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Hydrogen Exchange Equilibria in Thiols

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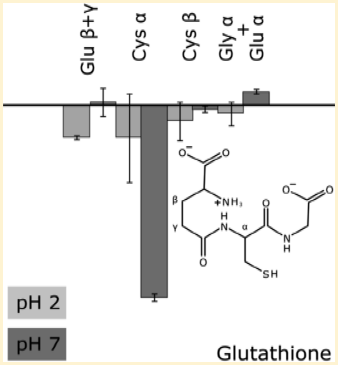
ABSTRACT: Cysteine, cysteinyl-glycine, glutathione, phenylalanyl-cysteinyl-glycine, and histidyl-cysteinyl-glycine were dissolved in acidic and neutral D_2O in the presence of the radical generator 2,2′-azobis(2-methylpropionamidine) dihydrochloride and radical mediator compounds (benzyl alcohol and 2-propanol). An exchange of H-atoms by D-atoms took place in these peptides due to intramolecular H-abstraction equilibria. NMR measurements allow one to follow the extent of H−D exchanges and to identify the sites where these exchanges take place. Significant exchanges occur in acidic media in GSH at positions Glu-β and Glu-γ, in Phe-Cys-Gly at positions Phe *ortho*, Phe-β, Cys-α, Cys-β, and Gly-α, and in His-Cys-Gly at positions His H1, His H2, His β, Cys β, and Gly α. In neutral media, exchanges occur in Cys-Gly at position Cys β and in GSH at position Cys α. Phe-Cys-Gly and His-Cys-Gly were not examined in neutral media. Sites participating in the radical exchange equilibria are highly dependent on structure and pH; the availability of electron density in the form of lone pairs appears to increase the extent of exchange. Interestingly, and unexpectedly, 2D NMR experiments show that GSH rearranges itself in acidic solution: the signals shift, but their patterns do not change. The formation of a thiolactone from Gly and Cys residues matches the changes observed.



INTRODUCTION

The formation of peptide and protein thiyl radicals has attracted attention during the last decades.[1−9] This is mostly because thiols in general, and glutathione (GSH) in particular, are very important in human metabolism: GSH is involved in detoxification, and it provides reducing equivalents to maintain the redox state of the cell. Furthermore, it is thought to play an important role in the process of aging, particularly in connection with warding off effects of partially reduced oxygen species or partially oxidized nitrogen species.[10,11]

In vivo, glutathione thiyl radical ($GS^\bullet$) can be produced from the reaction of GSH with $HO^\bullet$,[12] $NO_2^\bullet$, $CO_3^{\bullet-}$,[13] or protein radicals;[14] ONOOH, H_2O_2, and $O_2^{\bullet-}$ can be excluded because their reactions are either too slow, or they react with other cellular components.[13,15,16]

Recent pulse radiolysis experiments showed that our understanding of the reactivity of the GSH and cysteine thiyl radicals is incomplete.[17] Although deamination and desulfurization are probable fates of a cysteine thiyl radical,[5,18,19] there seems to be a plurality of coexisting radical species centered at sulfur, α-carbons, and β-carbons, which are present as intermediates.[17,19] While we found that hydrogen transfer occurs from $^{\alpha}$CH and $^{\beta}$CH to $S^\bullet$, kinetic methods do not tell us on which amino acid an α- or β-radical is located. To determine these sites, we carried out ^{1}H NMR experiments in D_2O analogous to those conducted with cysteamine[20] to determine which hydrogen atoms are abstracted and to what extent. Briefly, radicals generated from the thermal decomposition of an azo-compound abstract a hydrogen atom from the thiol (reactions 1 and 2).[21,22] Alcohols act as a radical chain extender (reaction 3):

$$R^1{-}N{=}N{-}R^1 \rightarrow 2R^{1\bullet} + N_2 \quad (1)$$

$$R^{1\bullet} + R^2SH \rightleftharpoons R^2S^\bullet + R^1H \quad (2)$$

$$R^2S^\bullet + HR^3OH \rightleftharpoons R^2SH + {}^\bullet R^3OH \quad (3)$$

At optimal concentrations of radical chain initiator (α,α'-azodiisobutyramidindihydrochloride, AAPH), chain extender (alcohol), and target molecule (thiol), the radical chain (eq 3) will be long, and $R^2S^\bullet$ can be generated many times. The concentration of all reactants must be optimized: at high concentrations of chain carrier (i.e., alcohol), eq 3 can shift too far to the thiyl side; the concentration of thiyl radicals may be too low to form enough products, or if the lifetime of thiyl radicals is too short, no intramolecular hydrogen transfer takes place. On the other hand, if the alcohol concentration is too low, the chain length is too short to produce sufficient products. The decrease of H-signal intensity, determined by ^{1}H NMR, is used to deduce where H has been replaced by the NMR-invisible D (^{2}H) from the solvent via protolysis of the thiol group (reactions 4 and 5):

$$^{H}R^2S^\bullet \rightleftharpoons {}^\bullet R^2SH \quad (4)$$

$${}^\bullet R^2SH + D_2O \rightleftharpoons {}^\bullet R^2SD + HDO \quad (5)$$

$${}^\bullet R^2SD \rightleftharpoons {}^{D}R^2S^\bullet \quad (-4)$$

where phenyl radicals might be formed, benzyl alcohol could not be used as a chain extender and instead d^6-isopropanol was used. Hydronation (protonation by all isotopes of hydrogen) hinders deamination of the residues. As this may happen at higher pH, we carried out experiments only in acidic and neutral media.

It should be noted here that these experiments provide positive proof only: if the required reaction conditions are not

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met, no products are found. Until now, only competing intermolecular reactions have been studied with this technique.[20] Under our conditions, competing inter- and intramolecular reactions take place, which makes it much more challenging to perform and interpret such experiments.

While in the past thiyl radicals were perceived as relatively unreactive,[23−26] our results indicate and confirm[6,27−29] that such radicals are harmful in that they can abstract hydrogen atoms from nearby C−H entities.

Somewhat surprisingly, we found that glutathione rearranges in acidic media. Although C−H connectivities and signal integrals are retained, the signal positions shift significantly in the course of time.

Evidence for hydrogen transfer within glutathione after formation of thiyl radicals is also presented in the accompanying paper by Mozziconacci, Williams, and Schöneich (this issue, DOI: 10.1021/tx3000494).

EXPERIMENTAL PROCEDURES

Materials. All chemicals were used as delivered. L-Cysteine (2-amino-3-sulfanylpropanoic acid, Cys, 99.5%) was obtained from Fluka {Buchs, Switzerland), cysteinyl-glycine ([(2-amino-3-sulfanyl-propanoyl)amino]acetic acid, cysteinyl-glycine (Cys-Gly), 98%} was from Bachem (Bubendorf, Switzerland), GSH {(2*R*)-2-amino-5-({(2*R*)-1-[(carboxymethyl)amino]-1-oxo-3-sulfanylpropan-2-yl}-amino)-5-oxopentanoic acid, 97%} was from ABCR (Karlsruhe, Germany), and phenyl-cysteinyl-glycine [({2-[2-amino-3-phenylpropanoyl)amino]-3-sulfanylpropanoyl}amino)acetic-acid, phenylalanyl-cysteinyl-glycine (Phe-Cys-Gly), 96%] and histidyl-cysteinyl-glycine {[(2-{(2-amino-3-(1*H*-imidazol-4-yl)propanoyl]amino}-3-sulfanylpropanoyl)amino]acetic acid, histidyl-cysteinyl-glycine (His-Cys-Gly), 97%} were obtained from Peptide 2.0 (Chantilly, VA). Deuterium oxide (99.96% ^{2}H) and deuterium chloride (35% in ^2H_2O, 99.5% ^{2}H) were purchased from Cambridge Isotope Laboratories Inc. (Andover, MA), sodium phosphate (96%) and 2-propanol-1,1,1,3,3,3-^{2}H6 (d^6-isopropanol, 99% ^{2}H) were from Aldrich (St. Louis, MO), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH, 98.0%) and tetramethylammonium chloride (99%) were from Fluka (Buchs, Swizerland), and 2-phenyl ethanol (benzyl alcohol, 99.8%) was from Riedel-de Haën (Seelze, Germany).

Sample Preparation and Measurements. The pD of the D_2O solutions was pD 1, pD 2, or neutral; they were deaerated by at least 3-fold evacuation with subsequent shaking under argon at atmospheric pressure and then transferred to a glovebox with an inert atmosphere. The solutions contained 2 mM cysteine or peptide, the radical initiator AAPH (20 mM), and a radical propagator (20 mM benzyl alcohol for Cys, Cys-Gly, and GSH, and 20 mM d^6-isopropanol for His-Cys-Gly and Phe-Cys-Gly). $N(CH_3)_4Cl$ (0.2 mM) was added to solutions of His-Cys-Gly and Phe-Cys-Gly as a NMR reference, and for all other samples, the phenyl group of the benzyl alcohol served as reference. Samples were prepared at two different acidities: some had high acidity (50 mM DCl), to prevent deamination; five others were at neutral pD prepared with 100 mM phosphate in DCl, to obtain results that were physiologically relevant.

NMR tubes were filled within the glovebox and sealed with rubber stoppers and Parafilm. Sets of 10 identical samples were prepared and stored in a N_2-filled glovebox to prevent any reaction with O_2. Pairs of samples were then sequentially removed from the glovebox for measurements with 250 (for GSH) or 300 MHz (all other samples) instruments from Bruker BioSpin GmbH (Fällanden, Switzerland). ^{1}H NMR spectra were recorded with a relaxation delay of 15 s. For ^{1}H−^{1}H correlation NMR spectroscopy (H,H−COSY) measurements, an anaerobic GSH solution (10 mM) in deuterium chloride (10 mM) without radical initiator was transferred to a glovebox and distributed over NMR tubes, which were sealed with stopper and Parafilm. Samples were removed from the glovebox immediately after preparation and after 6 days and analyzed with the 300 MHz instrument. Experiments carried out in the presence of O_2 gave comparable results: because the concentration of O_2 (approximately 260 μM) is much smaller than that of GSH (2 mM), the concentration of products formed from O_2 would have been below the detection limit.

Data Treatment. Reference compounds allowed alignment and normalization of the signals investigated. The free induction decay signals of the NMR recording were brought to the frequency/amplitude domain, and integrals of samples were evaluated. Differences between the signals directly after mixing and after several days were plotted. Increases originate from NMR signals from fission products of AAPH.

RESULTS

The GS$^\bullet$ at neutral pD abstracts the hydrogen at the Cys α site. During 96 h, the NMR integral of the α-H decreases to ca. 50% (Figure 1). No other exchanges are observed (Figure 2).

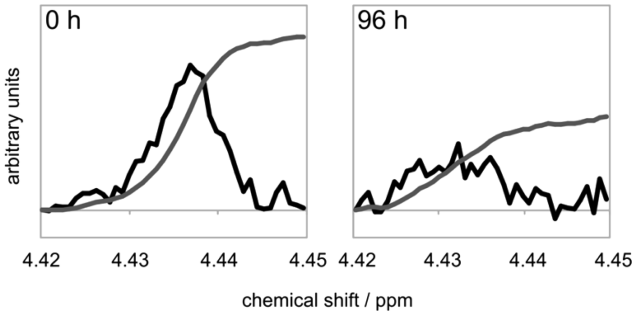

Figure 1. NMR signal (black line) and integral (gray line) for the hydrogen at the GSH (2 mM) Cys α site at pD 7 immediately after preparation (left) and after 96 h (right) at 24 °C.

Surprisingly, at acidic pD, the β- and γ-sites of Glu exchange H$^\bullet$ with the thiyl radical, but no other reactions can be seen (Figure 2). No H$^\bullet$ exchange is observed in cysteine (Figure 2), and the changes are, at both pD 1 and pD 7, not significant. However, when a glycine residue is present at the C terminus, the Cys β-carbon of Cys-Gly shows significant H/D exchange at neutral pD, while no significant changes are observed at other sites. No exchange activity is observed in Cys-Gly at low pD (Figure 2).

Exchange also occurs in His-Cys-Gly and Phe-Cys-Gly, but the exchange patterns are different (Figure 2): with the exception of the His and Phe α sites (which would correspond to the γ-site of Glu in GSH), nearly all carbon atoms appear to exchange. In both tripeptides, exchanges involving the Gly α- and Cys β-hydrogen are the most significant along with those at His-H1 for His-Cys-Gly and *o*-Phe for Phe-Cys-Gly. For the Cys α hydrogen of His-Cys-Gly, the results are too inaccurate to allow a conclusion. Most important is the degree of exchange: although His-Cys-Gly and Phe-Cys-Gly are similar, in the sense that they both contain an aromatic system, His-Cys-Gly exchanges are approximately twice as large as those of the Phe-tripeptide.

Unexpectedly, new peaks were found in the ^{1}H NMR spectrum of GSH after 6 days in acidic solution, with or without radical initiators being present. H,H−COSY experiments revealed that the C−H framework of GSH had remained unchanged. Moreover, these experiments allowed assignment of the product peaks to the original peaks (Figure 3), which enabled us to correct the original peaks for shifts caused by the rearrangement. All cross-peaks of the 2D NMR diagram were assigned; therefore, the formation of additional products can be excluded. Thus, we only observe two processes: fast replacement of H by D, which occurs in the range of days, and a slow rearrangement of GSH, which occurs in the range of weeks. Therefore, the results for GSH in acidic solutions are only of a qualitative nature.

DISCUSSION

The method of exchanging H atoms with D atoms has been illustrated earlier.[9,20,22] The decrease in the integral in Figure 1

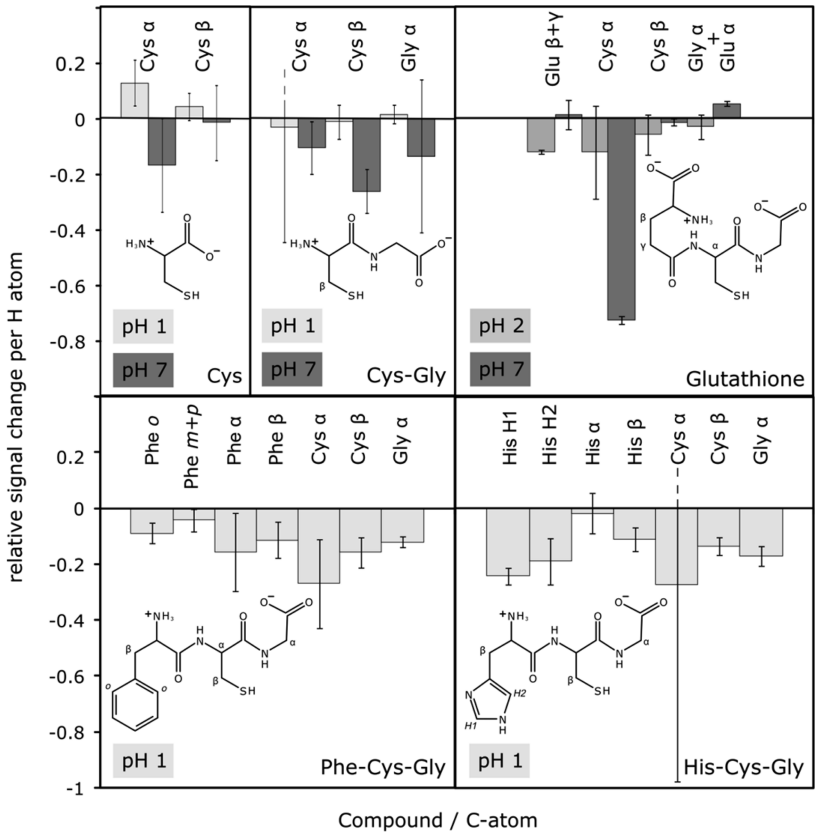

Figure 2. NMR signal change per H atom at the GSH sites (with 2σ error bars) during radical exposure. Cys and Cys-Gly at pD 1 (20 days) and 7 (15 days). In the case of GSH at pD 2 (192 h) and pD 7 (96 h), the glycine and glutamic acid α sites and glutamic acid β and γ sites could not be separated due to signal overlap. His-Cys-Gly at pD 1 (53 d), H1 designates the H atom linked to the carbon atom between the nitrogen atoms. Phe-Cys-Gly at pD 1 (53 d), the NMR signals for H atoms at *meta* and *para* positions could not be resolved. Sites with significant exchange are marked with their letter of position in the structures given.

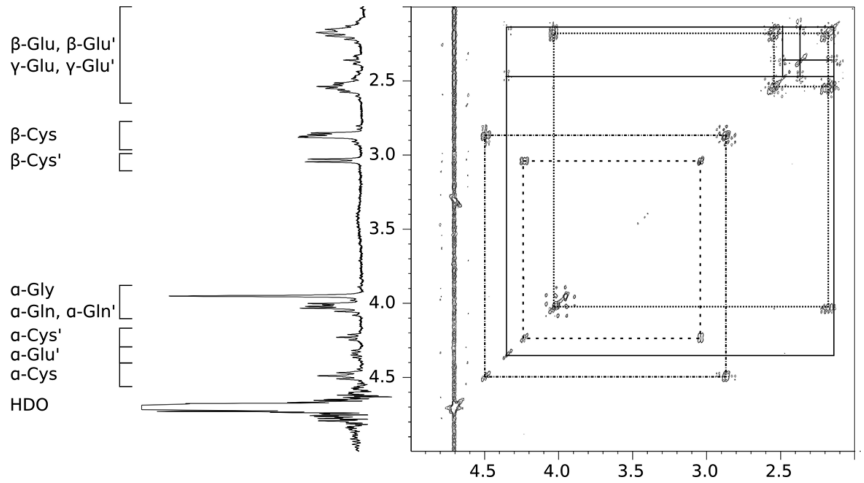

Figure 3. Two-dimensional H NMR of GSH in D_2O in 0.1 M DCl after 6 days without radical initiator. GSH undergoes an unidentified rearrangement in acidic media. NMR results show that the connectivity of C and H atoms remains. All three residues of GSH are distinguishable by their coupling scheme: Glu possesses three coupling protons, Cys only two, and the proton of Gly does not couple to any other proton. Two-dimensional H NMR allows us to attribute the number of coupling protons; therefore, emerging signals can be connected to the transformed residues Glu′, Cys′, and Gly′, respectively. The couplings are represented with the following line types: Cys, wide dash; Cys′, dash-dot; Glu, solid; Glu′, fine dash; and Gly and Gly′, none (no coupling). Corresponding signals were added for evaluation of the H/D exchange.

shows that such an exchange takes place in GSH. Noise and relaxation effects cause the signals to be of inhomogeneous quality. We therefore present our results with an error of two standard deviations, to avoid misinterpretation. Our experiments show that H/D exchange takes place in all investigated peptides at acidic pD significantly in Cys, GSH, His-Cys-Gly, and Phe-Cys-Gly but not in Cys-Gly. His-Cys-Gly and Phe-Cys-Gly were not examined at neutral pD, but Cys, Cys-Gly, and GSH show significant exchange, in agreement with a preceding publication,[17] where we determined rates and equilibria of intramolecular H abstraction that involve thiyl radicals but could not locate the H atoms concerned. Our findings are in agreement with recent γ-radiolysis

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studies on amino acids and peptides.[30] The present work assigns the radicals a specific site in the molecule. The different distribution patterns between α- and β-centered carbon radicals shown here and before[17] are not in contradiction: the amount of incorporation of deuterium atoms into GSH depends not on the persistence of the radicals, on their concentration, or on the fast equilibration of carbon-centered radicals in GSH, but on the total turnover rate, the rate constant of which is equal to $k_{forward} \cdot k_{reverse} \cdot (k_{forward} + k_{reverse})^{-1}$, with $k_{forward}$ and $k_{reverse}$ being the reactions of eq 4. The hydronic exchange eq 5 can be neglected due to its high rate constant. The visibility of the carbon-centered radicals in the UV spectrum depends on both the equilibrium constant $k_{forward}/k_{reverse}$ and the molar absorptivity; the equilibration rate is equal to the sum of the rate constants $k_{forward} + k_{reverse}$, and the persistence of a carbon-centered radical increases with decreasing $k_{reverse}$. Therefore, neither a high equilibration rate constant nor a high persistence of carbon-centered radicals necessarily leads to a high turnover rate constant. Efficient H/D exchange, the basis of a significant change in the NMR signal, needs both a large $k_{forward}$ and a large $k_{reverse}$. As intramolecular H abstraction in thiyl radicals is not limited to GSH, these observations are of major interest.

Our experiments showed where H abstraction takes place in vitro and could occur in vivo, but they do not show a discernible pattern. Proximity is not an important factor because Cys β is abstracted in Cys-Gly, and Cys α is abstracted in GSH (and possibly in Cys), at pD 7. It is clear that abstraction is dependent on the pD, that is, the abstraction patterns are different under acidic and neutral conditions. Whereas at pD 7 abstraction of the hydrogen atom at the Cys α site can be explained by proximity and formation of a π-system, the abstraction pattern at pD 2 is very surprising: abstraction of hydrogen atoms at the Glu β or γ position does not seem very intuitive and lacks an obvious driving force. However, experiments with His-Cys-Gly and Phe-Cys-Gly show abstractions at equivalent positions. Second, we also observe that compounds that contain nitrogen atoms (or, more general, hetero atoms with lone pairs) exchange more than others. Comparison of His-Cys-Gly with Phe-Cys-Gly shows that there is more H/D exchange in His-Cys-Gly than in Phe-Cys-Gly, although both structures are very similar: the Cys-Gly part is the same, and the first residue contains an aromatic system. However, we observe more exchange, especially near the N terminus of His-Cys-Gly. We speculate that the availability of electron density (or at least a lone pair) in general enhances H/D exchange by lowering the activation energy for H abstraction.[9,31] The participation of electron density in the exchange process would also explain the exchange observed in the otherwise rather inert benzene system of the Phe residue.

It was previously assumed that sulfur radicals were relatively stable. We have shown that such radicals produce intramolecularly and intermolecularly (reaction 6) C-centered radicals.[6,9,32] These radicals react further, as mentioned, to irreversibly form stable deamidated and desulfurized products.[5,19] Thus, the concentration of thiyl radicals decreases rapidly with time.

$$R^2S^\bullet + R^4CH \rightleftharpoons R^2SH + R^4C^\bullet \quad (6)$$

These results also shine new light on our photolysis study of *S*-nitrosoglutathione (GSNO).[33] If photolysis of GSSG or GSNO produces GS$^\bullet$,[34] then it should recombine rapidly with NO$^\bullet$ that was produced from GSNO in the same process or added to the solution beforehand. This we did not observe, and we therefore claimed that GS$^\bullet$ does not react with NO$^\bullet$.[33] Given the rapid intramolecular exchange of H (eq 4), our observation may now make sense: reaction at the C-centered radicals, which produces C−NO, pulls the equilibrium further to the C-centered side, which inhibits the recombination of GS$^\bullet$ and NO$^\bullet$. However, it was shown by γ-radiolysis and competition kinetics that NO$^\bullet$ disappeared at a rate that was commensurate with a rapid recombination of GS$^\bullet$ and NO$^\bullet$, and a GSNO-like build-up was directly measured when GS$^\bullet$ was produced by pulse radiolysis in NO$^\bullet$-containing solution.[35] However, these investigations did not show that only GSNO was produced: the rate constant, measured by competition kinetics, may be accurate for formation of both S−NO and C−NO bonds, approximately 3×10^9 M^{-1} s^{-1}.[36] Moreover, the m/z for all possible nitrosation products produced from the redistribution would be the same, rendering them inseparable by electrospray mass spectroscopy, if indeed those unknown products would actually separate under the chromatography conditions chosen. Thus, the matter stays unresolved. Most intriguingly, the fate of thiyl radicals seems to be dependent on the way they were generated: oxidatively from thiols, they seem to form disulfides exclusively, but photolysis of disulfides yields hemithioacetals,[37] and one-electron reduction of disulfides leads to considerable amounts of carbon-centered radicals,[17] probable precursors of such thiohemiacetals. It also remains unresolved whether the products found for the reaction of "thiyl radicals" with nitrogen monoxide are likewise dependent on the chemical system chosen for thiyl radical production. In principle, in vivo formation of nitrosoglutathione may proceed via reaction of GS$^\bullet$ with NO$^\bullet$, of NO_2^- with GSH, or of a metal−nitrosyl complex with GSH. The thermodynamics of these reactions have recently been presented.[38] It is surprising that the in vivo mechanism of nitrosation has not yet been elucidated.

Additional chemical implications of our findings are that peptides can cross-link irreversibly intra- or intermolecularly. This would interfere with protein turnover and, therefore, increase stress to the cell. C-centered radicals could also serve as initiators for peroxidation of proteins. These considerations emphasize that S-centered radicals are not harmless.

Although both GS$^\bullet$ and $O_2^{\bullet-}$ may be formed "accidently" in vivo, there is no enzymatic pathway to deal with GS$^\bullet$, while the disposal of $O_2^{\bullet-}$ by superoxide dismutases is very efficient. ^{H}GS$^\bullet$, or $^\bullet$GSH, is rapidly reduced by monohydrogen ascorbate and urate.[39,40] Previously, sulfur radials were thought to disappear via reaction with RSH, resulting in RSSR$^{\bullet-}$, which reacts with O_2 to form $O_2^{\bullet-}$.[41] In addition, we showed that carbon-centered radicals are formed and the kinetics of their formation.[17] Here, we show which sites are vulnerable.

The story of thiyl radicals in general, and of the GS$^\bullet$ in particular, does not appear to be finished yet. Many processes, such as H abstraction, deamination, or desulfurization,[5,19] determine the final fate of thiyl radicals. To capture important stages of processes in diseases and in aging, these underlying processes must be understood, and observation of β- and γ-radicals is a first step.

As for the acidic rearrangement of GSH, there are a variety of possible structures. The acid-catalyzed formation of a thiolactone (Figure 4) is in agreement with the NMR data and the

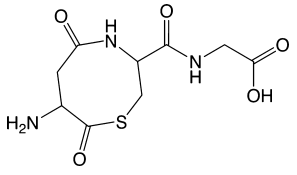

Figure 4. We suggest this thiolactone as the product of the unknown rearrangement under acidic conditions.

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experimental conditions, but we do not exclude the possibility that other structures produce identical NMR signals.

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Notes

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Amino acid side chain numbering: The Greek letters refer to the position of the C-atom from the amine group in the amino acid residue. IUPAC numbering is in Roman numbers, starting at the C terminus of the amino acid chain; different amino acids in a peptide chain are distinguished by an increasing number of primes: The 2-site according to IUPAC is equal to Cys α in Cys, to Gln α in GSH, and to Gly α in Cys-Gly, Phe-Cys-Gly, and His-Cys-Gly. The 3-site is equal to Cys β in Cys and to Gln β in GSH. The 2′- and 3′-sites are equal to Cys α and Cys β in GSH, Cys-Gly, Phe-Cys-Gly, and His-Cys-Gly.

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ABBREVIATIONS

AAPH, α,α'-azodiisobutyramidindihydrochloride; Cys-Gly, cysteinyl-glycine; $GS^{\bullet}$, glutathione thiyl radical; GSH, glutathione; GSNO, *S*-nitrosoglutathione; H,H−COSY, $^{1}H-^{1}H$ correlation NMR spectroscopy; His-Cys-Gly, histidyl-cysteinyl-glycine; His H1 and His H2, inequivalent H atoms on histidine ring according to Figure 2; Phe-Cys-Gly, phenylalanyl-cysteinyl-glycine

NOMENCLATURE (FORMULA: *VENERABLE NAME*, IUPAC NAME)

$CO_3^{\bullet-}$, *carbonate radical*, trioxidocarbonate(•1−); H_2O_2, *hydrogen peroxide*, dioxidane or dihydridodioxygen; HON═NOH, *hyponitrous acid*, diazenediol; $NO^{\bullet}$, *nitric* oxide, nitrogen monoxide or oxidonitrogen(•); $NO_2^{\bullet}$, nitrogen dioxide, or dioxidonitrogen(•); ONOOH, *peroxynitritrous acid*, (hydridodioxido)oxidonitrogen; $O_2^{\bullet-}$, *superoxide*, dioxide(•1−)

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