Significance of the intramolecular transformation of glutathione thiyl radicals to α-aminoalkyl radicals. Thermochemical and biological implications

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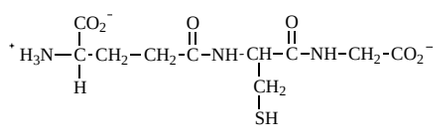
Product studies have been undertaken on the $OH^{\bullet}$ radical-induced oxidation of glutathione in N_2O-saturated aqueous solutions. Ammonia has been found to be a prominent product with *G* values around $2.5–2.9 \times 10^{-7}$ J mol^{-1} from pH 6 to 10.5. The ammonia is considered to be a product of the disproportionation reaction of the α-amino carbon-centred radicals, formed *via* the intramolecular transformation of glutathione thiyl radicals. At pH *ca.* 4–6, the ammonia yield decreases due to the fact that the transformation reaction slows down with decreasing pH and eventually comes into competition with bimolecular recombination. From the pH dependence of the ammonia yield curve, the equilibrium constant between the glutathione thiyl radical and the α-amino carbon-centred radical is deduced to be $>10^4$. The strength of the C–H bond α to the NH_2 and CO_2^- groups is thus <343 kJ mol^{-1}. The corresponding bond energy of the C–H bond α to the NH_2 and CO_2H groups is estimated to be <329 kJ mol^{-1}. Based on the ammonia formation, consumption of free SH groups and the HPLC chromatograms obtained at different pH values after γ-irradiation of N_2O-saturated glutathione solutions, the overall reaction mechanism concerning the fate of glutathione thiyl radicals is proposed. This mechanism and its kinetics indicate that the intramolecular transformation is one of the principal pathways of self-removal of glutathione thiyl radicals, which is formed in various repair processes, in both anaerobic and aerobic conditions.

Introduction

The tripeptide glutathione acts as an important electron or hydrogen atom donor towards oxidizing species.[1–3] It is found in all cells of higher animals where its level can be as high as 10 mmol dm^{-3}.[4] The structure of glutathione is shown below.



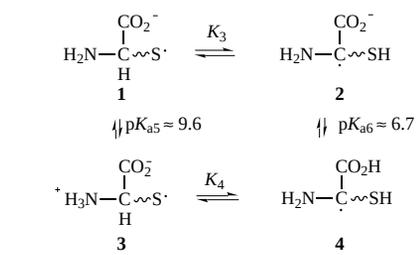
GSH

Note that glutathione contains a glutamic acid residue joined in an unusual peptide linkage involving its γ-carboxyl rather than the α-carboxyl group. The free radical chemistry of glutathione has been studied for a long time. It is generally accepted that glutathione thiyl radicals ($GS^{\bullet}$) derived from repair processes conjugate as $GSSG^{\bullet -}$ with GS^-.[5]

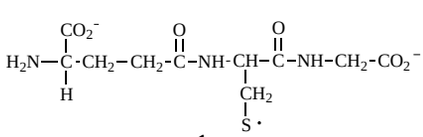
$$GSH \overset{pK_{a1} \approx 9.2}{\rightleftharpoons} GS^- + H^+ \qquad (1)$$

$$GS^{\bullet} + GS^- \rightleftharpoons GSSG^{\bullet -} \qquad (2)$$

Reaction (2) serves as a chemical link between the oxidizing and reducing function of the semi-oxidized thiol. The rate constants k_2 and k_{-2} were measured to be 5×10^8 dm^3 mol^{-1} s^{-1} and 2×10^5 s^{-1}, respectively, which yields an equilibrium constant of 2.5×10^3 dm^3 mol^{-1} for reaction (2).[6–8] However, at physiological pH, with the GSH sulfydryl $pK_{a1} \approx 9.2$,[5] only a very small portion of $GS^{\bullet}$ is conjugated as $GSSG^{\bullet -}$.[3] Recent studies on the kinetics of one-electron oxidation of thiols revealed that glutathione thiyl radicals (radical **1** in Scheme 1) undergo an intramolecular hydrogen transfer to form reducing α-aminoalkyl radicals **2**.[8,9] In Scheme 1, the carbon-centred radicals **2** and **4** represent the α-aminoalkyl radicals at the glutamyl residue.



where **1** and **2** represent the following structures:



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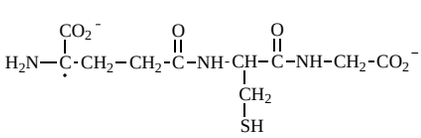

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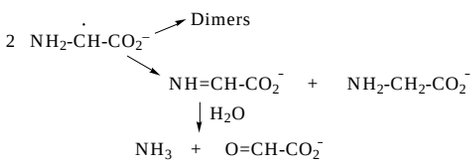
Scheme 1

The thermodynamic driving force for the transformation is that the C–H bond at the α-position to the deprotonated amino and carboxyl groups is substantially weaker than the S–H bond. Therefore, equilibrium (3) is driven far to the right.[8] The hydrogen transfer equilibrium is pH dependent due to the protonation of the α-amino group. Species **3** has a pK_a of 9.6. The pK_a of the α-amino carbon-centred radical **4** is probably close to 6.7, and protonation occurs at the carboxyl group (radical **4**), as measured by EPR experiments.[10] The α-ammonium pK_a of

this radical is even lower (−8.4) according to thermochemical estimation.[11] Kinetic studies by pulse radiolysis give a rate constant of 1.8×10^5 s^{-1} at pH 10.5,[8] and an OH^- dependent rate constant of 5×10^9 dm^3 mol^{-1} s^{-1} for the forward reaction of equilibrium (3).[9] This implies that at physiological pH 7.5, the effective rate constant of reaction (3), k_3', is around 10^3 s^{-1}. The equilibrium constant of reaction (3) was estimated to be at least 10^3.[8] Direct measurement of k_{-3} is not feasible due to the difficulties of initially producing radical **2** in the system. Nevertheless, this equilibrium appears to be a good alternative to equilibrium (2) for removing GS$^\bullet$ from various repair processes in biological systems.

Earlier studies of OH radical oxidation of the simpler α-amino acids glycine and alanine in aqueous solutions led to the identification of α-amino carbon-centred radicals.[2] The radicals thus formed mainly disproportionate or combine.



Scheme 2

The imino acid derivative produced in the disproportionation reaction of the α-amino carbon-centred radical hydrolyses spontaneously, liberating ammonia. Thus the ammonia formed as a result of oxidative deamination provides a measure by which the extent of formation of α-amino carbon-centred radicals can be quantified.

The oxidative deamination subsequent to γ-irradiation of glutathione in aqueous solution has not, to our knowledge, yet been investigated. The equilibrium between glutathione thiyl radical and the α-aminoalkyl radical can be observed through measurements of the stable reaction products. In the present work, extensive product studies were undertaken on the γ-radiolysis of glutathione in N_2O saturated aqueous solutions. Special attention was paid to the pH dependence of ammonia formation and glutathione thiol group consumption. The predominant formation of ammonia with a G value around 2.5–2.9×10^{-7} J mol^{-1}, and the low consumption of free thiol group in the pH range 6–10.5, indicate that equilibrium (3) plays a very important role in the free radical chemistry of glutathione. The thermochemistry of the α-amino C–H bond as well as the overall reaction mechanism concerning the fate of thiyl radicals in biological systems are also discussed.

Experimental

Glutathione (GSH) and its oxidized form (GSSG), 5,5′-dithiolbis-2-nitrobenzoic acid (DTNB, also called Ellman's reagent), NH_4Cl, K_2HPO_4 and KH_2PO_4 were obtained from Aldrich. All chemicals were of the highest purity commercially available and were used as received.

γ-Radiolysis was performed in a ^{60}Co γ-source (AECL Gammacell 220), with a dose rate of 0.16 Gy s^{-1}. A Fricke dosimeter[12] was used to check the dose.

All solutions were prepared using millipore-deionized water and buffered in 10^{-2} mol dm^{-3} phosphate medium. The pH of the solution was adjusted by adding phosphoric acid and sodium hydroxide. The solutions were deoxygenated by bubbling with Ar gas, and subsequently saturated with N_2O. Since oxygen was suspected to be critical, the purging gas was bubbled through an alkaline pyrogallol solution to reduce the oxygen level as much as possible. In N_2O saturated solutions, $G(OH^\bullet) = 5.6 \times 10^{-7}$ J mol^{-1}, $G(H^\bullet) = 5.7 \times 10^{-8}$ J mol^{-1}, $G(H_2O_2) = 7 \times 10^{-8}$ J mol^{-1}, and the radicals arise in the reactions below:

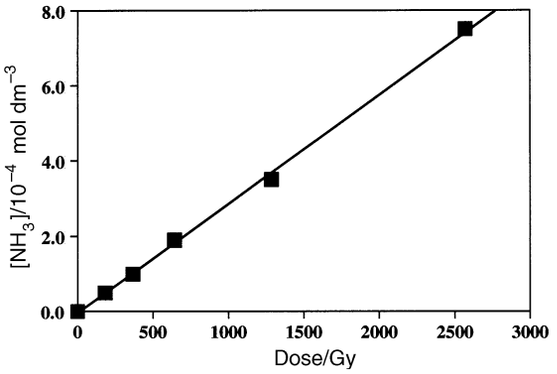


Fig. 1 Ammonia yield upon the γ-irradiation of N_2O-saturated solutions containing 5×10^{-3} mol dm^{-3} glutathione at pH 7.3 as a function of dose

$$H_2O \longrightarrow OH^\bullet, e_{aq}^-, H^\bullet, OH^-, H_2O_2, H_2$$

$$e_{aq}^- + N_2O \longrightarrow OH^\bullet + OH^- + N_2$$

Hydroxyl radicals and hydrogen atoms attack glutathione mainly at sulfur, with rate constants of (1–2) × 10^{10} dm^3 mol^{-1} s^{-1}.[2]

$$GSH + OH^\bullet \longrightarrow GS^\bullet + H_2O$$

$$GS^- + OH^\bullet \longrightarrow GS^\bullet + OH^-$$

Hydrogen atoms participate in two reaction pathways.

$$GSH + H^\bullet \longrightarrow GS^\bullet + H_2$$

$$GS^- + 2H^\bullet \longrightarrow G^\bullet + H_2S$$

The glutathione carbon-centred radical G$^\bullet$ thus formed is rapidly repaired by GSH to form GS$^\bullet$.

$$GSH + G^\bullet \longrightarrow GS^\bullet + GH$$

Thus the total $G(GS^\bullet)$ derived from the primary radicals is $G(OH^\bullet) + G(H^\bullet) \approx 6.2 \times 10^{-7}$ J mol^{-1}.

The ammonia yield after γ-irradiation was measured by a sensitive membrane electrode, Model 8002-8 Ammonia Probe, from ABB Kent-Taylor. The probe is specified to have a linear NH_3 response range from 5×10^{-6} to 5×10^{-2} mol dm^{-3}, which was checked by measuring against standard NH_4Cl solutions. Standard control samples were used in each measurement to avoid errors from drifting of the probe. The consumption of free SH groups after irradiation was analysed by means of Ellman's method.[13] The H_2S formation was measured by comparing free SH group consumption before and after degassing the irradiated solutions at low pH. Samples were also analysed by HPLC with a reverse phase column (Nucleosil 100-5C18) in combination with UV detection at 210 nm. The mobile phase contained 2.5% MeOH and 0.1 mol dm^{-3} phosphate adjusted to pH 3 with phosphoric acid.

All experiments were carried out at room temperature (21 ± 2 °C).

Results and discussion

Ammonia formation

Hydroxyl radical oxidation of glutathione yields ammonia. The dose-dependent formation of NH_3 in a N_2O-saturated aqueous solution containing 5×10^{-3} mol dm^{-3} GSH at pH 7.3 is shown in Fig. 1. The ammonia formation is linear *versus* dose with a slope of 2.9×10^{-7} J mol^{-1}, which represents $G(NH_3)$ at this pH.

The pH dependence of ammonia formation was measured in

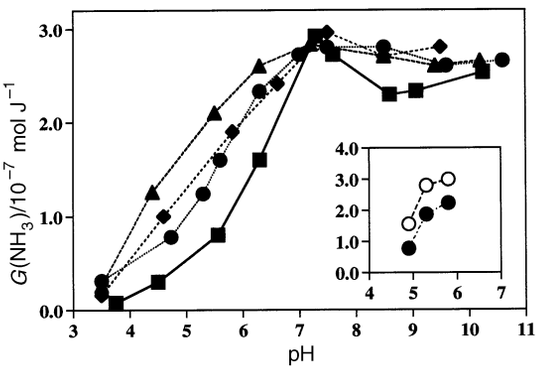


Fig. 2 pH dependence of $G(NH_3)$ in the γ-irradiation of an N_2O-saturated aqueous solution containing (■) 5×10^{-3} mol dm^{-3}, (●) 2×10^{-3} mol dm^{-3}, (◆) 1×10^{-3} mol dm^{-3}, (▲) 2×10^{-4} mol dm^{-3} GSH. Inset: pH dependence of $G(NH_3)$ in identical samples of 2×10^{-3} mol dm^{-3} GSH upon 640 Gy irradiation with dose rate of (●) 0.153 Gy s^{-1}, and (○) 0.011 Gy s^{-1}.

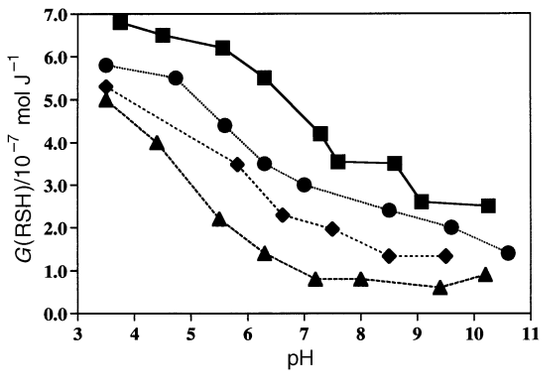


Fig. 3 pH dependence of consumption of the free thiol group G(-RSH) in the γ-irradiation of N_2O-saturated aqueous solution of (■) 5×10^{-3} mol dm^{-3}, (●) 2×10^{-3} mol dm^{-3}, (◆) 1×10^{-3} mol dm^{-3}, (▲) 2×10^{-4} mol dm^{-3} GSH as measured by Ellman's reaction

the pH range 3.5–10.5 at different glutathione concentrations. The doses were chosen such that the GSH consumption never exceeded 30%. The curves in Fig. 2 have approximately sigmoidal shapes. As can be seen, there is a persistent formation of ammonia at alkaline and neutral pH with G values around 2.5–2.9. On the assumption that ammonia was formed exclusively by way of oxidation of α-amino carbon-centred radicals **2** and/or **4** in a bimolecular radical process, these G values imply that at least 80–95% of the initial OH radicals are converted into α-amino carbon-centred radicals **2** and/or **4** *via* the thiyl radical transformation. The ammonia production decreases dramatically at acidic pH. The decrease in ammonia formation can be interpreted as being governed either by thermodynamic or by kinetic factors. If the pK_a of radical **4** were much lower than the pH at which the ammonia yield is halved (*i.e.*, pK_a < 5.5), the shape of $G(NH_3)$ *versus* pH would reflect the pH-dependent equilibrium of radical **3** and radical **2**. However, as the pK_a of the glycine radical, which should be similar to that of **4**, is reported to be 6.7,[10] and the decrease of ammonia yield occurs below this pH value, we have to infer kinetic reasons. As is shown in Scheme 1, below the pK_a of the carbon-centred radical **4**, we reach the thermodynamic pH-independent equilibrium (4). Kinetically, equilibrium (4) is established more slowly as the pH decreases. At a certain pH, equilibration will come into competition with thiyl radical bimolecular recombination reactions. To ascertain the causes of ammonia depletion, the following experiments were performed. The ammonia formation was measured on identical samples, irradiated to the same doses at different dose rates. As shown in the inset of Fig. 2, when a 14 times lower dose rate was applied in the γ-irradiation, a higher $G(NH_3)$ was observed at pH around 5–6. This clearly demonstrates that the decrease of ammonia formation is the outcome of the competition between thiyl radical bimolecular reactions and its intramolecular transformation reaction. Possible radical combination reactions are as follows.

$$GS^{\bullet} + GS^{\bullet} \longrightarrow GSSG \quad (7)$$

$$\mathbf{2}\ (\text{or}\ \mathbf{4}) + \mathbf{2}\ (\text{or}\ \mathbf{4}) \longrightarrow GSH + NH_3 + \alpha\text{-keto acid} \quad (8)$$

$$GS^{\bullet} + \mathbf{2}\ (\text{or}\ \mathbf{4}) \longrightarrow GSH + NH_3 + \alpha\text{-keto acid} \quad (9)$$

$$GSSG^{\bullet -} + GS^{\bullet} \longrightarrow GSSG + GS^{-} \quad (10)$$

$$GSSG^{\bullet -} + \mathbf{2}\ (\text{or}\ \mathbf{4}) \longrightarrow GSSG + GSH \quad (11)$$

As shown in Fig. 2, the half yields on the ammonia formation curves were shifted to higher pH when the glutathione concentration increased. In trying to explain this phenomenon, computer simulations were performed. Since the decrease of ammonia formation is related to the thiyl radical recombination reactions, we have to attribute this to reactions involving both GS$^{\bullet}$ and GSH. Equilibrium (2) is obviously among these reactions. However, the relation between glutathione concentrations and the ammonia formation curves is difficult to explain wholely in terms of equilibrium (2), as shown by simulations. Since in the pH region 4–6 equilibrium (2) is shifted far to the left it should thus not have a strong effect on ammonia formation. The system composed of equilibria (1)–(6), and reactions (7)–(11) was simulated, taking radical recombination rate constants $2k$ to be around 10^8–10^9 dm^3 mol^{-1} s^{-1}, and assuming protonation equilibria to be rapidly established. The simulation indicated no GSH concentration dependence of ammonia formation in the pH region 4–6, unless equilibrium (12) was included, assuming equilibrium constant $K_{12} \approx 1$ dm^3 mol^{-1}.

$$GS^{\bullet} + GSH \rightleftharpoons GSSGH^{\bullet} \quad (12)$$

At neutral and acidic pH, equilibrium (12) has been invoked in several papers.[14,15] The rate constant k_{12} was measured to be 2×10^8 dm^3 mol^{-1} s^{-1} by Abedinzadeh *et al.*,[15] which means that equilibrium (12) is rapidly established. From our assumed equilibrium constant $K_{12} \approx 1$ dm^3 mol^{-1}, we arrive at p$K_a \approx 5.7$ for the equilibrium between GSSGH$^{\bullet}$ and GSSG$^{\bullet -}$. This is close to the value observed for 1,4-dithiothreitol, from which an intramolecular disulfide radical RSSRH$^{\bullet}$ has a p$K_a \approx 5.3$.[16]

Based on the ammonia formation curve shown in Fig. 2, we can discuss equilibria (3) and (4). We note that below pH 6.7, the approximate pK_a of α-amino carbon-centred radicals, ammonia formation is still at its plateau value (see inset in Fig. 2). This implies that at least 90% of the glutathione thiyl radicals have been transformed to α-amino carbon-centred radicals *via* equilibrium (4). We assume that $K_4 \geq 10$. Equilibrium constants K_3 and K_4 are related to pK_{a5} and pK_{a6}:

$$pK_3 = pK_4 - pK_{a5} + pK_{a6}$$

As a result, $K_3 \geq 10^4$ is obtained. This value is at least 10 times larger than previously estimated ($K_3 \geq 10^3$).[8,9] The thermochemical implications of these equilibrium constants for the α-amino C–H bond will be discussed later.

Other products

The consumption of the free thiol group G(-RSH) subsequent to irradiation at different glutathione concentrations in N_2O saturated solution at various pH was also measured with Ellman's reagent. The curves are shown in Fig. 3. It is seen that G(-RSH) has a curvature opposite to that of the ammonia formation curves. It is about full yield [$G(OH^{\bullet}) + G(H^{\bullet}) +$

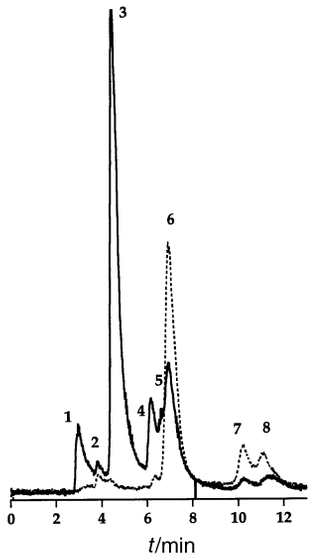


Fig. 4 HPLC chromatograms recorded after γ-irradiation of 5×10^{-3} mol dm^{-3} GSH N_2O saturated aqueous solution at pH 9, before (solid line) and after (dotted line) reacting with DTNB

$2fG(H_2O_2)$, where *f* is the extent of completion of reaction (13), see below] at pH 3.5, and decreases markedly at neutral and alkaline pH. Similarly to the ammonia yield, *G*(-RSH) decreases at lower pH with decreasing glutathione concentration. Low yields for the free thiol consumption at neutral and alkaline pH are expected, since according to the bimolecular decay mode, both the dimers and the α-keto acids formed in the disproportionation reactions have a free thiol group, which will react with Ellman's reagent. In Fig. 3, the level of the *G*(-RSH) curve increases with increasing glutathione concentration. Part of this increase is attributed to reaction (13), GSSG formation by hydrogen peroxide oxidation of glutathione.

$$H_2O_2 + 2GSH \longrightarrow GSSG + 2H_2O \qquad (13)$$

In the γ-radiolysis of water, H_2O_2 is formed with a *G* value of 0.7. At high GSH concentration, *e.g.* 5×10^{-3} mol dm^{-3}, reaction (13) can go to completion in *ca.* 2–3 h.[17] The irradiation time chosen for the formation of 1×10^{-3} mol dm^{-3} primary radicals was 3 h, and it is obvious that reaction (13) goes to completion during the irradiation and before the product analysis. At low concentrations of GSH and short irradiation times, reaction (13) is retarded ($f \ll 1$).

Irradiated samples of N_2O saturated 2×10^{-3} and 5×10^{-3} mol dm^{-3} GSH solutions at different pH were also analysed by HPLC to check the distribution of the products. Whether or not the substances characterized by the separated peaks possess a free thiol group was examined in the following experiments. The irradiated samples were subjected to HPLC analysis before and after treatment with excess DTNB (Ellman's reagent) for *ca.* 1 h. Typical chromatograms of irradiated 5×10^{-3} mol dm^{-3} GSH in N_2O-saturated solution at pH 9 are compared in Fig. 4. Since both DTNB and the product of its reaction with thiol, thionitrobenzoic acid, have much lower polarity, their peaks are far away from those of the glutathione species and are not included in the Figure. It can be seen that eight peaks were observed with retention times of 3.2, 3.8, 4.3, 6.1, 6.7, 7, 10.3 and 11.3 min, and marked as peaks 1–8, respectively. After treatment with DTNB, peaks 1, 3, 4 and 5 disappeared from their positions, while peaks 2, 6, 7 and 8 remained. Peaks 3 and 6 are identified as GSH and GSSG by comparison with standards. Since Ellman's reaction of GSH produces GSSG as a stable product, peak 6 (GSSG) increased in size. When other free thiol species (RSH) are involved, mixed disulfides RSSR′ are formed. Peaks 7 and 8 also increased in size, indicating that these species have no free -SH functions but are instead mixed disulfide species. The latter might be formed if the species characterized by peaks 1, 4 and 5 were to combine with GSH to form RSSG. They could also be multi-sulfur compounds such as GSSSG and GSSSSG. The size of peak 4 correlates to the ammonia yield over the entire pH region, and it is reasonable to assign this peak to an α-keto acid in the glutamyl residue of glutathione, according to the postulated reaction mechanism. The disappearance of peak 4 after treatment with DTNB supports this identification, since glutathione α-keto acid has a free SH group. Authentic glutathione α-keto acid is not available. Further work is needed to finally characterize this product. Peak 5 was only seen at pH > 8.5, and is probably a dimer of the α-amino carbon-centred radicals **2** and/or **4**. The dimer might be formed as a minor product at alkaline pH, where $G(NH_3)$ is slightly decreased (see Fig. 2). The size of peaks 1, 2, 7 and 8 decreases at neutral and acidic pH. They also decrease with decreasing GSH concentration. To explain the formation of these peaks, reactions (14)–(18) are postulated.

$$GSSG^{\bullet -} + H^+ \longrightarrow GSS^{\bullet} + GH \qquad (14)$$

$$GSSGH^{\bullet} \longrightarrow GSS^{\bullet} + GH \qquad (15)$$

$$GSS^{\bullet} + GSS^{\bullet} \longrightarrow GSSSSG \qquad (16)$$

$$GSS^{\bullet} + GS^{\bullet} \longrightarrow GSSSG \qquad (17)$$

$$GSS^{\bullet -} + \mathbf{2}\ (\text{or } \mathbf{4}) \longrightarrow GSSH + NH_3 + \alpha\text{-keto acid} \qquad (18)$$

Peaks 1 and 2 are assigned to be GSSH and GH, respectively. It is important to keep in mind that reactions (14) and (15) are rather slow, since the product yields from them are minor. By including these reactions in the scheme, the curvatures of $G(NH_3)$ and *G*(-RSH) *versus* pH in Figs. 2 and 3 are also better accounted for.

Formation of H_2S with *G* values around 0.5 was observed after irradiation of N_2O saturated aqueous solutions of glutathione at pH > 7. This minor product is attributed to the hydrogen atom displacement of GSH (see Experimental section).

Mass balance

The loss of glutathione *G*(-GSH) after irradiation of glutathione solutions can be quantified from the HPLC chromatograms. All *G* values obtained from γ-irradiation of N_2O-saturated 5×10^{-3} mol dm^{-3} GSH solutions are listed in Table 1.

It can be seen that at acidic pH the *G*(-GSH) has approximately full yield if reaction (13) is taken into consideration. At neutral and alkaline pH, where ammonia is formed, *G*(-GSH) decreases. The differences in *G*(-RSH) between acidic and neutral/alkaline pH are close to the values of $G(NH_3)$. This is also expected, since a part of GSH is regenerated through the disproportionation process. The total mass balance for radical comsumption is given by $[G(\text{-GSH}) + G(NH_3)]$, or $[2G(NH_3) + G(\text{-RSH})]$, as listed in Table 1. $G_{total} = G(OH^{\bullet}) + G(H^{\bullet}) + 2G(H_2O_2) = 7.6$ should be equal to $[G(\text{-GSH}) + G(NH_3)]$ or $[2G(NH_3) + G(\text{-RSH})]$. At acidic pH, within experimental errors, the G_{total} is well balanced. It is noticed, though, that high G_{total} values up to 9 or 10 were obtained at some neutral and alkaline pH. Possible explanations can be reactions (19) and (20).

$$GSSG^{\bullet -} + H^+ \longrightarrow GSSH + G^{\bullet} \qquad (19)$$

$$G^{\bullet} + GSH \longrightarrow GS^{\bullet} + GH \qquad (20)$$

Short chains like this would increase the total glutathione consumption yield to a certain extent.

Table 1 G values (in 10^{-7} J mol^{-1}) for γ-irradiation of N_2O-saturated 5×10^{-3} mol dm^{-3} GSH solution

pH	$G(NH_3)$	G(-RSH)	G(-GSH)	$2G(NH_3) + G$(-RSH)	$G(NH_3) + G$(-GSH)
3.8	0.08	6.8	6.5	7	6.6
4.5	0.3	6.5	7	7.1	7.1
5.6	0.8	6.2	7.7	7.8	8.5
6.3	1.6	5.5	7.2	8.7	8.8
7.3	2.9	4.2	5.7	10	8.6
7.6	2.7	3.5	5.5	9	8.2
8.6	2.3	3.5	5.8	8	8.1
9.1	2.3	2.6	5.9	7.3	8.2
10.3	2.5	2.5	6.8	7.6	9.3

Table 2 C–H bond energies of relevant compounds

Species	C–H bond energy/kJ mol^{-1}
CH_4	439[a]
NH_2CH_3	389[a]
$CH_3CO_2^-$	402[b]
CH_3CO_2H	406[b]
$NH_2CH_2CO_2^-$	≤343[c]
$NH_2CH_2CO_2H$	≤329[c]

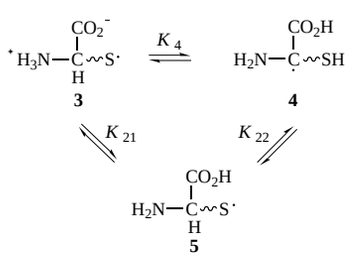
[a] From ref. 19. [b] From ref. 20 and references therein. [c] From this work.

Thermochemical implications of the α-amino C–H bond

The minimum value of equilibrium constant K_3 is 10^4, as concluded above. The free energy change for the transformation reaction (3), derived from $\Delta G^\circ_3 = -RT \ln K_3$, is thus more negative than −23 kJ mol^{-1}. The strength of a typical alkyl S–H bond in the gas phase is 366 kJ mol^{-1}.[18] Assuming similar entropies and hydration free energies for the radicals **1** and **2**, the strength of the C–H bond at the α-position to both amino (-NH_2) and carboxyl (-CO_2^-) groups appears to be no higher than *ca*. 343 kJ mol^{-1}. The same applies to the equilibrium (4). If K_4 is at least 10, the free energy of reaction (4) is more negative than −5.7 kJ mol^{-1}. Now, the free energies of formation, $\Delta_f G^\circ$(aq), for the glycine species, $NH_2CH_2CO_2H$ and $^+NH_3CH_2CO_2^-$, are given as −340 and −371 kJ mol^{-1} respectively.[11]

$$^+H_3NCH_2CO_2^- \rightleftharpoons H_2NCH_2CO_2H \qquad (21)$$

Thus, ΔG°_{21} is *ca*. 31 kJ mol^{-1}. We assume a similar free energy difference between glutathione thiyl radicals **3** and **5**, as shown in Scheme 3.



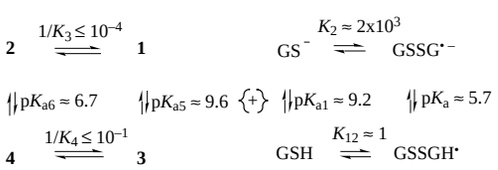
Scheme 3

From $\Delta G^\circ_4 = \Delta G^\circ_{21} + \Delta G^\circ_{22}$, we obtain the free energy of reaction (22) to be *ca*. −37 kJ mol^{-1}. Again, assuming similar entropies and hydration free energies for radicals **5** and **4**, the C–H bond energy at the α-position to both amino (-NH_2) and carboxylic (-CO_2H) groups is estimated to be no higher than *ca*. 329 kJ mol^{-1}.

The low C–H bond energy is due to the joint stabilization of the carbon-centred radicals by the -NH_2 and -CO_2^- or -CO_2H groups at the α-position. Table 2 lists a set of bond energies which were measured experimentally.[19,20] The stabilization energies of -NH_2, -CO_2H and -CO_2^- taken separately, on the α-carbon-centred radicals, are *ca*. 54 and 38 kJ mol^{-1}, respectively. With both -NH_2 and -CO_2H, or -NH_2 and -CO_2^- present, the stabilization energy on the α-carbon-centred radicals become *ca*. 109 and 96 kJ mol^{-1}, respectively. Thus the captodative effect on the stabilization of α-carbon radical centre is more than 25 kJ mol^{-1} by the NH_2 and CO_2H groups jointly, and more than 8.4 kJ mol^{-1} by NH_2 and CO_2^- groups jointly. This agrees well with theoretical calculations of Armstrong *et al*.[11,21]

Reaction mechanism and biological implications

The formation curves of ammonia as shown in Fig. 2 clearly demonstrate the significance of the equilibria (3) and (4) (between glutathione thiyl radicals and the α-amino carbon-centred radicals) in the free radical chemistry of glutathione. The reaction mechanism over the entire pH range is made up of several equilibria, as can be summarized in Scheme 4.



Scheme 4

The radicals produced in the system either disproportionate or recombine to form the respective stable products.

The fate of thiyl radicals in cells has attracted great interest recently, because of the discovery of a general equilibrium between the thiyl and the carbon-centred radical.[22] Our work presented so far clearly shows that in anaerobic conditions, thiyl radicals disappear mainly through intramolecular transformation to form α-amino carbon-centred radicals, which subsequently form ammonia and glutathione α-keto acids.

When oxygen is involved, as it is in most cells, reactions (23)–(25) have to be considered.

$$GS^{\bullet -} + O_2 \rightleftharpoons GSOO^\bullet \qquad (23)$$

$$GSSG^{\bullet -} \text{ (or } GSSGH^\bullet) + O_2 \longrightarrow GSSG + O_2^{\bullet -} \text{ (or } HO_2^\bullet) \qquad (24)$$

$$\mathbf{2} \text{ (or } \mathbf{4}) + O_2 \longrightarrow NH_3 + \alpha\text{-keto acid} + O_2^{\bullet -} \qquad (25)$$

Equilibrium (23) is rapidly established with $k_{23} = 2 \times 10^9$ dm^3 mol^{-1} s^{-1} and $k_{-23} = 6 \times 10^5$ s^{-1}. Reactions (24) and (25) are one-way reactions with rate constant of *ca*. 1×10^9 dm^3 mol^{-1}s^{-1}. (A value of 4.8×10^8 dm^3 mol^{-1} s^{-1} was measured by Al-Thannon *et al*. for the cysteine radical anion with O_2.[23,24]) Thus the kinetics controlling the fate of $GS^\bullet$ radicals in cells ultimately bear on the competing reactions (3) and (24), where the effective rates can be written as:

$$k'(3) = k_3 [1 + 10^{(pK_{NH3} - pH)}]^{-1}$$

$$k'(24) = k_{24} K_2 [O_2] [GSH] [1 + 10^{(pK_{SH} - pH)}]^{-1}$$

When $k'(3) = k'(24)$, taking $pK_{SH} \approx pK_{NH_3} \approx 9.5$, $k_3 \approx 2 \times 10^5$ s^{-1}, $K_2 \approx 2500$ dm^3 mol^{-1}, and $k_{24} \approx 10^9$ dm^3 mol^{-1} s^{-1}, we arrive at $[O_2][GSH] \approx 10^{-7}$ mol^2 dm^{-6}. As can be seen, the important parameters controlling the fate of thiyl radicals in cells are oxygen and glutathione concentration. When $[O_2][GSH] < 10^{-7}$ mol^2 dm^{-6}, equilibrium (3) becomes the main pathway of glutathione thiyl radical consumption in the system. The distribution of glutathione in cells is not homogeneous, but concentrations of 1–2 mmol dm^{-3} are common. The oxygen concentration within living cells is also variable. It can decrease from cell membrane to the oxygen-consuming mitochondria.[25] Thus, when both low glutathione and oxygen concentrations are involved, equilibrium (3) is easily recognized as one of the principal pathways of removing glutathione thiyl radicals in biological systems.

Glutathione contains a glutamic acid residue joined in an unusual peptide linkage involving a γ-carboxyl rather than an α-carboxyl group. This unique structure makes the weak α-amino hydrogen available to the glutathione thiyl radicals derived from various repair processes.

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