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Author manuscript

*Free Radic Biol Med.* Author manuscript; available in PMC 2016 November 22.

Published in final edited form as:

*Free Radic Biol Med.* 2015 March ; 80: 158–163. doi:10.1016/j.freeradbiomed.2014.12.006.

## Protein thiyl radical reactions and product formation: a kinetic simulation

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### Abstract

Protein thiyl radicals are important intermediates generated in redox processes of thiols and disulfides. Thiyl radicals efficiently react with glutathione and ascorbate, and the common notion is that these reactions serve to eliminate thiyl radicals before they can enter potentially hazardous processes. However, over the past years increasing evidence has been provided for rather efficient intramolecular hydrogen transfer processes of thiyl radicals in proteins and peptides. Based on rate constants published for these processes, we have performed kinetic simulations of protein thiyl radical reactivity. Our simulations suggest that protein thiyl radicals enter *intramolecular* hydrogen transfer reactions to a significant extent even under physiologic conditions, i.e. in the presence of 30  $\mu$ M oxygen, 1 mM ascorbate and 10 mM glutathione. At lower concentrations of ascorbate and glutathione, frequently observed when tissue is exposed to oxidative stress, the extent of irreversible protein thiyl radical-dependent protein modification increases.

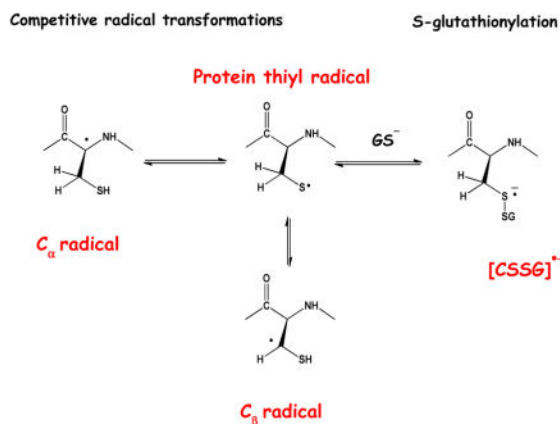
### Graphical abstract

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## Keywords

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

## Introduction

Protein thiols play an important role in signaling pathways, enzyme activation and inactivation, and the detoxification of reactive oxygen species[1–4]. Key to these pathways are redox reactions, where thiol oxidation proceeds via two-electron and one-electron oxidation, yielding sulfenic acid (RSOH) and thiyl radical ( $RS^{\bullet}$ ), respectively. The redox chemistry of thiols, and pathways for sulfenic acid and thiyl radical formation has been covered in many articles[1–10] and shall not be reviewed in detail here. Instead, the current article will focus on reaction pathways of protein thiyl radicals and include several novel reactions of thiyl radicals for which experimental evidence has been provided in recent years. We will provide kinetic simulations, based on recent experimental data, which predict the fate of protein thiyl radicals under biologic conditions. These calculations will provide estimates for product yields of thiyl radical reactions, which may need to be taken into account when considering the role of protein thiyl radicals in biologic systems. Our kinetic simulations were motivated by several recent discussions on the potential fate of protein thiyl radicals under conditions of oxidative stress. In this regard, it is important to note that thiyl radicals, via reaction with nitric oxide (NO), have been implicated in the formation of S-nitrosocysteine (S-NO-Cys)[11], i.e., an important product of thiol-dependent signaling. However, further research is required in order to evaluate to what extent this mechanism is physiologically significant[12]. Moreover, thiyl radical-dependent regulation of nucleotide exchange has been documented for a series GTPases[13–17]. While it is commonly assumed that antioxidants such as glutathione (GSH) and ascorbate ( $HAsc^{-}$ ) prevent deleterious *intra*- and *intermolecular* reactions of protein thiyl radicals, our kinetic simulations will show that, due in part to chain reactions, quite significant yields of reaction products other than thiols or disulfides may form from these protein thiyl radicals. This would be especially the case at decreased, suboptimal levels of GSH and/or ascorbate, and under conditions where protein thiyl radicals may not be fully accessible to these antioxidants.

Chemically, protein thiyl radicals can be generated via a manifold of pathways but physiologically only a few of them may be significant. Protein thiyl radical generation has been documented for the reaction of hydrogen peroxide with heme proteins, including hemoglobin[18, 19]. Analogous pathways may operate with non-heme iron and other protein-associated redox-active transition metals present under conditions of iron overload[20] or neurodegenerative diseases[21]. Protein thiyl radicals form through electron transfer between Cys and protein tyrosyl radicals[22], where tyrosyl radicals are generated through one-electron oxidation of Tyr. The latter can be induced through peroxynitrite-decomposition in the presence of CO<sub>2</sub>, generating <sup>•</sup>CO<sub>3</sub><sup>-</sup> and <sup>•</sup>NO<sub>2</sub>. For example, peroxynitrite-induced tyrosyl radicals were detected in human blood plasma[23]. However, the extent to which tyrosyl radicals will involve in electron-transfer with protein Cys residues will depend on the rate-constant for intramolecular electron transfer, which has to compete with the reduction of the tyrosyl radical by GSH and ascorbate. Protein thiyl radicals have been involved in mechanisms leading to S-nitrosation, and specifically in mechanisms of nucleotide exchange of various GTPases[11–17].

Theoretical calculations predicted that thiyl radicals should react with <sup>α</sup>C-H bonds of most amino acids in proteins when located in flexible or β-sheet structures but not in α-helices[24–27]. Our kinetic NMR experiments confirmed that *intermolecular* hydrogen atom transfer (HAT) reactions of the general type displayed in Scheme 1, equilibrium 2, occur between thiyl radicals and several amino acids[28]. However, these HAT reactions occurred not only with the <sup>α</sup>C-H bonds but also with the C-H bonds of the amino acid side chains[29]. Radiation chemistry, ESR, NMR and mass spectrometry experiments then provided evidence for *intramolecular* HAT reactions in model peptides[30–32] as well as in glutathione (GSH)[33–38], where HAT was observed between the Cys thiyl radical and the <sup>α</sup>C-H bonds of γ-Glu, Cys and Gly. More recently, pulse radiolysis experiments with Cys and several model compounds provided rate constants for *intramolecular* HAT reactions with the <sup>α</sup>C-H and the <sup>β</sup>C-H bond (Scheme 1, equilibria 2 and 1)[39]. The consequence of these *inter-* and *intramolecular* HAT equilibria is the intermediary generation of significant fractions of carbon-centered radicals, which are at the origin of various products. When oxygen is available, these carbon-centered radicals can react with oxygen to yield peroxy radicals, where peroxy radical formation may lead to irreversible protein damage, including fragmentation[40]. However, under physiological conditions the availability of oxygen may be limited for various reasons. First, oxygen concentrations can range from ca. 3–70 μM[41–43], depending on the nature of the tissue. Second, oxygen diffusion into the interior of proteins may be limited[44] and peroxy radical formation may be slow when carbon-centered radicals are generated within buried domains of proteins, for example through HAT reactions induced by thiyl radicals. Under these conditions, carbon-centered radicals may undergo additional reactions such as the addition to aromatic amino acids[45].

In vitro photochemical studies with several model peptides[31–35, 46, 47] and proteins[48–50], including IgG1 and IgG2, have demonstrated that thiyl radicals can generate intermediary carbon-centered radicals on proteins in a sequence-specific manner. While thiyl radicals were not generated through physiological processes in these studies, they are

relevant to physiology as they demonstrate what can happen when thiyl radicals are generated on proteins. In the absence of oxygen, the reversible generation of carbon-centered radicals has led to the conversion of L-Ala to D-Ala in a model peptide[32], and to the sequence-specific generation of D-amino acids on IgG1[51]. Hence, thiyl radical generation on proteins is a viable source for D-amino acid generation, and such reactions have to be taken into account when simulating thiyl radical reaction pathways in proteins (see below).

## Kinetic simulations

The chemistry according to scheme 1 has been simulated and the results are summarized in Table 1. We evaluated the yield of several products and reaction channels starting from a hypothetical protein thiyl radical. The published rate constants were used where available. We assumed, that reaction rates for *intramolecular* hydrogen transfer (reactions 1–3) are the same for free Cys[39] and for Cys located within the protein and that the repair of the protein thiyl radicals by ascorbate (reactions 12 and 13) proceeds with the same rate constant as the repair of glutathione thiyl radicals by ascorbate (reaction 14)[52]. In our simulation we further assume that there is a 50% probability to form D-Cys upon repair of the  $^{\alpha}\text{C}^{\bullet}$ -radical ( $k_2 = k_3$ ), *i.e.* that there are no geometrical restraints on the orientation of the Cys. Reaction 10 and 11 are well known[53, 54], and represent the main detoxification route of “radical repair processes” by thiols, e.g. reactions involving thiyl radicals [55]. The equilibrium between the thiyl radical the disulfide radical anion is documented not only for GSH, but also for other thiols, including Cys residues in proteins[56]. We, therefore, conclude that such a reaction is also to be expected between protein thiyl radicals and GSH (reaction 4a) as well as for glutathione thiyl radicals and the protein thiolate (reaction 4b). Therefore, there is a chain reaction possible for the epimerization of protein cysteine residues via reactions –2, 3, 5, 4b and 4a. In order to simplify the simulation, the protonation equilibria of GSH and protein thiol have not been explicitly simulated. Instead, we calculated the amount of thiolate in equilibrium with  $\text{pK}(\text{GSH})=9.2$  and  $\text{pK}(\text{protein thiol})=8.4$ , respectively, and used the resulting concentrations to evaluate the equilibration reactions 4a, 4b and 10. As a further simplification, HAT reactions with amino acids other than Cys are not considered. The protein concentration was arbitrarily chosen to be 1 mM. This number does not refer to one particular protein but constitutes an assumed total concentration of *all* proteins carrying at least one solvent exposed thiol. The reaction of thiyl radicals with oxygen has been discussed in a number of publications; we base our simulation on the rate constants of Zhang et al. [9]. The following rate constants were used for the kinetic simulations:  $k_1 = 1 \times 10^5 \text{ s}^{-1}$  [39],  $k_{-1} = 1.5 \times 10^{-5} \text{ s}^{-1}$  [39],  $k_2 = 8 \times 10^4 \text{ s}^{-1}$  [39],  $k_{-2} = 7 \times 10^5 \text{ s}^{-1}$  [39],  $k_3 = 7 \times 10^5 \text{ s}^{-1}$  [39], and  $k_{-3} = 8 \times 10^4 \text{ s}^{-1}$  [39],  $k_{4a} = k_{4b} = k_5 = 1.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  (by analogy[57]),  $k_{-4a} = k_{-4b} = k_{-5} = 8 \times 10^6 \text{ s}^{-1}$  (by analogy[57]),  $k_6 = k_8 = 2.2 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  (by analogy[9]),  $k_{-6} = k_{-8} = 6.2 \times 10^5 \text{ s}^{-1}$  (by analogy[9]),  $k_7 = k_9 = 2 \times 10^3 \text{ s}^{-1}$  (by analogy[9]),  $k_{10} = 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  [53],  $k_{-10} = 2.5 \times 10^5 \text{ s}^{-1}$  [53],  $k_{11a}$  [55] =  $k_{11b}$  (by analogy[55]) =  $2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ ,  $k_{12} = k_{16} = 1 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$  (estimate), and  $k_{13} = k_{14} = k_{15} = 5 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$  (by analogy[52]).

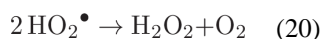
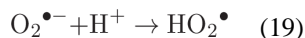
## Thermodynamic considerations

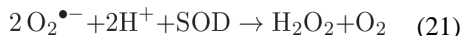
The energetics of Scheme 1 are evaluated with known electrode potentials at pH 7:  $E^{\circ'}$  ( $\text{asc}^{\bullet-}$ ,  $\text{H}^+/\text{Hasc}$ ) = +0.28 V[58],  $E^{\circ'}$  ( $\text{HRS}^{\bullet}$ ,  $\text{H}^+/\text{HRSH}$ ) =  $E^{\circ'}$  ( $\text{GS}^{\bullet}$ ,  $\text{H}^+/\text{GSH}$ ) = +0.92 V[59];  $E^{\circ'}$  ( $\text{O}_2$ ,  $\text{O}_2^{\bullet-}$ ) = -0.35 V[60],  $E^{\circ'}$  ( $\text{RSSG}/\text{RSSG}^{\bullet-}$ ) = -1.3 V[61],  $E^{\circ'}$  ( $^{\alpha}\text{RSH}$ ,  $\text{H}^+/\text{HRSH}$ ) = +0.98 (based on  $K = 0.1$  for the equilibrium  $\text{HRS}^{\bullet} \rightleftharpoons ^{\alpha}\text{RSH}$ [33]) and  $E^{\circ'}$  ( $^{\beta}\text{RSH}$ ,  $\text{H}^+/\text{HRSH}$ ) = +0.92 (based on  $K = 1$  for the equilibrium  $\text{HRS}^{\bullet} \rightleftharpoons ^{\beta}\text{RSH}$ [39]). As more  $^{\beta}\text{RSH}$  radicals are formed than  $^{\alpha}\text{RSH}$  from  $\text{HRS}^{\bullet}$ , we assume that  $E^{\circ'}$  ( $^{\beta}\text{RSH}$ ,  $\text{H}^+/\text{HRSH}$ ) <  $E^{\circ'}$  ( $^{\alpha}\text{RSH}$ ,  $\text{H}^+/\text{HRSH}$ ).

The cycle consisting of reactions 2, 3, 5, 4b and 4a is, of course, thermoneutral. The reaction of a thiyl radical with dioxygen, reaction 8, is an equilibrium[62], and compared with the fast and favorable reaction of thiyl radicals with  $\text{Hasc}^-$ , not very important. The reaction of a carbon-centered radical with  $\text{O}_2$  is considered to be irreversible, due to follow-up reactions (reactions 12 and 16). As follows from the Table 1,  $\text{Hasc}^-$ , even at a concentration of only 0.10 mM, limits protein damage more effectively than 10 mM GSH. The energetics for both superoxide and ascorbyl radical formation are quite favourable, but the former reaction requires the presence of a substantial concentration of GSH to form  $\text{RSSG}^{\bullet-}$ , which then reacts with  $\text{O}_2$ , while the latter, repair by  $\text{Hasc}^-$ , is a fast straightforward bimolecular reaction.

## Discussion

The formation of GSSG has frequently been used as proof for repair of protein radicals by GSH. With our simulations we differentiate between the following reaction channels: (a) direct repair by GSH via reaction 11, (b) repair by ascorbate via reactions 13,14 and 15, (c) formation of thioaldehyde via reaction 12 (significant yields of thioaldehyde or its hydrolysis product, the aldehyde, were detected when thiyl radicals were generated photochemically[31]), (d) epimerization via reaction (3), (e) peroxidation via reaction (16) and (f) oxidation of the protein thiol via reaction 7. In all cases studied protein thiol oxidation, reaction channel (f), was negligible and, therefore, is not referenced to in Table 1. Because of reactions 18 – 22 (*in vivo*,  $\text{H}_2\text{O}_2$  is predominantly generated *via* catalysis by SOD, reaction 21), the experimentally determined GSSG formation will be much higher than indicated by the simulation results on channel (a) only.





We, therefore, added a line in our Table, that indicates the experimentally expected yield. This number probably is underestimated. Reactions 23 and 24 (where reaction 24 is catalyzed by glutathione peroxidase) probably also occur nearly quantitatively.



In the absence of ascorbate, the protein peroxy radicals will probably oxidize GSH via



A kinetic simulation with as many reactions as ours is inherently prone to substantial errors: rate constants of very rapid reactions, as used in our simulations here, typically have errors larger  $\pm 10\%$ , even if these rate constants are reproducible. Additionally, some rate constants were taken from analogous reactions. There are several competition situations which influence yields, for example  $k_1 + k_2$  vs.  $k_{-4a}$ , which determines the minimum damage *via* formation of carbon-centered radicals, or  $k_{11}$  vs.  $k_{12}$ , which strongly influences the maximum production of thioaldehyde. Also, the assumed total concentration of protein (*i.e.* 1 mM) influences the chain reaction, *i.e.* the yield of reaction -4b. We calculated several sets of probable combinations, based on the spread of rate constants published in the literature. For most starting concentrations, uncertainties are below  $\pm 20\%$ .<sup>1</sup> Higher uncertainties, up to  $\pm 60\%$ , were found in simulations for complete absence of ascorbate. Overall, we believe our results to be semiquantitatively correct, the simulations give important insight into the relevant reaction pathways.

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<sup>1</sup>Every rate constant used has an uncertainty and contributes to an error propagation in the calculations. The total error is dependent on all the errors of the rate constants used and to the weight of each individual reaction in the reaction network. We chose a pragmatic approach to estimate errors: we first analysed the robustness of the simulations to changes of single rate constants. Then, we concentrated our analysis on reactions with a high influence on the overall error. With the spread of reported rate constants of such reactions, we calculated the cumulative maximum impact on our result, the yields. The result represents a range where the true result is expected. The true error, however, may be even higher: many rate constants have been determined only once, and we suspect that in such cases the error may be larger than the statistical reproducibility usually given. Because of this, and because we neglected "minor influence reactions" in our determination, we estimate our simulation to have a total error of double the range derived above.

The simulations let us expect a substantial yield of GSSG for typical *in vitro* experiments in the absence of ascorbate (200  $\mu\text{M}$   $\text{O}_2$ , 10 mM GSH), in line with the commonly used reaction scheme based solely on reactions 2, 4a, 4b, 10, and 11a[63]. However, this does *not* imply quantitative *repair*. Our model suggests the formation of additional products, mainly protein aldehydes from the hydrolysis of thioaldehydes and protein-bound D-cysteine (or other D-amino acids[51]). Such species would not be found in standard assays. *In vivo*, the concentration of oxygen is typically around 3–70  $\mu\text{M}$ [41–43], much lower than in air saturated solutions. Under such conditions the reaction chain gets much longer and we expect a distinct increase of the formation of D-Cys. In the presence of ascorbate, damage to the protein strongly decreases and the uncertainties of the simulation sharply drop because the determining influence of ascorbate on the half-life of thiyl radicals. In the presence of 30  $\mu\text{M}$  oxygen, the yield of damage per initial protein thiyl radical decreases from 2.5 irreversibly damaged protein molecules per initiating protein radical in the absence of ascorbate, *i.e.* a chain length of over two, to 0.1 damaged protein molecules in the presence of 1 mM ascorbate. Ascorbate suppresses the propagation step of the chain reaction, repair by 100  $\mu\text{M}$  ascorbate is about one order of magnitude more efficient than repair by 10 mM GSH.

## Conclusion

An important result of our simulations is the fact, that thiyl radical formation may lead to protein damage even in the presence of 10 mM GSH and 1mM ascorbate. Under these conditions, a simulated yield of ca. 0.5 equivalents of GSSG per initial protein thiyl radical would suggest that protein damage might be negligible; nevertheless, our simulated yields for thioaldehyde, D-Cys and protein peroxy radical account for ca. 12% protein damage under such conditions. These yields increase as the concentrations of GSH and ascorbate are lowered, where specifically the ascorbate concentration has a significant effect.

## Acknowledgments

Support of this work by the ETH Zürich and the NIH (P01AG12993) is gratefully acknowledged.

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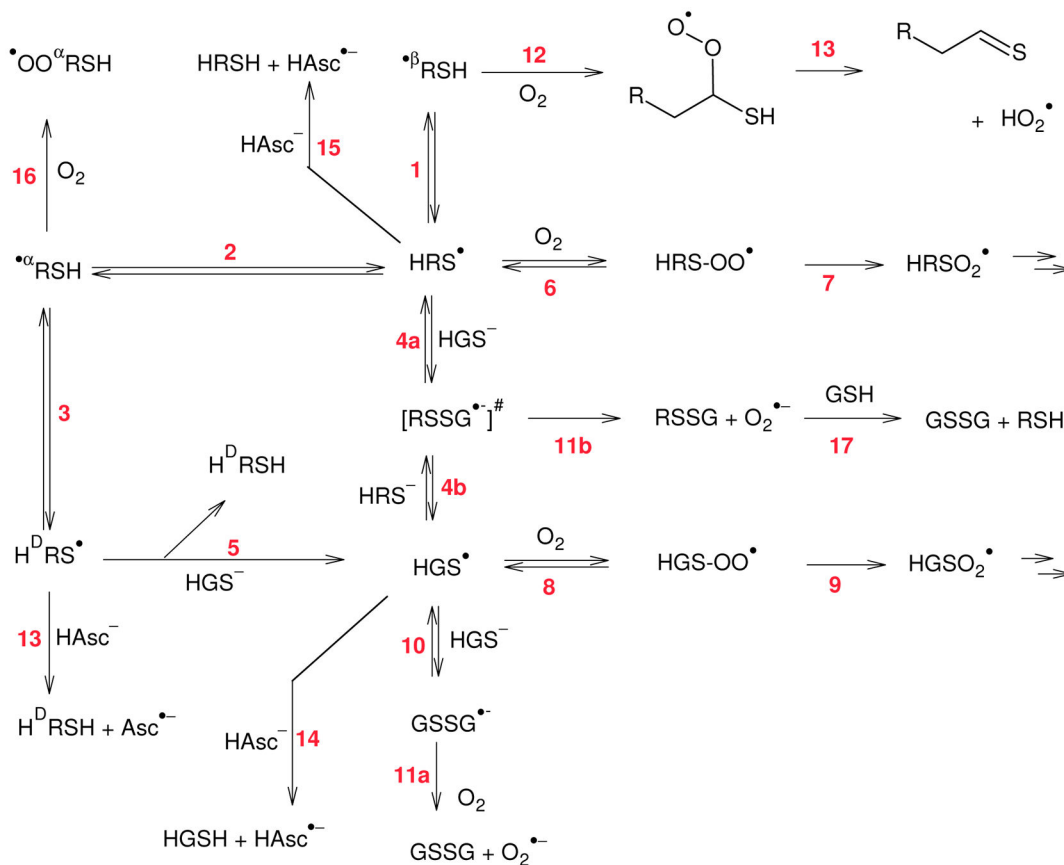


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### Highlights

- Recent experimental evidence suggests the possibility of intramolecular hydrogen atom transfer reactions of thiyl radicals in proteins
- Kinetic simulations including experimental rate constants for hydrogen atom transfer reactions suggest that thiyl radical reactions in proteins may lead to additional products different from disulfides
- The extent to which such hydrogen atom transfer reactions may occur under physiological conditions will depend on the presence of oxygen and antioxidants
- The potential for intramolecular hydrogen atom transfer reactions will increase upon a decrease of antioxidant levels, such as observed under conditions of oxidative stress



**Scheme 1. Reactions of protein thiol radicals considered in the kinetic simulations**

Abbreviations used:

HAsc<sup>-</sup>, ascorbate; HAsc<sup>•-</sup>, ascorbyl radical; HRS<sub>SH</sub>, intact protein; H<sup>D</sup>R<sub>SH</sub>, protein with incorporated D-Cys; HRS<sup>-</sup>, protein thiolate; HRS<sup>•</sup>, protein thiyl radical; <sup>•β</sup>R<sub>SH</sub>, alpha-mercaptoalkyl radical of protein; <sup>•α</sup>R<sub>SH</sub>, backbone radical of protein at Cys; <sup>•OO</sup><sup>α</sup>R<sub>SH</sub>, backbone peroxy radical; HRS-OO<sup>•</sup>, oxygen adduct to a protein thiyl radical; HRSO<sub>2</sub><sup>•</sup>, protein sulfenyl radical; HGSH, Glutathione; HGS<sup>•</sup>, Glutathione thiyl radical; #, This intermediate is invoked by analogy to known chemistry

**Table 1**

Simulated yields of products as a function of experimental parameters

Conditions	pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4
[GSH]/mM		10	10	10	10	0	10	10
[Hasc-]/mM		1	0.1	0	0.1	0	0.1	0
[O <sub>2</sub> ]/μM		30	30	30	30	30	200	200
Calculated yield/initial protein thiyyl radical (in %)								
Product yield	GSSG <sup>a</sup>	0.2	12	30	0	12	32	32
	(Thio-)aldehyde <sup>b</sup>	3.6	34	62	18	32	52	52
	D-Cys in Protein	7.5	16	133	39	20	33	33
	Protein peroxy radical	0.5	2	7.9	23	8	13	13
	Ascorbyl radical <sup>c</sup>	96	78	0	79	47	0	0
	O <sub>2</sub> <sup>-d</sup>	3.7	20	44	18	44	84	84
	GSSG(total) <sup>e</sup>	51	64	64	0	70	94	94
	Protein damage (sum)	12	52	203	80	60	98	98

<sup>a</sup> via oxidation of GSSG<sup>-</sup>

<sup>b</sup> via oxidation of alpha-mercaptoalkyl radicals of Cys by O<sub>2</sub>

<sup>c</sup> via reaction with GS<sup>•</sup> and RS<sup>•</sup>, reacts with O<sub>2</sub> to form O<sub>2</sub><sup>•-</sup>

<sup>d</sup> via a) and b), dismutates to form H<sub>2</sub>O<sub>2</sub>

<sup>e</sup> via a), and oxidation by protein hydroperoxide, H<sub>2</sub>O<sub>2</sub>, and reduction of protein peroxy radicals either by ascorbate or GSH