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 intramolecular hydrogen-transfer reactions with amino acid residues in peptides and proteins. These reactions can be experimentally demonstrated through covalent hydrogen–deuterium exchange when experiments are carried out in D2O. To this end, hydrogen-transfer reactions have been observed between cysteine thiyl radicals and glycine, alanine, serine, valine and leucine 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 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 cysteine through S-nitrosation, S-glutathiolation or sulfenic acid formation plays an important role in signalling processes [2,3]. Thiyl radicals (RS•) have been implicated in at least one possible mechanism of S-nitrosation, i.e. the radical–radical reaction (reaction 1) [4–6]. In theory, thiyl radicals can also serve as the origin for S-glutathiolation (reactions 2 and 3, where GS− denotes the thiolate form of glutathione) and sulfenic acid formation (reactions 4 and 5).

\[
\begin{align*}
\text{RS}^+ + \text{NO} &\rightarrow \text{RSNO} \\
\text{RS}^+ + \text{GS}^- &\rightarrow [\text{RSSG}]^+ \\
[\text{RSSG}]^- + \text{O}_2 &\rightarrow \text{RSSG} + \text{O}_2^- \\
\text{RS}^+ + \text{O}_2 &\rightarrow \text{RSOO}^* \\
\text{RSOO}^* + \text{R}SH &\rightarrow \text{R}SOO^* + \text{R}SOH \\
\text{RSH}/\text{RS}^- + \text{Ox}^* &\rightarrow \text{RS}^+ + \text{OxH}/\text{Ox}^- 
\end{align*}
\]

(1) (2) (3) (4) (5) (6)

Thiyl radicals easily form via hydrogen transfer from cysteine to a variety of carbon- and oxygen-centred radicals, or via electron transfer from the thiolate form of cysteine to any suitable one-electron acceptor (reaction 6) [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 centres 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 of the order 10−7–10−8 M−1·s−1 [11,12]. These are several orders of magnitude lower compared with hydrogen-transfer processes from thiols to carbon-centred radicals from alcohols and ethers (reaction −7), which generally proceed with rate constants of the order 105–108 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 BDEs (bond-dissociation energies) between the S–H bond of cysteine, BDE(S–H, cysteine) = 367 kJ·mol−1 [13], and the C–H bonds of aliphatic alcohols and ethers, e.g. BDE(C–H) = 393 kJ·mol−1 for methanol [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 (whereas in pyruvate formate lyase, thiyl radicals add to the carbonyl group of pyruvate [17,21]).

\[
\text{RS}^+ + \text{-O-CH(R')}^- \rightarrow \text{RSH} + \text{-O-C}(\text{R'})^- 
\]

(7/−7)

A more efficient hydrogen abstraction by thiyl radicals would be expected from substrates containing C–H bonds of lower homolytic BDEs. The “C–H bonds of amino acid residues in peptides and proteins can have significantly lower homolytic BDEs, depending on peptide and protein conformation. For example, for fully optimized structures of model peptides, BDE(C–H, glycine)helix = 348 kJ·mol−1 [22], whereas for an α-helix, BDE(C–H, glycine)helix ≈ 361 kJ·mol−1 [22], i.e. values which are smaller than BDE(S–H, cysteine) (367 kJ·mol−1) [13]. Moreover, peptides and proteins offer the opportunity for intramolecular...
Hydrogen abstraction by thiol radicals from C–H bonds in peptides

The intermolecular reactions of thiol radicals with amino acids within model peptide structures, N-acetylamino acid amides and diketopiperazines, proceed with rate constants of the order 10³–10⁵ M⁻¹·s⁻¹ [23]. Considering the significantly lower BDEs of the αC–H bonds of amino acid residues compared with the C–H bonds of alcohols and ethers, the similar rate constants suggest that hydrogen-transfer reactions by thiol radicals are not controlled by thermodynamics alone. In fact, polar effects promote the reactions of thiol radicals with alcohols and ethers [10,24], but may be of lower significance for the reactions of thiol radicals with amino acid residues in peptides. It has been concluded that the αC–H bonds of amino acid residues are deactivated by inductive effects, especially in reactions with highly oxidizing radicals such as the hydroxyl radical (HO•) and chlorine radical (Cl•), but such inductive effects are less likely to control the reactions of less oxidizing radicals (such as thiol radicals) [25].

Experimental evidence for intramolecular hydrogen-transfer reactions between thiol radicals and nearby amino acid residues in peptides and proteins was obtained through covalent hydrogen–deuterium exchange [26–29], as outlined in Figure 1 for a model peptide containing the -Cys-Ala- subsequence. Generally, thiol radicals were generated through the photolytic cleavage of disulfide bonds and the ensuing incorporation of deuterium quantified by MS.

Reaction 8 represents the initial intramolecular hydrogen transfer from the αC–H bond of alanine in position n +1 relative to the cysteine thiol radical, generating a carbon-centred radical and cysteine. In ²H₂O, the S–H bond is rapidly converted into an S–²H bond (reaction 9), which reacts with the carbon-centred radical (reaction 10). Pulse radiolysis experiments have provided rate constants for the reversible hydrogen transfer between cysteine thiol radicals and alanine for the model peptide N-acetyl-Cys-Ala-Ala-Ala-Ala-Ala-Ala (reactions 10/−10), where k₁₀ ≈ 10⁴ M⁻¹·s⁻¹ and k⁻₁₀ ≈ 10⁵ M⁻¹·s⁻¹ [30]. Importantly, equilibrium 10/−10 bears the opportunity for the inversion of the chiral centre at the αC-position of alanine, and conversion of L-alanine into D-alanine was experimentally detected after photolysis of the disulfide bond-containing dipeptide (Leu-Gly-Ala-Cys-Ala-Gly-Leu)₂ [29]. Substitution of glycine for alanine in the model peptide N-acetyl-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 with that with alanine, i.e. k₁₁ ≈ 10⁵ M⁻¹·s⁻¹ and k⁻₁₁ ≈ 10⁶ M⁻¹·s⁻¹ [30]. Hence K₁₀ (= k₁₀/k⁻₁₀) ≈ K₁₁ (= k₁₁/k⁻₁₁), which may be expected considering the rather similar αC–H BDEs of glycine (348–350 kJ·mol⁻¹ [13,31]) and alanine (345 kJ·mol⁻¹ [31]), whereas the absolute rate constants differ significantly.

\[
\begin{align*}
N\text{-acetyl-Cys}(S^*)[\text{Ala}_{5}\text{Asp}] & = \\
N\text{-acetyl-Cys}(\text{SH})[\text{Ala}(\text{C}^*)\text{Ala}_{4}\text{Asp}] & (10/−10) \\
N\text{-acetyl-Cys}(S^*)[\text{Gly}_{5}\text{Asp}] & = \\
N\text{-acetyl-Cys}(\text{SH})[\text{Gly}(\text{C}^*)\text{Gly}_{4}\text{Asp}] & (11/−11)
\end{align*}
\]
Radical–radical combination products between peptide radicals and acetyl radicals during the photochemical decomposition of $[^{2}H_6]$acetone in the presence of Gly-Gly-Cys-Gly-Gly-Leu

$D$ represents $^2H$ (deuterium).

Additional evidence for the intermediate formation of $\alpha C^\bullet$ radicals was derived from complementary experiments, where thyl radicals were generated via the photolysis of acetone in the presence of cysteine-containing peptides [29]. The photolysis of acetone (CH$_3$COCH$_3$) yields $^\bullet$CH$_3$ and CH$_3$CO$^\bullet$; when $^\bullet$CH$_3$ and CH$_3$CO$^\bullet$ were allowed to react with Leu-Gly-Ala-Cys-Ala-Gly-Leu in $^2$H$_2$O, significant incorporation of deuterium was detected in all amino acids of the subsequence Cys$_4$-Ala$_5$-Gly$_6$. The photolysis of deuterated acetone (C$_2$H$_3$COC$_2$H$_3$) leads to $^\bullet$C$_2$H$_3$ and C$_2$H$_3$CO$^\bullet$, which were allowed to react with Gly-Gly-Cys-Gly-Gly-Leu. Both carbon-centred radicals are expected to react primarily with the cysteine thiol group, yielding thyl radicals, which equilibrate with $\alpha C^\bullet$ radicals (Figure 2; reactions 12/−12). MS 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 C$_2$H$_3$CO$^\bullet$ with (i) the thyl radical of Gly-Gly-Cys-Gly-Gly-Leu (Figure 2; reaction 13), and (ii) the $\alpha C^\bullet$ radical at the glycine residue at position $n+1$ from cysteine (Figure 2; reaction 14).

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

**Hydrogen abstraction by thyl radicals from C–H bonds in proteins**

The intramolecular hydrogen abstraction by thyl radicals in a protein was studied experimentally through the photolysis of insulin [27]. Insulin possesses three disulfide bonds, of which two connect the A- and B-chains, and one is an intrachain disulfide bond located on the A-chain. It should be noted that the photolysis of insulin can generate thyl radicals not only via direct homolysis of one or more of the disulfide bonds, but also via photo-induced electron transfer from a tyrosine residue to the disulfide bond, resulting in cleavage of the disulfide bond into thyl radical and thiolate. Importantly, when insulin was subjected to photoradiation in $^2$H$_2$O, only six amino acids incorporated significant amounts of deuterium (where the letter and number in parentheses indicate the location on A- or B-chain respectively): Leu(B$_6$), Gly(B$_8$), Ser(B$_9$), Val(B$_{18}$), Gly(B$_{20}$) and cysteine (A$_{20}$).

This rather selective hydrogen–deuterium exchange is best rationalized by the secondary structure of insulin, which may (i) limit the access of thyl 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 glycine is located in an $\alpha$-helix (BDE = 361 kJ·mol$^{-1}$ [22]) or in a $\beta$-sheet (BDE = 402 kJ·mol$^{-1}$ [22]), compared with a glycine residue within a fully optimized structure (BDE = 348 kJ·mol$^{-1}$ [22]).

More recently, we have extended our studies to radical reactions with cysteine residues on (i) GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and (ii) SERCA (sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase). In preliminary experiments, both proteins were exposed in $^2$H$_2$O to radicals generated through (i) the thermal decomposition of AAPH (2,2′-azobis-2-methylpropanimidamide dihydrochloride), and (ii) 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 (C. Schöneich, O. Mozziconacci and V.S. Sharov, unpublished work),

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**Figure 2** | Radical–radical combination products between peptide radicals and acetyl radicals during the photochemical decomposition of $[^{2}H_6]$acetone in the presence of Gly-Gly-Cys-Gly-Gly-Leu

$D$ represents $^2H$ (deuterium).
Figure 3 | The reaction of oxygen with peptide radicals

\[ \text{D represents } ^2\text{H (deuterium).} \]

Figure 3: The reaction of oxygen with peptide radicals

D represents $^2$H (deuterium).

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 rapidly 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-centred radicals (•CH-), peroxyl radicals (ROO•), alcoxyl radicals (RO•), phenoxyl radicals (e.g. tyrosyl radicals, TyrO•), and the carbonate radical (CO$_3$•–) [32,34]. Except for NO, what all of these radicals have in common is that they oxidize thiols to thiyl radicals. Moreover, among the 20 essential amino acids represented in mammalian proteins, cysteine represents the amino acid most easily attacked by all of these radicals. In fact, superoxide does not react at a measurable rate with any of these amino acids except for cysteine, and the rate constant for this reaction is low ($\sim 10^2$–$10^3$ M$^{-1}$ s$^{-1}$) [35]. The same is true for tyrosyl radicals; most carbon-centred radicals react with thiols with rate constants of the order $10^4$–$10^5$ M$^{-1}$ s$^{-1}$ [7], whereas reactions with the other amino acids are comparatively slow. Hence, a protein cysteine residue will always represent a preferred target for these radicals, and, except for most carbon-centred radicals, the reactivity of cysteine will be enhanced through deprotonation of the thiol. Therefore thiols with low pK$_a$ values constitute hotspots for free radical oxidation. Once formed, a peptide or protein thyl radical then has the opportunity to abstract a hydrogen atom from a nearby aliphatic amino acid. Although these reactions are reversible, the data presented above provide evidence that the resulting carbon-centred radicals can react via additional pathways.

In Figure 2, such possibility is represented by reaction 14, where the carbon-centred radical combines with an acetyl radical. In tissue, carbon-centred radicals may react with oxygen, generating amino acid peroxyl radicals (Figure 3; reaction 15). Such peroxyl radicals on protein backbones may ultimately lead to protein fragmentation [36]. We note that also thyl radicals add oxygen, generating thyl peroxyl radicals (reaction 16). However, oxygen addition to thyl radicals is reversible with a relatively high rate constant for oxygen elimination (reaction $-16$; $k_{-16} = 6.3 \times 10^5$ s$^{-1}$ for thyl radicals from 2-mercaptoethanol) [37].

Reaction 17 displays the reaction of a carbon-centred radical with cysteine, and reaction 18 is the hydrogen abstraction by the ensuing thyl radical from an amino acid. In this sequence, the thiol is restored, and has functioned as a catalyst for the reaction of the carbon-centred radical with the amino acid. An analogous catalytic function of thiols has been described previously for synthetic organic reactions with alcohols and ethers, referred to as ‘polarity-reversal catalysis’ [10].

\[ \text{•CH-} + \text{RSH}\rightarrow\text{-CH}_2\text{-} + \text{RS}^* \quad (17) \]

\[ \text{RS}^* + \text{-NH-CH(R')-CO-} \rightleftharpoons \text{RSH} + \text{-NH-*C(R')-CO-} \quad (18) \]

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

Conclusions
The intramolecular reaction of peptide and protein thyl radicals with C–H bonds of surrounding amino acids is reversible and leads to intermediary carbon-centred 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, cysteine and cysteine thyl radicals can function as catalysts for protein damage.

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References


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