32, 34]. Except NO, all of these radicals have in common, that they oxidize thiols to thiyl radicals. Moreover, among the 20 essential amino acids represented in mammalian proteins, Cys represents the amino acid most easily attacked by all these radicals. In fact, superoxide does not react at a measurable rate with any of these amino acids except Cys, and the rate constant for this reaction is slow (ca. 10^2-10^3 $M^{-1}s^{-1}$) [35]. The same is true for tyrosyl radicals; most carbon-centered radicals react with thiols with rate-constants on the order of 10^6-10^8 $M^{-1}s^{-1}$ [7] while reactions with the other amino acids are comparatively slow. Hence, a protein Cys residue will always represent a preferred target for these radicals, and, except for most carbon-centered radicals, the reactivity of Cys will be enhanced through deprotonation of the thiol. Therefore, especially thiols with low pK_a values constitute hot spots for free radical oxidation. Once formed, a peptide or protein thiyl radical has then the opportunity to abstract a hydrogen atom from a nearby aliphatic amino acid. Though these reactions are reversible, the data presented above provide evidence that the resulting carbon-centered radicals can react via additional pathways. In Scheme 2, such possibility is represented by reaction 14, where the carbon-centered radical combines with an acetyl radical. In tissue, carbon-centered radicals may react with oxygen, generating amino acid peroxyl radicals (Scheme 3; reaction 15). Such peroxyl radicals on protein backbones may ultimately lead to protein fragmentation [36]. We note that also thiyl radicals add oxygen, generating thiyl peroxyl radicals (reaction 16). However, oxygen addition to thiyl radicals is reversible with a relatively high rate constant for oxygen elimination (reaction -16; $k_{-16} = 6.3\times10^5$ s^{-1} for thiyl radicals from 2-mercaptoethanol) [37].

Reaction 17 displays the reaction of a carbon-centered radical with Cys, and reaction 18 the hydrogen abstraction by the ensuing thiyl radical from an amino acid. In this sequence, the thiol is restored, and has functioned as a *catalyst* for the reaction of the carbon-centered radical with the amino acid. An analogous catalytic function of thiols has been described

before for synthetic organic reactions with alcohols and ethers, referred to as “polarity-reversal catalysis” [10].

$$-\ ^{\bullet}CH-\ +RSH \rightarrow -\ CH_2-\ +RS^{\bullet} \quad (17)$$

$$RS^{\bullet}+-\ NH-\ CH\,(R')-\ CO-\ \rightleftharpoons\ RSH+-\ NH-\ ^{\bullet}C\,(R')-\ CO- \quad (18)$$

In proteins, such catalytic role of Cys residues may have significant consequences such as fragmentation and/or aggregation. Moreover, the possible inversion of a chiral center, i.e. the conversion of an L-amino acid to a D-amino acid, such as demonstrated for L-Ala, may be of relevance to protein conformation and immunogenicity. Future experiments must show to what extent especially proteins with low pK_a thiols are susceptible to the mechanisms described above. Considering that one possible mechanism for the formation of S-nitrosothiols involves the reaction of protein thiyl radicals with NO [4-6], and that physiologic concentrations of NO are low, the competitive reaction of such thiyl radicals with amino acid C-H bonds must be considered.

Conclusion

The *intra*molecular reaction of peptide and protein thiyl radicals with C-H bonds of surrounding amino acids is reversible, and leads to intermediary carbon-centered radicals. The extent of such reactions will depend on peptide and protein sequence and structure, and has the potential for irreversible modifications of peptides and proteins such as epimerization and/or fragmentation. Through hydrogen transfer reactions from other amino acids, Cys and Cys thiyl radicals can function as catalysts for protein damage.

Acknowledgments

We gratefully acknowledge financial support from Amgen Inc and the NIH (P01AG12993).

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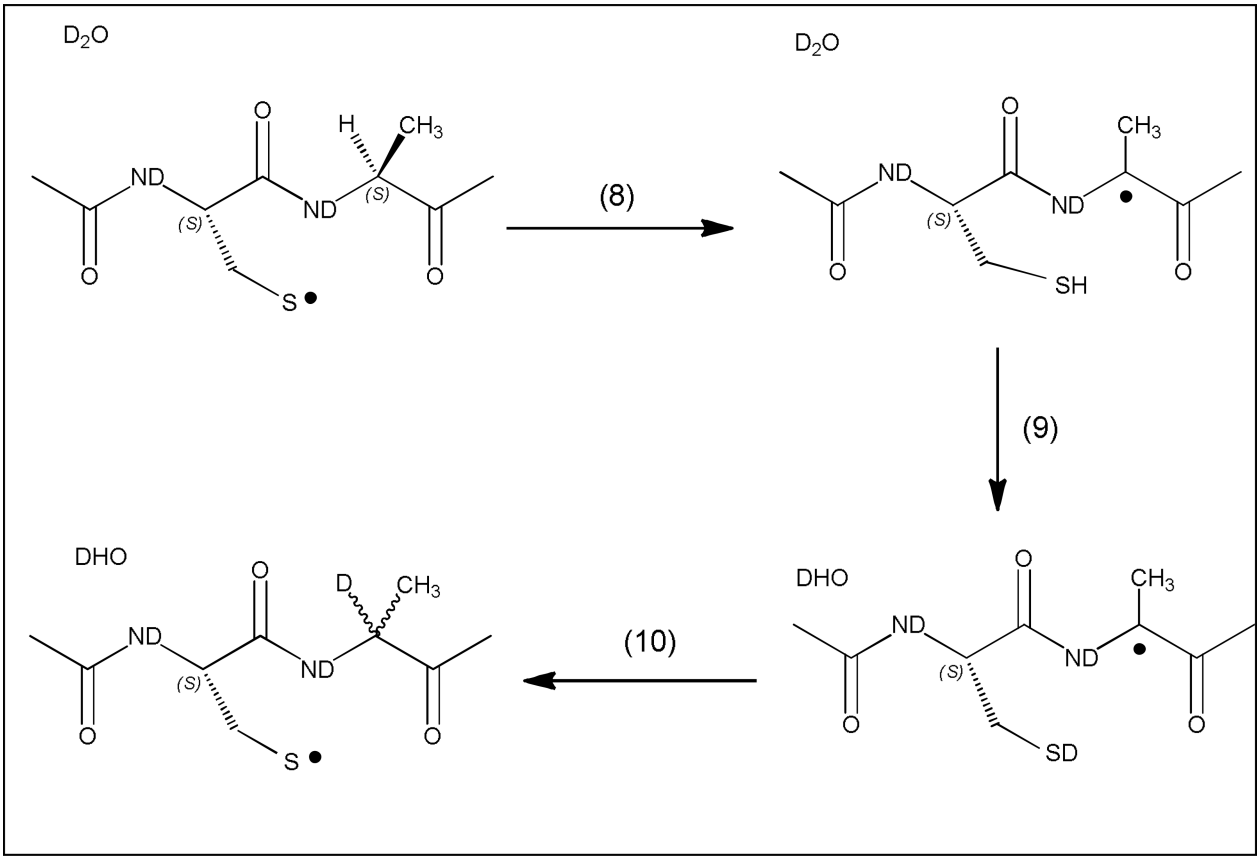
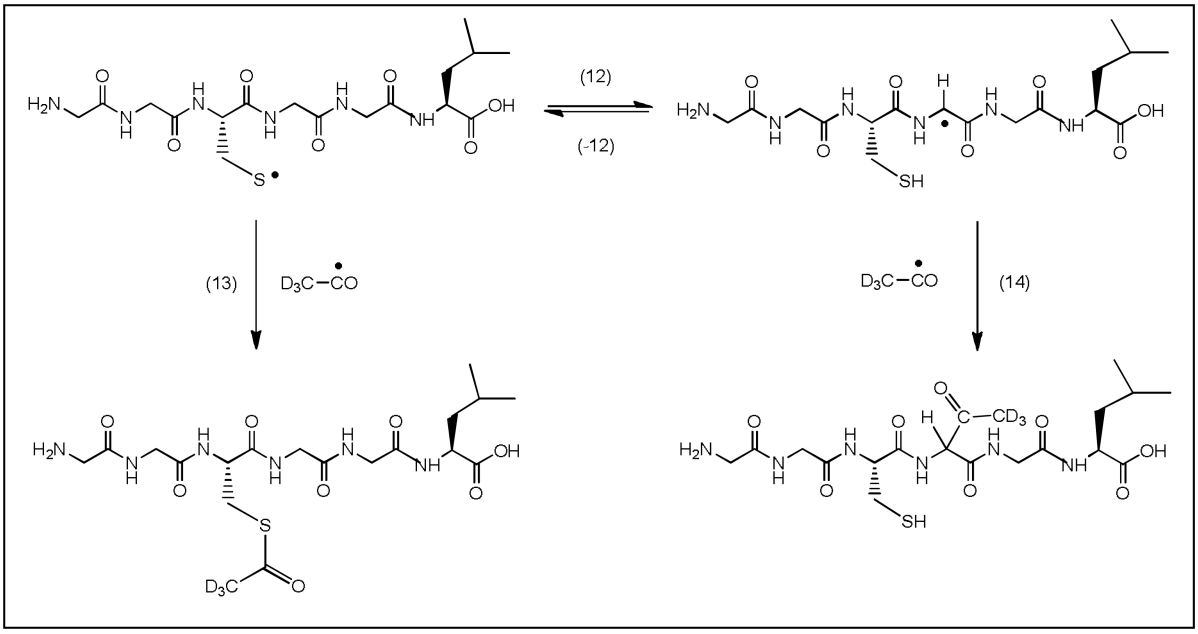
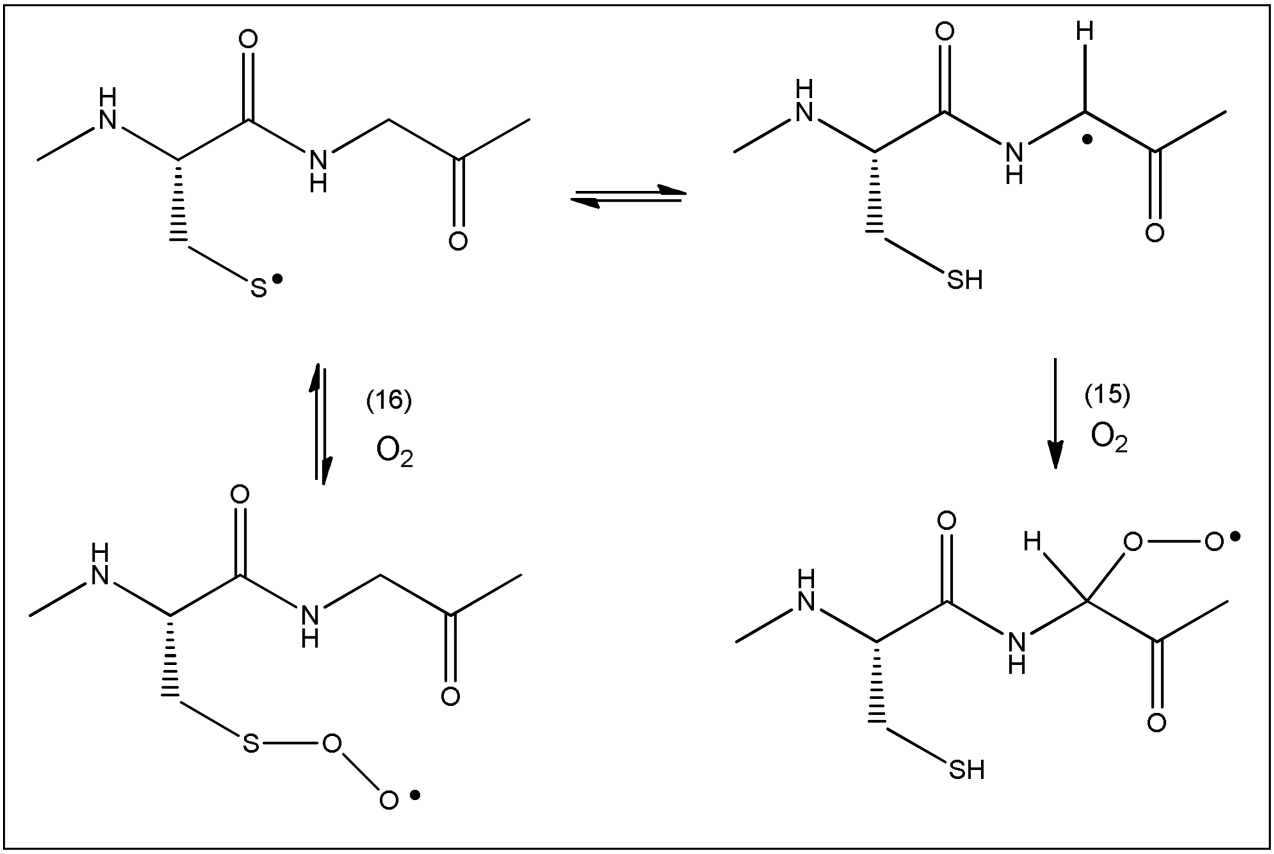


Scheme 1.
Reversible intramolecular hydrogen transfer between a Cys thiyl radical and an $^{\alpha}$C-H bond in a peptide



Scheme 2.

Radical-radical combination products between peptide radicals and acetyl radicals during the photochemical decomposition of acetone-d_6 in the presence of Gly-Gly-Cys-Gly-Gly-Leu.



Scheme 3.
The reaction of oxygen with peptide radicals