Intramolecular addition of cysteine thiyl radicals to phenylalanine in peptides: formation of cyclohexadienyl type radicals

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The *intra*-molecular addition of peptide cysteine thiyl radicals to phenylalanine yields alkylthio-substituted cyclohexadienyl radicals for the peptides Phe–Cys and Phe–Gly–Cys–Gly, *i.e.* even when Phe and Cys are separated by a Gly residue, and presents a possible free radical pathway to thioether-containing peptide and protein cross-links.

Thiyl radicals are important reactants in several enzymes,[1] and form *in vivo* during conditions of oxidative stress.[2] For a long time, thiyl radicals have been considered as rather unreactive species. However, more recently several reactions of thiyl radicals with biomolecules have been described, such as catalysis of *cis–trans* isomerization of unsaturated fatty acids[3] and hydrogen abstraction from polyunsaturated fatty acids,[4] thymine[5] and peptide C_α–H and side chain C–H bonds.[6,7] In peptides and proteins, *intra*-molecular hydrogen abstraction reactions may compete successfully against *inter*-molecular repair by glutathione or ascorbate. Here, we describe the addition of a peptide thiyl radical to the aromatic ring of phenylalanine (Phe) as a novel, biologically potentially significant process.

We first describe the preparation of our starting thiyl radical by pulse radiolysis and, subsequently, the formation of an *intra*-molecular bond between sulfur and the phenyl moiety.

The thiyl radical was prepared as follows: pulse radiolysis[8–10] (1.8 MeV, 20 ns pulse width, FWHM, sample thickness: 1 mm, window thickness: 0.5 mm) of an Ar-saturated (pH 4.0) aqueous solution of 5×10^{-4} M phenylalanylcysteine disulfide, $(PheCysS)_2$, and 0.4 M *tert*-butanol leads to the transient formation of an optical absorbance with $\lambda_{max} = 410$ nm, which is fully developed at *ca.* 0.4 μs after the pulse and disappears within 4 μs (Fig. 1A). The absorbing species is the well-characterized three-electron bonded disulfide radical anion $[PheCysS\cdots SCysPhe]^{\cdot -}$ (**1**), generated through reactions (1) and (3).[11–13]

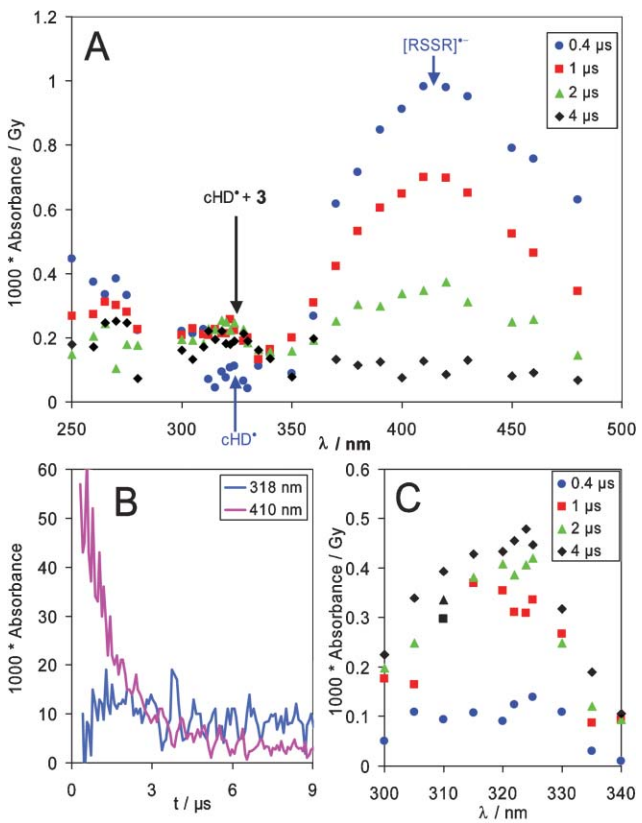

Fig. 1 Spectra after pulse irradiation of Ar-saturated solutions of 5×10^{-4} M $(PheCysS)_2$ and 0.4 M *tert*-butanol (dose ~ 45 Gy). Panels A and B: pH 4.0, panel C: pH 1.7. At 1 μs after the pulse, alkylthio-substituted cyclohexdienyl radicals (species **3**, $\lambda_{max} = 324$ nm, $\varepsilon_{324} \approx 5000$ M^{-1} cm^{-1}), but not benzyl radicals ($\lambda_{max} = 258$ nm, $\varepsilon_{258} \approx 14000$ M^{-1} cm^{-1}) are observed (panels A and C).

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$$H_2O \rightarrow e^-_{aq}, H^\cdot, HO^\cdot \quad (1)$$

$$HO^\cdot + (CH_3)_3COH \rightarrow H_2O + {}^\cdot CH_2C(CH_3)_2OH \quad (2)$$

$$e^-_{aq} + (PheCysS)_2 \rightarrow [PheCysS\therefore SCysPhe]^{\cdot -}\ (\mathbf{1}) \quad (3)$$

$$e^-_{aq} + H^+ \rightarrow H^\cdot \quad (4)$$

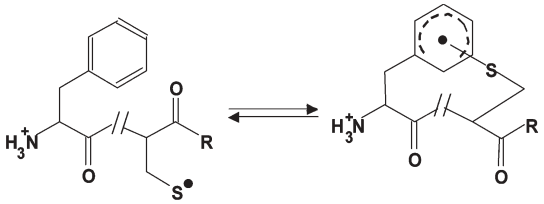
$$H^\cdot + (PheCysS)_2 \rightarrow [(PheCysS\therefore SCysPhe)H]^\cdot\ (\mathbf{2}) \quad (5)$$

$$\mathbf{2} \rightarrow PheCysSH + PheCysS^\cdot \quad (6)$$

$$\mathbf{1} + H^+ \rightarrow \mathbf{2} \quad (7)$$

$$Phe + RS^\cdot \rightarrow Phe(-CH^\cdot-C_6H_5) + RSH \quad (8)$$

$$Phe + H^\cdot \rightarrow Phe(-CH_2-(C_6H_6{}^\cdot)) \ (cHD^\cdot) \quad (9)$$



3 (10)

A quantitative analysis of the transients based on published data is mandatory for later mechanistic conclusions. The hydrated electron (e^-_{aq}) participates in reactions 3 and 4; the yield† of reaction 3 is $G(e^-_{aq,3}) = 2.2$.[13] At 0.4 μs after the pulse the absorbance A_{410} is $(1.0 \pm 0.1) \times 10^{-3}$ absorbance units (AU)/Gy. Based on $\varepsilon_{410} \approx 4200\ M^{-1}\ cm^{-1}$,[12] this value corresponds to $G(\mathbf{1}) = 2.3$, in agreement with $G(e^-_{aq,3})$. The yield of H• atoms, based on reactions 1 and 4 is $G(H^\bullet) = 1.2$.‡ All reactions of H• are completed at 0.4 μs after the pulse.[13,14]

About 85% of the H• atoms react with the disulfide moiety of $(PheCysS)_2$ (reaction 5) to yield **2** that decomposes (reaction 6) into the corresponding thiyl radical ($\lambda_{max} = 330$ nm, $\varepsilon_{330} \approx 320\ M^{-1}cm^{-1}$)[15] and a thiol.[16] The remaining 15% of the H• atoms ($G = 0.18$) add to Phe (reaction 9)[17] to yield cyclohexadienyl radicals (cHD•) which absorb maximally at $\lambda_{max} = 320$ nm ($\varepsilon_{320} \approx 5000\ M^{-1}\ cm^{-1}$)[17] with a shoulder at 310 nm.[17] At 0.4 μs after the pulse (Fig. 1A) the absorbance at 320 nm with $A_{320} = 1.2 \times 10^{-4}$ AU Gy^{-1} corresponds quantitatively to $G\varepsilon l$(cHD•) + $G\varepsilon l$(RS•) = $(0.9 + 0.3) \times 10^{-4}$ AU Gy^{-1}. Hence, at 0.4 μs after the pulse, the experimental spectrum is quantitatively rationalized by the formation of **1**, cHD• and PheCysS•.

In the following, radical anion **1** converts into PheCysS•, which undergoes an *intra*-molecular addition of the thiyl radical to the Phe moiety. Experimental evidence for these processes is summarized below. At pH 4, the H^+-assisted decomposition[15] of **1** proceeds within *ca.* 4 μs (reactions 7 and 6, where reaction 7 is rate-determining[15])§ and is accompanied by a build-up of an absorbance with $\lambda_{max} = 324$ nm and a shoulder around 315 nm (Figs 1A and 1B). The maximal absorbance at 324 nm is 3×10^{-4} AU Gy^{-1} and is reached within *ca.* 2 μs after the pulse. The similarity of this spectrum to the published one of cHD•,[17] and the fact that PheCysS• radicals are the only product of reactions 7 and 6, suggests an *intra*-molecular (*vide infra*) addition of the thiyl radical to Phe, which generates an alkylthio-substituted cyclohexadienyl radical. Based on the spectral properties, this transient *cannot* be a benzyl radical: benzyl radicals display absorbance maxima[18] around 258 nm ($\varepsilon_{258} \approx 14000\ M^{-1}\ cm^{-1}$), 307 nm ($\varepsilon_{307} \approx 3300\ M^{-1}\ cm^{-1}$) and 318–320 nm ($\varepsilon \approx 5500\ M^{-1}\ cm^{-1}$). Hence, for a benzyl radical the intensity of the 260 nm absorbance must be *ca.* 3-fold higher compared to the absorbance in the 320 nm region. However, that is not what we observe. At times ⩾ 1 μs after the pulse, we quantify an absorbance ratio of $A_{260}/A_{324} \approx 1.0$. This quantitative evaluation suggests that benzyl radicals are, at most, a minor component of the radical products at times ⩾ 1 μs after the pulse.

Reaction 10 shows the possibility of *ortho*-, *meta*- and *para*-addition of the Cys thiyl radical to the Phe moiety in a generalized intermediate structure **3**. Based on an estimated $\varepsilon_{324} \approx 5000\ M^{-1}\ cm^{-1}$ (by analogy to cHD•), the maximal yield of **3** amounts to *ca.* $G = 0.6$. Two controls were performed. First, at 0.4 μs after pulse irradiation of an N_2O-saturated solution, pH 4, of 5×10^{-4} M $(PheCysS)_2$ and 0.4 M *tert*-butanol, no 410 nm transient was observed, but, instead, a small amount of cHD• ($A_{320} = 8 \times 10^{-5}$ AU Gy^{-1}). N_2O converts hydrated electrons into HO• radicals, which are scavenged by *tert*-butanol (reaction 2), and eliminates reaction 3. Importantly, only negligible yields ($A_{324} = 4 \times 10^{-5}$ AU Gy^{-1}) of **3** are observed within 2 μs after the pulse, consistent with the lack of thiyl radicals. Second, when $(GlyCysS)_2$ was irradiated, no absorbance increase at 324 nm was detected—the absorption increase at 324 nm is therefore related to the Phe moiety.

At pH ⩽ 1.7, all hydrated electrons are converted to H•,[10] and thiyl radicals are generated within 0.4 μs.[15] Fig. 1C shows a small yield of cHD• at 0.4 μs after pulse irradiation of $(PheCysS)_2$, followed by a significant formation of **3** over 2 μs, which confirms the experimental results obtained at pH 4.

Abstraction of a benzylic hydrogen by thiyl radicals has been reported (reaction 8)[5] but given the *absence* of a strong absorption band near 260 nm, the benzyl radical[18] is not a *primary* reaction product. We suspect that reaction 10 is reversible, and that the yields of **3** are determined by this equilibrium. At the applied radiolytic doses, **3** disappears predominantly *via* radical recombination, *i.e.*, *via* approximate second-order kinetics with $k/\varepsilon \approx 2 \times 10^6\ s^{-1}$. However, at lower, *physiologically* more relevant radical concentrations slower processes, such as reaction 8 or electron transfer followed by deprotonation to a benzyl radical, can occur. In fact, benzyl radical formation during the *inter*-molecular reaction of thiyl radicals with Phe was demonstrated by means of H/D-exchange experiments.[5]

Pulse irradiation of an Ar-saturated solution, pH 4, of 2.5×10^{-4} M of the disulfide-linked peptide $(Phe–Gly–Cys–Gly)_2$ and 0.5 M *tert*-butanol gave results comparable to those with $(PheCysS)_2$: a low initial yield of cHD• ($A_{320} = 1.2 \times 10^{-4}$ AU Gy^{-1}), followed by the formation of **3** ($\lambda_{max} = 322$ nm, shoulder at 315 nm) with a maximal yield of 2.4×10^{-4} AU Gy^{-1} at 322 nm (Fig. 2A). However, **3** was not obtained in Ar-saturated solutions, pH 4, of 0.5 M *tert*-butanol and the individual amino acids, 5×10^{-4} M Phe and 2.5×10^{-4} M $CysS_2$ (Fig. 2A). This experiment confirms the *intra*-molecular addition of the Cys thiyl radical to Phe.

The formation of **3** from $(Phe–Gly–Cys–Gly)_2$ was monitored at 318 nm at pH 1 (Fig. 2B), where it was best resolved. Again, *ca.* 15% of the H• atoms add to Phe, as revealed by the fast initial rise of A_{318}, followed by the slower formation of **3** over *ca.* 1.5 μs. On longer time scales, **3** disappears *via* approximate scond-order

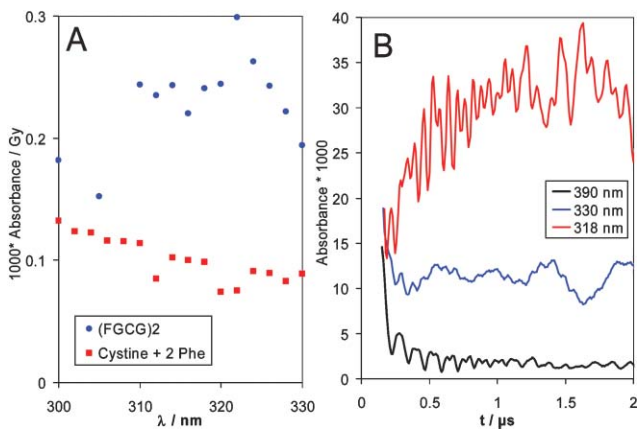


Fig. 2 Panel A: formation of cyclohexadienyl radicals **3** by *intra*- [blue dots, 2.5×10^{-4} M $(Phe–Gly–Cys–Gly)S_2$] but not by *inter*-molecular (red dots, 2.5×10^{-4} M cystine and 5×10^{-4} M Phe) reaction of cysteine thiyl radicals with Phe. Spectra were taken 2 μs after the pulse of 45 Gy on an Ar-saturated solution, pH 3.8, containing 0.4 M *tert*-butanol. Panel B: kinetic traces of $(Phe–Gly–Cys–Gly)S_2$ reduced by e^-_{aq}/H• recorded at 318 nm (**3**), 330 nm (λ_{max} of thiyl radical) and 390 nm (species **2**). Solutions (Ar sat, pH 2, 0.4 M *tert*-butanol) were irradiated with ~ 45 Gy.

kinetics with $k/\varepsilon \approx 8 \times 10^6$ s^{-1}, indicating that also the radical intermediates for this peptide react predominantly *via* radical combination.

The described model reactions are of great *biological significance*. For (Phe–Gly–Cys–S$^{\bullet}$)–Gly, the *intra*-molecular addition occurs with $t_{1/2} \approx 0.5$ μs, outcompeting addition of O_2 to the Cys thiyl radical ($k \approx 2.2 \times 10^9$ M^{-1} s^{-1};[19] $t_{1/2} \approx 10$ μs, based on a biologically relevant tissue concentration of $[O_2] \approx 30$ μM).[20] Moreover, cyclohexadienyl radicals react at a nearly diffusion-controlled rate with O_2, and the resulting peroxyl radicals may eliminate $HO_2^{\bullet}$ to rearomatize the ring.[21] Such a reaction sequence leads to a covalent thioether cross-link. Evidence for such a reaction has come from product studies of the oxidative addition of thiols to anthracene.[22] In proteins, such cross-links may stabilize non-native conformations, or lead to protein aggregation and consequently compromise activity. A naturally occuring thioether cross-link, tyrosylcysteine, was identified in the enzyme galactose oxidase, but mechanistically its formation has not been well characterized.[23] The reaction characterized in this paper may offer a facile route to such biologically relevant thioether cross-links. Heo *et al.*[24,25] provided another recent example for the biological significance of the reaction between Cys thiyl radicals and Phe, playing an important role in the nitric oxide-dependent guanine nucleotide exchange of Ras proteins. Here, an initial thiyl radical at Cys^{118} is suggested to oxidize Phe^{28}, which ultimately oxidizes guanine nucleotide diphosphate. While the detailed mechanisms have not yet been established, nitric oxide-dependent guanine nucleotide exchange clearly does not operate in mutant Ras proteins, where either Cys or Phe have been replaced by Ser or Leu, respectively.

For experimental reasons, we studied the reaction of Cys thiyl radicals with Phe with two model peptides where Phe and Cys are located in close sequential proximity. However, in proteins such proximity in sequence is not necessarily required for an effective reaction as long as the two reactants are close in space, as underlined by the reaction described for Ras proteins (*vide supra*).

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Notes and references

† Based on $k_3 = 2.3 \times 10^{10}$ M^{-1} s^{-1}[13] and $k_4 = 1.6 \times 10^{10}$ M^{-1} s^{-1},[13] the yield of e^-_{aq} available for reaction with 5×10^{-4} M $(PheCysS)_2$ at pH 4 amounts to $0.78\ G_i(e^-_{aq}) = 0.78 \times 2.75 = 2.2$. The yield of H$^{\bullet}$ available for reaction with $(PheCysS)_2$ amounts to $G_i(H^{\bullet}) + 0.22\ G_i(e^-_{aq}) = 0.6 + 0.22 \times 2.75 = 1.2$. The radiation chemical yield G refers to the number of species reacted/generated per 100 eV absorbed energy; $G = 1.0$ corresponds to 0.1036 μM generated/reacted species per 1 J absorbed energy.
‡ Based on the rate constants for the direct reaction of H$^{\bullet}$ with the free amino acids Phe ($k = 7.1 \times 10^8$ M^{-1} s^{-1})[13] and $CysS_2$ ($k = 8 \times 10^9$ M^{-1} s^{-1}),[14] only 85% of H$^{\bullet}$ will react with the disulfide moiety while 15% of H$^{\bullet}$ will directly add to the side chain of Phe.
§ Similar experiments performed at different pH values, 4.3, 3.6, and 2.3, demonstrate that higher proton concentrations accelerate the decomposition of radical anion **1**.

1 J. Stubbe and W. A. van der Donk, *Chem. Rev.*, 1998, **98**, 705.
2 D. Jourd'heuil, F. L. Jourd'heuil and M. Feelisch, *J. Biol. Chem.*, 2003, **278**, 15720.
3 C. Ferreri, A. Samadi, F. Sassatelli, L. Landi and C. Chatgilialoglu, *J. Am. Chem. Soc.*, 2004, **126**, 1063.
4 Ch. Schöneich, U. Dillinger, F. von Bruchhausen and K.-D. Asmus, *Arch. Biochem. Biophys.*, 1992, **292**, 456.
5 T. Nauser and Ch. Schöneich, *Chem. Res. Toxicol.*, 2003, **16**, 1056.
6 T. Nauser and Ch. Schöneich, *J. Am. Chem. Soc.*, 2003, **125**, 2042.
7 T. Nauser, J. Pelling and Ch. Schöneich, *Chem. Res. Toxicol.*, 2004, **17**, 1323.
8 For a description of the pulse radiolysis setup, see ref. 9, and for an overview over radiation chemical techniques, see ref. 10.
9 A. Daiber, T. Nauser, N. Takaya, T. Kudo, P. Weber, C. Hultschig, H. Shoun and V. Ullrich, *J. Inorg. Biochem.*, 2002, **88**, 343–352.
10 C. von Sonntag, *The Chemical Basis of Radiation Biology*, Taylor & Francis, London, 1987.
11 M. Göbl, M. Bonifačić and K.-D. Asmus, *J. Am. Chem. Soc.*, 1984, **106**, 5984.
12 S. P. Mezyk, *Chem. Phys. Lett.*, 1995, **235**, 89.
13 G. V. Buxton, C. L. Greenstock, W. P. Helman and A. B. Ross, *J. Phys. Chem. Ref. Data*, 1988, **17**, 513.
14 P. Neta and R. H. Schuler, *Radiat. Res.*, 1971, **47**, 612.
15 M. Z. Hoffman and E. Hayon, *J. Phys. Chem.*, 1973, **77**, 990.
16 M. Z. Hoffman and E. Hayon, *J. Am. Chem. Soc.*, 1972, **94**, 7950.
17 N. N. Lichtin and A. Shafferman, *Radiat. Res.*, 1974, **60**, 432.
18 H. C. Christensen, K. Sehested and E. J. Hart, *J. Phys. Chem.*, 1973, **77**, 983.
19 By analogy to thiyl radicals from 2-mercaptoethanol: X. Zhang, N. Zhang, H.-P. Schuchmann and C. von Sonntag, *J. Phys. Chem.*, 1987, **98**, 6541.
20 M. C. Hogan, *J. Appl. Physiol.*, 1999, **86**, 720.
21 J. W. Taylor, G. Ehlker, H.-H. Carstensen, L. Ruslen, R. Field and W. H. Green, *J. Phys. Chem. A*, 2004, **108**, 7193.
22 A. L. J. Beckwith and B. S. Low, *J. Chem. Soc.*, 1961, 1304.
23 S. J. Firbank, M. S. Rogers, C. M. Wilmot, D. M. Dooley, M. A. Halcrow, P. F. Knowles, M. J. McPherson and S. E. V. Phillips, *Proc. Natl. Acad. Sci. USA*, 2001, **98**, 12932.
24 J. Heo, K. C. Prutzman, V. Mocanu and S. L. Campbell, *J. Mol. Biol.*, 2005, **346**, 1423–1440.
25 J. Heo and S. L. Campbell, *J. Biol. Chem.*, 2005, **280**, 12438–12445.

