'OH Radical Induced Decarboxylation of γ-Glutamylmethionine and *S*-Alkylglutathione Derivatives: Evidence for Two Different Pathways Involving *C*- and *N*-Terminal Decarboxylation

Krzysztof Bobrowski,[a,*] Christian Schöneich,[b,*] Jerzy Holcman[c] and Klaus-Dieter Asmus[b,*]

[a] *Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Rakowiecka 36, 02-532 Warsaw, Poland*

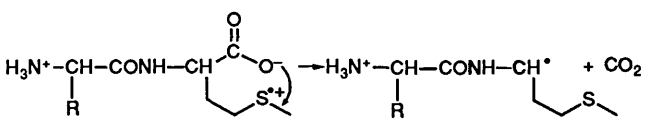
[b] *Hahn-Meitner-Institut Berlin, Bereich S, Abteilung Strahlenchemie, Glienicker Str. 100, 1000 Berlin 39, Germany*

[c] *Chemistry Department, Risø National Laboratory, 4000 Roskilde, Denmark*

The hydroxyl radical induced oxidation of γ-glutamylmethionine and *S*-alkylglutathione derivatives (alkyl = CH_3, C_2H_5, C_4H_9, C_6H_{13}, C_9H_{19}) in aqueous solution results in significantly different decarboxylation yields upon variation of the peptide concentration, pH and chain length of alkyl substituents adjacent to the sulphur. Mechanistically, the decarboxylation is considered to proceed *via* two different routes: (*i*) electron transfer between oxidized sulphur, $>S^{\bullet+}$, and the *C*-terminal carboxyl group (pseudo-Kolbe mechanism) whenever both reactants are located within the same peptide unit, and (*ii*) interaction between an 'OH adduct, $>{}^{\bullet}S{-}OH$, and a protonated amino group which is positioned α to a carboxyl group (*N*-terminal decarboxylation). The latter mechanism also occurs if both reaction centres are not located within the same peptide unit.

The hydroxyl radical constitutes the strongest oxidant among the reactive oxygen species responsible for various deleterious effects in a biological environment.[1] On a molecular level such effects include, *e.g.*, lipid peroxidation, membrane alteration, DNA strand breakages as well as protein degradation and inactivation.[1] Mechanistic studies of the 'OH radical induced decarboxylation of sulphur-containing amino acids[2,3] and peptides[4] have revealed rather complex reaction mechanisms which depend on a variety of parameters. The 'OH radical attacks primarily the sulphur leading to a reactive $>{}^{\bullet}S{-}OH$ adduct which subsequently converts to a monomeric radical cation $>S^{\bullet+}$ or its dimeric complex $(>S\therefore S<)^{+}$. In previous studies of methionine-containing peptides we have shown that effective decarboxylation occurs if both the sulphide function and the *C*-terminal carboxyl group are located in the same peptide unit (*e.g.* in glycylmethionine, Gly-Met). Separation of these two key functionalities into different amino acids prevents decarboxylation (*e.g.* in Gly-Met-Gly).[4] A second prerequisite for decarboxylation is the presence of an 'activating group' (–OH, –NHR)[4,5] in the α-position to the carboxyl function resulting in stabilization of the arising *C*-centred radical [reaction (1)].

These findings have been rationalized in terms of an intramolecular, probably 'outer sphere', electron transfer from the deprotonated carboxyl group to the monomeric sulphur radical cation [reaction (**1**)][4] (analogous to the 'pseudo-Kolbe mechanism'[6,7]).

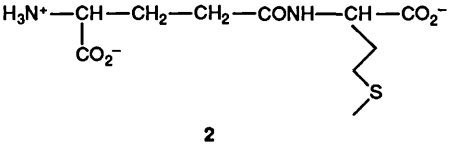


1 (1)

This decarboxylation process (1) has to compete with irreversible deprotonation of $>S^{\bullet+}$ α to the sulphur. The relative probabilities of all possible reaction routes depend significantly on the electronic inductive properties of side chain substituents R (in compound **1**) as well as on the number, location and interaction of charges in the molecule which affect the deprotonation kinetics.[4]

Particularly high CO_2 yields (oxidized sulphur: CO_2 = 1:1) were found upon oxidation of γ-glutamylmethionine (γ-Glu-Met **2**),[4] a peptide which contains not only a *C*-terminal but also an *N*-terminal carboxyl group. Here the question arises whether decarboxylation might also occur from the *N*-terminal carboxyl group which derives 'activation' from the α-amino group but is not located in the methionine unit, *i.e.* does not fulfil a seemingly important prerequisite for decarboxylation of simple X-Met peptides (X = Gly, Ala, Val, Leu).

Evidence for direct participation of an amino function located α to a carboxyl group has been obtained in the 'OH induced decarboxylation of methionine.[2,8] It seems reasonable to expect a similar amino group participation in the decarboxylation mechanism of γ-Glu-Met in addition to the pseudo-Kolbe induced decarboxylation from the *C*-terminal carboxyl group observable in the simple X-Met peptides. In order to obtain more information on intermediates and mechanistic details of the decarboxylation processes from both sites we have now conducted a systematic investigation of 'OH radical induced decarboxylation of γ-Glu-Met (**2**) and *S*-alkylglutathione derivatives (**3**).



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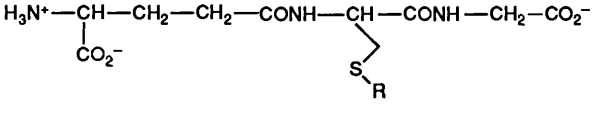

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The latter class of compounds excludes decarboxylation from the *C*-terminal carboxyl group *via* the intramolecular electron transfer mechanism according to reaction (1) (sulphur and carboxyl function are not located in the same amino acid moiety) while decarboxylation from the *N*-terminal glutamic acid moiety should be unaffected. These studies thus provide a means of separating the two processes.

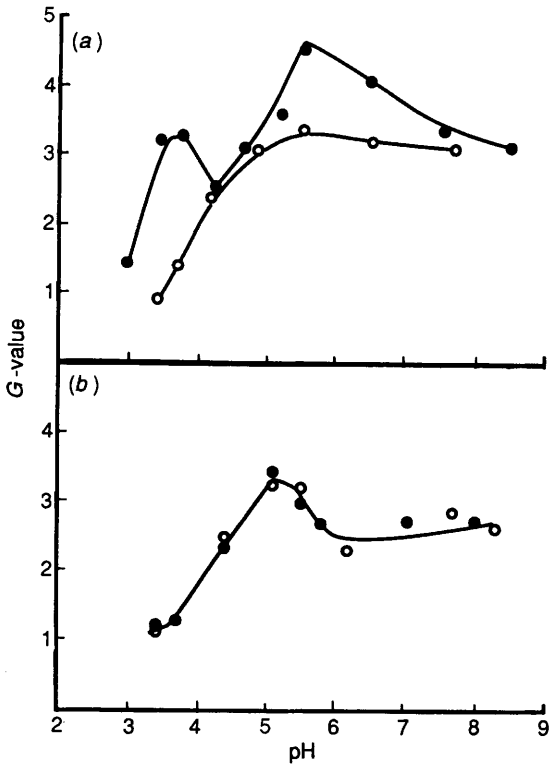


Fig. 1 (*a*) pH-Dependence of CO_2 (●) and of the $PNAP^{\bullet-}$ yield (○) in γ-Glu-Met, for N_2O-saturated solutions of 10^{-3} mol dm^{-3} peptide. (*b*) pH-Dependence of the CO_2 (●) and of the $PNAP^{\bullet-}$ yield (○) in N_2O-saturated solutions of 10^{-3} mol dm^{-3} *S*-methylglutathione.

Experimental

The *S*-alkyl derivatives of glutathione, *S*-methylglutathione (G–S–CH_3), *S*-ethylglutathione (G–S–C_2H_5), *S*-butylglutathione (G–S–C_4H_9), S-hexylglutathione (G-S-C_6H_{13}) and S-nonylglutathione (G–S–C_9H_{19}) were obtained from Sigma. γ-Glutamylmethionine (γ-Glu-Met) was obtained from Bachem, *p*-nitroacetophenone (PNAP) and methylviologen hydrate ($MV^{2+}\cdot H_2O$) were purchased from Aldrich. All compounds were of the purest commercially available grade and were used as received. Reagent grade NaOH and $HClO_4$ were added to the solutions for the adjustment of pH. All solutions were made with deionized water ('Millipore-Q'-quality, 18 MΩ).

Solutions were generally prepared at peptide concentrations of 5×10^{-4}–4×10^{-3} mol dm^{-3}. Deoxygenation and N_2O-saturation was achieved by bubbling with N_2 for at least 30 min per 20 cm^{-3} sample and subsequent bubbling with N_2O (30 min per 20 cm^{-3}) which was passed over a Cu catalyst to remove traces of oxygen. In such solutions all radiolytically formed hydrated electrons are converted into hydroxyl radicals ($e_{aq}^- + N_2O \longrightarrow N_2 + OH^- + {}^\bullet OH$). These together with the directly generated $^\bullet OH$ radicals account for 90% of all reactive primary species (the remaining 10% are $H^\bullet$ atoms).

The pulse radiolysis experiments were performed with the 1.55 MeV Van de Graaff accelerator of the Hahn-Meitner-Institut, Berlin. Typically 1–2 Gy pulses (1 Gy = 1 J kg^{-1}) of 1 μs duration were used. Based on the radiation chemical yield $G(^\bullet OH) = 5.7$ (radicals per 100 eV absorbed energy) in N_2O-saturated solution this corresponds to a total radical concentration of (0.6–1.2) × 10^{-6} mol dm^{-3} per pulse. Details of technical set up and dosimetry are described elsewhere.[9]

Decarboxylation was initiated by irradiation in the field of a 6000 Ci ^{60}Co γ-source using a dose rate of 1000 Gy h^{-1} calibrated by Fricke dosimetry. Total absorbed doses were in the order of 50–350 Gy depending on the peptide concentration. Generally, less than 10% of the peptide was radiolytically converted to avoid reactions of the primary radicals with reaction products. Carbon dioxide analysis was performed using a Dionex 2010i ion chromatograph equipped with a HPICE-AS 1 column. Details of the method are described elsewhere.[10,11]

The p*K* values of the peptides were measured by pH titration using 10^{-3} mol dm^{-3} peptide solutions and 1 mol dm^{-3} NaOH or 1 mol dm^{-3} HCl. The actual pH values were measured with a Knick digital pH meter.

All experiments were carried out at room temperature.

Results

CO_2-Formation by γ-Radiolysis.—γ-Glu-Met. γ-Irradiation of N_2O-saturated, pH 5.5, solutions of 10^{-3} mol dm^{-3} γ-Glu-Met (**2**) results in the formation of CO_2, indicated by a high radiation chemical yield of $G = 4.55$. This yield corresponds to almost 100% of the $^\bullet OH$ radicals which react with the sulphur moiety (*i.e. ca.* 80% of the total yield of $^\bullet OH$, while the remaining 20% react *via* other pathways *e.g.* hydrogen abstraction from C–H bonds located α to the sulphur yielding –C$^\bullet$H–S–).[2] The pH dependence of $G(CO_2)$ shows a pronounced structure with two distinct maxima at pH *ca.* 3.7 and 5.5 (Fig. 1*a*). Starting from low pH $G(CO_2)$ is first seen to increase and to reach a first maximum with $G(CO_2) = 3.3$. Subsequently it decreases to a minimum at pH 4.25 ($G = 2.25$) and rises again to the second maximum ($G = 4.55$). Then $G(CO_2)$ steadily decreases again between pH 5.5 and 8.5. The half-value of the initial rising portion of the curve is located at pH *ca.* 3.2 which is between the p*K* values of the two carboxyl groups, measured to be $pK_I = 2.95$ and $pK_{II} = 3.75$.

Increase of the γ-Glu-Met concentration from 5×10^{-4} to 4×10^{-3} mol dm^{-3} results in a *ca.* 30% decrease of $G(CO_2)$ (Table 1) in contrast to Gly-Met and Gly-Gly-Met, where almost no concentration dependence was observed.[4]

S-alkylglutathione derivatives. γ-Irradiation of N_2O-saturated, pH 5.1, solutions of 10^{-3} mol dm^{-3} *S*-alkylglutathione derivatives (**3**) with R = CH_3, C_2H_5, C_4H_9, C_6H_{13} and C_9H_{19}, respectively, results in CO_2 yields which decrease with the length of the alkyl chain.

For better assessment of the decarboxylation yields, the $G(CO_2)$ values were related to the actual yields of primarily oxidized sulphur *G*(S-oxid.). The latter were calculated for each compound taking $G = 4.8$ as a reference value for the formation of oxidized sulphur through $^\bullet OH$ radical induced oxidation of methionine, and by applying standard competition kinetics, using the rate constants[12] $k_{^\bullet OH + Met(S)} = 1.8 \times 10^{10}$ dm^3 mol^{-1} s^{-1}, $k_{^\bullet OH + \gamma\text{-Glu}} = 2.3 \times 10^8$ dm^3 mol^{-1} s^{-1} and $k_{^\bullet OH + Gly} = 1.7 \times 10^7$ dm^3 mol^{-1} s^{-1}.* All results are expressed in terms of decarboxylation efficiency $f = G(CO_2)/G$(S-oxid.) as shown in Table 2.

Significant CO_2 yields, although generally lower than from γ-Glu-Met, were measured at varying pH in solutions of 10^{-3} mol dm^{-3} *S*-methylglutathione (G–S–Me). The respective pH-dependence shows only one maximum, at pH 5.1, with $G(CO_2) = 3.5$ (Fig. 1*b*).

Increase of G–S–Me concentration (from 5×10^{-4} mol dm^{-3}

* Since the rate constant of hydrogen abstraction by $^\bullet OH$ radicals from ethane (C_2H_6), $k = 1.8 \times 10^9$ mol^{-1} dm^3 s^{-1},[12] is lower than for hydrogen abstraction from a C–H bond located α to sulphur (–CH_2–S–CH_3), $k = 4.5 \times 10^9$ mol^{-1} dm^3 s^{-1},[2] the latter value was taken as the rate constant of hydrogen abstraction by $^\bullet OH$ radicals in *S*-methylglutathione. The rate constants for the other *S*-alkylglutathione derivatives are estimated by adding to $k_{^\bullet OH + C\text{-}H}$ for $GSCH_3$ the respective difference between rate constants measured for ethane and the alkane.

Table 1 Yields of $^{\bullet}OH$ radical induced CO_2 formation (expressed in G) in N_2O-saturated solutions of various concentrations of X-Met peptides (X = γ-Glu, Gly, Gly-Gly) and *S*-methylglutathione

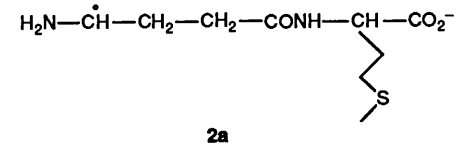
[Peptide]/mol dm^{-3}	γ-Glu-Met[a,b]	G-S-CH$_3$[a,c]	Gly-Met[d,e]	Gly-Gly-Met[d,e]
5×10^{-4}	4.2	2.5	1.3	1.4
1×10^{-3}	4.1	2.3[f]	1.3	1.5
2×10^{-3}	—	1.8	1.2	1.3
4×10^{-3}	3.2	1.7	1.2	1.4

[a] This work. [b] Measured at pH 5.4. [c] Measured at pH 6.1. [d] From ref. 4. [e] Measured at pH 5.6. [f] Taken from $G(PNAP^{\bullet -})$.

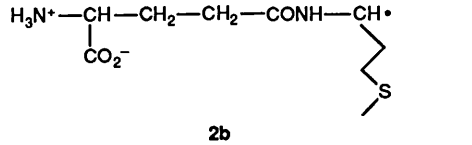
Table 2 Yields of $^{\bullet}OH$ radical induced formation (expressed in G) and efficiency of decarboxylation $f(CO_2) = G(CO_2)_{exp}/G(S\text{-oxid.})$ in N_2O-saturated solutions of 10^{-3} mol dm^{-3} *S*-alkylglutathione derivatives (G-S-R) at pH 5.1.

G-S-R	$G(CO_2)_{exp}$	$f(CO_2)$
R = CH_3	3.44	0.75
C_2H_5	2.32	0.55
C_4H_9	1.90	0.48
C_6H_{13}	1.54	0.42
C_9H_{19}[a]	1.00	*b*

[a] Measured at pH 7.7. [b] Rate constant for $^{\bullet}OH + C_9H_{20}$ not known.



2a



2b

to 4×10^{-3} mol dm^{-3}) results in a *ca.* 35% decrease of $G(CO_2)$ similar to the γ-Glu-Met system (Table 1).

Pulse Radiolysis.—*γ-Glu-Met.* According to the $^{\bullet}OH$ induced oxidation mechanism of methionine[2,8,13] and X-Met peptides,[4,14] decarboxylation of γ-Glu-Met may result in the formation of two types of radicals as indicated above [Structures (**2a** and **2b**)], depending on which of the carboxyl groups is cleaved.

Radical **2a**, as for α-amino radicals in general, should be a strong reductant[13] whereas this is not expected to apply for the *N*-carboxy derivative **2b**, where the electron withdrawing nature of the substituent lowers the reducing character (the ionization potentials of α-aminoalkyl radicals are known to increase with decreasing electron release by substituents at the nitrogen[15]). Reduction of moderately good electron acceptors such as *p*-nitroacetophenone (PNAP) ($E_{red} = -0.358$ V)[16] and methylviologen (MV^{2+}) ($E_{red} = -0.447$ V)[17] may accordingly serve as a probe for the formation of the comparatively more reducing radical **2a** which results from decarboxylation at the *N*-terminal γ-glutamine carboxyl group.

The reduced forms of both electron acceptors, $PNAP^{\bullet -}$ ($\varepsilon_{360} = 17\,600$ dm^3 mol^{-1} cm^{-1}, $\varepsilon_{545} = 2900$ dm^3 mol^{-1} cm^{-1})[18] and $MV^{\bullet +}$ ($\varepsilon_{600} = 11\,850$ dm^3 mol^{-1} cm^{-1})[19] are easily observed by time resolved optical detection.

No reduction of PNAP by the radical generated from decarboxylation of Gly-Met in N_2O-saturated 10^{-3} mol dm^{-3} solutions, pH 5.6, is observed. This confirms that H_3N^+–CH_2–CO–NH–C$^{\bullet}$H–CH_2–CH_2–S–CH_3, formed upon decarboxylation of this peptide, and most likely any other amido radical, *e.g.* **2b** from γ-Glu-Met, are only weak reductants.

Significant yields of $PNAP^{\bullet -}$ are obtained, however, with γ-Glu-Met and are thus attributable to the **2a** type radicals. The $PNAP^{\bullet -}$ formation according to reaction (2) has been measured over the pH region 3–8 in N_2O-saturated solutions containing 10^{-3} mol dm^{-3} γ-Glu-Met and 1.4×10^{-4} mol dm^{-3} PNAP, as depicted together with the respective pH dependence of $G(CO_2)$, in Fig. 1*a*

$$\mathbf{2a} + PNAP \longrightarrow \text{imine} + PNAP^{\bullet -} \qquad (2)$$

The curves exhibit different characteristics over the entire pH region, with $G(PNAP^{\bullet -})$ being generally lower than $G(CO_2)$. The yield of $MV^{\bullet +}$ ($G = 3.7$) formed in reaction (3) at the pH of highest decarboxylation yield (*i.e.* pH 5.5) is in good agreement with that of $PNAP^{\bullet -}$ ($G = 3.4$), indicating that both reactions (2) and (3) are fairly good probes for the detection of α-amino radicals **2a**.

$$\mathbf{2a} + MV^{2+} \longrightarrow \text{imine} + MV^{\bullet +} \qquad (3)$$

These findings are supported by pulse radiolysis of solutions containing 10^{-3} mol dm^{-3} γ-Glu-Met. At pH 5.6, for example, an absorption is observed which steadily increases towards the UV (spectrum not shown here), similar to the spectrum obtained upon pulse radiolysis of methionine at pH 5,[13] *i.e.* under conditions of exclusive formation of α-amino radicals. It is therefore assigned to **2a**.

Thioether radical cations (**4a**) tend to associate with lone pair orbitals of unoxidized sulphur in the general equilibrium (4).[20,21]

$$\underset{\mathbf{4a}}{>S^{\bullet +}} + >S< \rightleftharpoons \underset{\mathbf{4b}}{(>S\therefore S<)^+} \qquad (4)$$

Such adducts (**4b**) are characterized by broad absorption spectra peaking in the 400–600 nm region.[20–23] In order to obtain information about whether such processes can interfere with the formation of α-amino radicals, as was observed in case of methionine,[2,8,13] the yield of $(>S\therefore S<)^+$ in N_2O-saturated 10^{-3} mol dm^{-3} solutions of γ-Glu-Met was measured over the pH range 1–8 (Fig. 2). Comparison with the CO_2 yield in Fig. 1 demonstrates that $(>S\therefore S<)^+$ formation and the overall decarboxylation are not complementary in a simple competitive two-way mechanism since the combined yields of CO_2 and $(>S\therefore S<)^+$ are not constant and, furthermore, significantly exceed the amount of $^{\bullet}OH$ radicals formed at some pHs, *e.g.* pH 3.4. The pH dependences of $(>S\therefore S<)^+$ and $PNAP^{\bullet -}$ formation, however, are complementary and exhibit breaking points of both curves around pH 3.8–3.9 (Fig. 2).

S-Alkylglutathione derivatives. The $PNAP^{\bullet -}$ yields obtained upon pulse radiolysis of N_2O-saturated 10^{-3} mol dm^{-3} solutions of *S*-methylglutathione containing 1.5×10^{-4} mol dm^{-3} PNAP parallel the respective yields of CO_2 as shown in Fig. 1*b*. The formation of $MV^{\bullet +}$ [according to reaction (3)] was measured only at the pH (5.1) of maximum CO_2 formation. Its yield ($G = 3.4$) is in good agreement with that of $PNAP^{\bullet -}$ ($G = 3.3$), both representing the amount of α-amino radical **3a** formed.

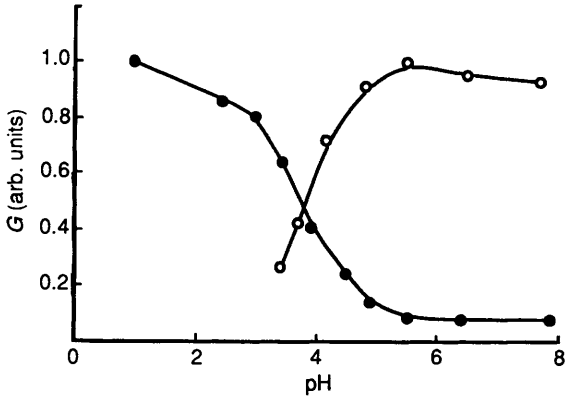
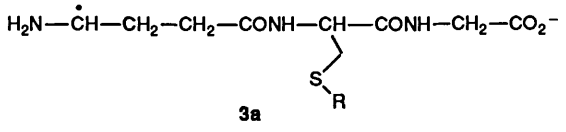


Fig. 2 Plots of the yields of $(>S\therefore S<)^+$ (●) and $PNAP^{\bullet -}$ (○), normalized to their respective maximum yields, as a function of pH in pulse irradiated N_2O-saturated solutions of 10^{-3} mol dm^{-3} γ-Glu-Met.



3a

Finally, pulse radiolysis of N_2O-saturated 10^{-3} mol dm^{-3} solution of *S*-ethylglutathione, pH 5.1, containing 1.5×10^{-4} mol dm^{-3} PNAP leads to the formation of $PNAP^{\bullet -}$ with G = 2.3 in excellent accordance with $G(CO_2)$ = 2.3 measured at the same pH.

Discussion

The results on the $^{\bullet}OH$ induced oxidation of γ-Glu-Met and *S*-alkylglutathione derivatives show that decarboxylation may occur not only from the *C*-terminal but also from the *N*-terminal carboxyl group, which derives activation from the α-amino group and is not located within the methionine unit. This finding differs from X-Met peptides (X = Gly, Ala, Val, Leu, *N*-acetyl) where decarboxylation was found to occur exclusively *via* a 'pseudo-Kolbe pathway, and only if carboxyl group and thioether were located in the same amino acid moiety.[4] It implies the existence of yet another mechanism of CO_2 formation. As will be discussed below this route appears to be influenced by (*i*) solute concentration, (*ii*) pH and (*iii*) electron inductive properties of substituents located at the sulphur.

The initial oxidation step in the overall mechanism is known to be an $^{\bullet}OH$ addition to the sulphur [eqn. (5)][22] yielding the adduct **5**. This species rapidly protonates to yield $(>S\therefore OH_2)^+$

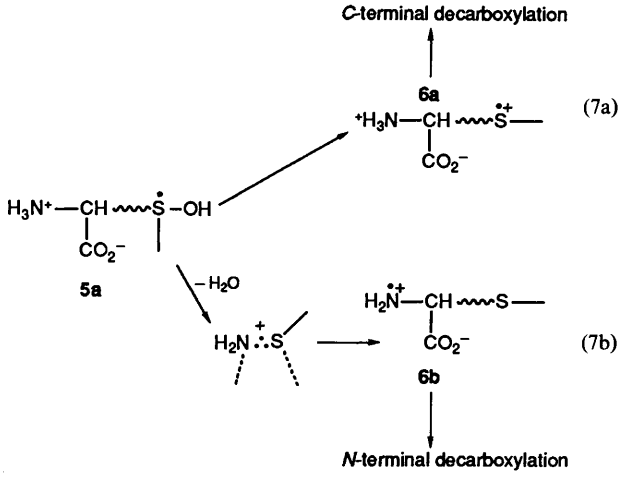
$$^{\bullet}OH + >S \longrightarrow >{}^{\bullet}S\text{–}OH \qquad (5)$$

which is essentially the molecular radical cation **4a** associated with a water molecule.[22,24] For convenience it is generally referred to as 'monomeric' $>S^{\bullet +}$ **4a** in the following:

$$\mathbf{5} + H^+ \longrightarrow (>S\therefore OH_2)^+ \qquad (6)$$

The proton may be taken from the bulk of the solution, but may also be delivered intramolecularly from the protonated amino group, as has been shown to occur in simple methionine,[8,25] Met-X-Met peptides (X = Gly, Ala, Gly-Met)[26] and alkylthio substituted amines [eqn. (7b)].[8] This latter process may, however, also occur intermolecularly, *i.e.* involve a second peptide molecule.

Direct pulse radiolysis evidence for the formation of an S∴N bonded intermediate (as in the case of methionine[8,25] and other peptides[15,26,27]) leading to **6b** has not been found. However, since it is not stabilized by a favourable 5-membered ring system as in methionine (in which it lives for only 200 ns), it may be too short-lived to be detectable in our present systems.



Another pathway which involves a second peptide molecule leads to the dimeric radical cation $(>S\therefore S<)^+$ **4b** [eqn. (8)],

$$\mathbf{5} + H^+ + >S \longrightarrow H_2O + \mathbf{4b} \qquad (8)$$

which exists in equilibrium with the 'monomeric' species $>S^{\bullet +}$ **4a** [eqn. (4)]. The chemical fate of **5** is thus linked to the equilibrium **4a** ⇌ **4b** and consequently to all reactions of **4a** and **4b**. In addition, the radical cation **4a** undergoes fast deprotonation [eqn. (9)] leading to the α-thioalkyl radical **7**.[13,21]

$$\mathbf{4a} \longrightarrow H^+ + >C^{\bullet}\text{–}S\text{–} \qquad (9)$$
$$\mathbf{7}$$

Comparison of the yields of CO_2 and α-amino radicals from γ-Glu-Met reveals that two reaction pathways lead to decarboxylation, yielding the respective radical species **2a** and **2b**. The α-amino radical **2b** is formed *via* the intramolecular electron transfer mechanism [reaction (1)] requiring the existence of $>S^{\bullet +}$, and the location of both reaction centres (sulphur and carboxyl) within the same peptide unit. In contrast, the formation of **2a** occurs even though sulphur and *N*-terminal carboxyl group are separated by a peptide bond. This is particularly evident in the oxidation of *S*-methylglutathione, where CO_2 and α-amino radical yields are equal over the entire pH range investigated. Since the yields of type **2a** α-amino radicals detected in γ-Glu-Met are similar to those in *S*-methylglutathione systems it appears that the two decarboxylation routes do not compete directly against each other (if this were the case, one might have expected relatively lower yields of α-amino radicals from γ-Glu-Met or higher yields from *S*-methylglutathione). This conclusion excludes the existence of one common species (e.g. $>S^{\bullet +}$) as the key intermediate for both pathways but points out the need for two different precursors, each of them favouring a distinct decarboxylation mechanism. In analogy to methionine[2,8] this second precursor (besides $>S^{\bullet +}$) is suggested to be the $>S^{\bullet}$–OH adduct **5** leading to species **6b** [reaction (7b)] which subsequently, and presumably in a concerted reaction,[3] decarboxylates *via* reaction (10).

$$\mathbf{6b} \longrightarrow [H_2N\text{–}CH(CO_2^{\bullet})\text{——}S\text{–}] \longrightarrow CO_2 + \mathbf{2a/3a} \qquad (10)$$

It is specifically noted that with the γ-Glu-Met and *S*-

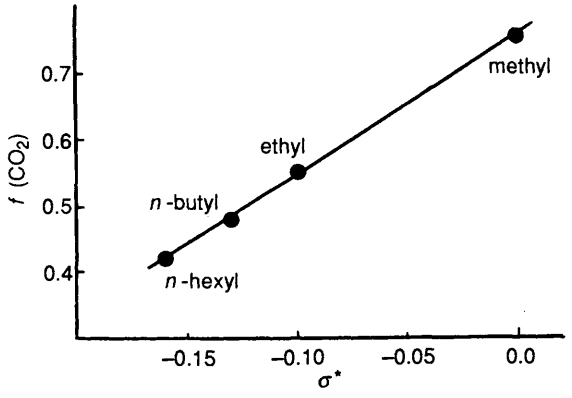


Fig. 3 Decarboxylation efficiency f (see text) *vs.* Taft inductive parameters (σ*) of the substituents located at sulphur in *S*-alkylglutathione derivatives at pH 5.1.

alkylglutathione derivatives intramolecular *N*-oxidation is not sterically assisted by the formation of a 5-membered ring as in methionine.[2,8] The effects on this reaction channel of the concentration of the solute, pH and chain length of substituents located at the sulphur atom (in *S*-alkylglutathione derivatives) are discussed below.

Influence of Concentration.—Practically no dependence of the CO_2 yield on peptide concentration is observed when decarboxylation occurs *via* the pseudo-Kolbe mechanism, *i.e.* in case of direct electron transfer from the *C*-terminal carboxyl group to $>S^{+\bullet}$ within the Met moiety[4] (data for Gly-Met and Gly-Gly-Met in Table 1). Even if the dimer radical cation $(>S.\therefore S<)^+$ is present the $>S^{+\bullet}$ reaction centre is nevertheless available through equilibrium (4).

In contrast, an increase in peptide concentration for γ-Glu-Met and *S*-methylglutathione systems results in a marked decrease of $G(CO_2)$ (Table 1) implying that in this case it is not $>S^{+\bullet}$ which initiates the *N*-terminal decarboxylation. As suggested above, the $>S^{\bullet}$–OH adduct is considered to be the responsible precursor. As an increase of peptide concentration enhances the effective rate of reaction (8) and, in turn, reduces the probability of reaction (7) and subsequent decarboxylation (10), this provides a reasonable rationale for the observed concentration dependence of the decarboxylation.

Considering γ-Glu-Met, any *N*-terminal decarboxylation is precluded once $>S^{+\bullet}$ is formed, and only the *C*-terminal process may then occur. This, however, still has to compete against deprotonation from $>S^{+\bullet}$ and from $(>S.\therefore S<)^+$. This satisfactorily explains the reduced overall CO_2 yield from γ-Glu-Met. One-electron *S*-oxidation converts γ-Glu-Met into an overall neutral species, like a one-electron oxidized N-Ac-Met. From the latter peptide it is known that such an overall neutral form undergoes *C*-terminal decarboxylation with an efficiency of only 54% relative to $>S^{+\bullet}$ formation.[4]

Variation of pH.—The pH dependence of the $(>S.\therefore S<)^+$ (**4b**) yield essentially reflects two parameters. First, the lower the pH, the higher the efficiency of the conversion of the $^{\bullet}OH$-adduct **5** by bulk protons to the three-electron-bonded dimer **4b**. Secondly, increasing protonation of the *C*-terminal carboxyl group lowers the redox potential of the latter, thus preventing efficient electron transfer to the sulphur-centred radical cation. Consequently, higher yields of **4b** are observed in very acid solutions. For the γ-Glu-Met system, in particular, the respective pH profile exhibits sigmoidal character with a break point at pH 3.9.

The competition between reactions (7a) (leading to **4b** or *C*-terminal decarboxylation) and (7b) (leading to *N*-terminal decarboxylation) is also directly reflected in the pH dependence of the α-amino radical (**2a**) yield. As shown in Fig. 2 it increases with pH and complements the **4b** formation. The break points at pH 3.9 are considered to reflect both thermodynamic (pK of carboxyl groups and of the α-amino radical **2a**) as well as kinetic parameters [reactions (6), (7) and (9)] and can therefore not be associated with one particular process.

Comparison of the pH profiles of the total CO_2 yields and the *N*-terminal decarboxylation (represented by the $PNAP^{\bullet-}$ yield) shows two pH regions, namely 4–5 and >8, where the *N*-terminal process appears to be the exclusive CO_2 source.

The contribution of *C*-terminal decarboxylation in γ-Glu-Met is given by the difference between the two curves in Fig. 1*a*. It is interesting to note that this process seems to be restricted to two separate pH regions with maximum efficiencies around pH 3.6 and 5.5, respectively. Starting from the low pH side the first increase in *C*-terminal decarboxylation is explained by the pK of the *C*-terminal carboxyl group; as discussed previously the deprotonated form $(-CO_2-)$ facilitates electron transfer to the sulphur-centred radical cation $(>S^{\bullet+})$. The subsequent decrease in CO_2 yield can be associated with competition between reactions (7a) and (7b) according to which the yield of $>S^{\bullet+}$, necessary for *C*-terminal decarboxylation, decreases with pH. (The $>S^{\bullet+}$ are formed from adduct **5a** *via* reaction (7a), *i.e.* by reaction with free protons, while reaction (7b) involves proton transfer from, and oxidation of, the amino group and is thus pH independent as long as the amino group is protonated.) As expected this decrease in *C*-terminal decarboxylation parallels the pH profile of the $(>S.\therefore S<)^+$ stabilization and complements that of the $PNAP^{\bullet-}$ formation (*N*-terminal decarboxylation). Superimposed on this trend is the effect of yet another parameter which is responsible for the second increase in *C*-terminal decarboxylation at pH > 5. It has been demonstrated[4] that the lifetime of $>S^{\bullet+}$ with respect to deprotonation (and consequently the probability of electron transfer from the *C*-terminal carboxyl group) increases if the influence of the second positive charge, *i.e.* at the *N*-terminal amino group, disappears. This, of course, occurs beyond the pK of the latter which, in oxidized peptides, has been found to be of the order of 5–6 (± 1).[4]

A quantitative analysis of the pH effects still awaits exact knowledge of the rate constant for deprotonation, equilibrium constant of $(>S.\therefore S<)^+$ and stability constant of $>S-^{\bullet}OH$ as well as the pKs of carboxyl and amino groups in the non-oxidized and oxidized state of the peptide.

Comparison of $G(CO_2)$ and $G(PNAP^{\bullet-})$ in *S*-methylglutathione shows no difference between the two yields within the pH range 3.2–8.0. Here, the only process leading to CO_2 is *N*-terminal decarboxylation, since the sulphur and the *C*-terminal carboxyl function are not located within the same peptide unit (a prerequisite for *C*-terminal decarboxylation). At present it is not possible to provide a conclusive explanation for the maximum at *ca.* pH 5.

Variation of Alkyl Chain Length in S-Alkyl Glutathione Derivatives.—The results listed in Table 2 indicate a strong dependence of CO_2 formation on the length of the *S*-alkyl chain (R) in *S*-alkylglutathione derivatives. The observed trend parallels the electron releasing power of R,[28] with the highest CO_2 yield being formed for R = CH_3, the lowest for R = C_9H_{19}. A Taft plot (Fig. 3) showing the efficiency of decarboxylation $f(CO_2) = G(CO_2)_{exp}/G(\text{S-oxid.})$ as a function of Taft's inductive parameters σ*† yields a straight line with $f(CO_2) = 0.75 + 2.7\,\sigma^*$.

† The Taft parameter for C_6H_{13} (σ* = 0.16) was approximated using Δσ* = 0.015 for each methylene group going from R = C_2H_5 to R = C_4H_9. Thus Δσ* = 0.03 was added to σ* C_4H_9 to obtain σ* C_6H_{13}.

Increasing electron density at the sulphur would facilitate protonation of species **5** *via* reaction (6) because of better resonance stabilization of $(>S.^{\cdot}.OH_2)^+$. Thus, increasing the electron releasing nature of the substituent R reduces the probability of *N*-terminal decarboxylation initiated by the interaction of $>S^{\bullet}-OH$ with $-NH_3^+$. It is noted that a similarly sensitive substituent effect has been observed for the influence of an *N*-terminal amino group on *C*-terminal decarboxylation in another series of methionine containing peptides.[4]

Conclusion

The present investigation on the decarboxylation of γ-Glu-Met and *S*-alkylglutathione derivatives has demonstrated that two mechanisms can lead to the formation of CO_2. These are electron transfer between $>S^{\bullet+}$ and the carboxyl group whenever both reaction centres are located within the same peptide unit, and interaction between an OH-adduct ($>S^{\bullet}-OH$) and a protonated amino function α to a carboxyl group. The latter mechanism is independent of the location of the two reaction centres in the peptide. In so far the arrangement within a sterically favourable 5-membered ring, as observed with methionine, is not a necessary prerequisite for decarboxylation.

The efficiency of *N*-terminal decarboxylation is strongly affected by solute concentration, pH and, particularly, by the electron inductive properties of substituents located at the sulphur atom.

The structure of the $^{\bullet}OH$ adduct, formation conditions and its stability are physically interesting aspects, since they could provide some insight into the process of 'oxygen activation', a biological mechanism, that is not yet well understood.

Biochemically it is interesting that the *N*-terminal decarboxylation process still occurs if the $>S^{\bullet}-OH$ adduct is embedded in the inner of a bulky molecule such as *S*-nonylglutathione, which could be taken as an approach to the interior of a protein molecule. It can thus be anticipated that our considerations may be generally applicable for the understanding of $^{\bullet}OH$ radical induced oxidation reactions within proteins which contain sulphur functions.

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