Oxidation of methionine peptides by Fenton systems: the importance of peptide sequence, neighbouring groups and EDTA

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We investigated the anaerobic oxidation of several Thr- and Met-containing di- and tri-peptides by Fenton systems, $(NH_4)_2Fe(SO_4)_2/H_2O_2$ and $[Fe^{II}(EDTA)]^{2-}/H_2O_2$, respectively, and compared the respective product patterns with those obtained after oxidation with free radiation chemically generated hydroxyl radicals. The products obtained by the $(NH_4)_2Fe(SO_4)_2/H_2O_2$ system did not show any significant resemblance to product patterns characteristic for free hydroxyl radicals. In contrast, the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ system generated a material balance which showed some similarity to the free hydroxyl radical-generated pattern. From a comparison of the relative reactivities of the various functional groups of the peptides with the quantities of products obtained, we conclude that for Thr-Met, in particular at pH 6.3, a direct attack of a fraction of reactive oxygen species at the Met sulfur caused the formation of a sulfuranyl radical intermediate. This then underwent intramolecular coupled proton/electron-transfer with the protonated *N*-terminus to yield nitrogen-centered radical cations. The latter subsequently suffered heterolytic fragmentation of the C_α–C_β bond of Thr to yield acetaldehyde. Such a pathway had previously been characterized for the oxidation of Thr-Met by free HO•. The occurrence of such intramolecular radical transformation is taken as evidence that neighbouring group effects can operate during metal-catalysed peptide (and possibly protein) oxidation.

Introduction

There is a growing interest in the mechanistic characterization of the metal-catalysed oxidation of biological macromolecules such as proteins and DNA. Such pathways are of general importance for (*i*) biological systems exposed to conditions of oxidative stress,[1] (*ii*) the manufacturing of recombinant proteins and polynucleotides in the biotechnology industry[2] and (*iii*) the biochemical mapping of protein and DNA structure through site-specific cleavage *via* iron–EDTA complexes.[3–8] In the latter systems iron–EDTA complexes have been immobilized either directly at the protein[3,4] (*e.g.* through conjugation with a cysteine residue) or at a target molecule which then specifically binds to a distinct domain of the protein (*e.g.* binding of a trifluoroperazine–EDTA–iron entity to calmodulin[5,8]). For such metal-catalysed oxidation of proteins site-specific mechanisms play an important role.

There are as yet many unresolved problems pertinent to these reactions. The chemical oxidants which are ultimately responsible for the site-specific oxidation reactions have not been identified in detail. The participation of hydroxyl radicals (HO•) has been suggested.[9] However, some laboratories argue against the formation of free hydroxyl radicals in metal-catalysed processes, based on findings during the oxidation of organic compounds by hydrogen peroxide in the presence of various iron complexes.[10] In addition, there is no systematic evaluation of the influence of neighbouring amino acids on the metal-catalysed oxidation of a target residue. Since the significance of neighbouring group effects has been demonstrated for many redox reactions of small organic molecules,[11] we should expect a similar importance for the redox reactions of peptides and proteins. Recently, we have shown that sulfur-centered reactive intermediates, formed *via* the reaction of radiation chemically produced free hydroxyl radicals (HO•) with Met residues in di-, tri- and tetra-peptides, promote decarboxylation reactions depending on the nature of the neighbouring amino acids around Met.[12–14] As another example for the operation of neighbouring group effects, we have demonstrated that the addition of hydroxyl radicals to the Met sulfur of Thr-Met and Ser-Met resulted in an efficient cleavage of the side chains of the *N*-terminal residues Thr or Ser, respectively (Scheme 1; reactions 1–4).[15]

This mechanism requires the formation of the hydroxy sulfuranyl radical **1a** and does not occur (or occurs with significantly less efficiency) with initially formed sulfur-centered radical cations. The cyclic intermediate **2**, an 8-membered ring, represents a sulfur–nitrogen three-electron-bonded radical cation which has been identified by means of pulse radiolysis and its characteristic absorption with λ_{max} = 385 nm,[15] and is analogous to previously reported sulfur–nitrogen three-electron bonds of methionine and some model compounds.[16,17] Its short lifetime ($t_{1/2}$ = 310 ns) precludes further isolation for chemical analysis.

The objective of the present work was to evaluate the potential importance of such neighbouring group effects for the metal-catalysed oxidation of peptides. It was of particular interest to study whether intramolecular transformations such as those shown for the hydroxyl radical induced process in reactions 1–4 would also occur during metal-catalysed oxidation by hydrogen peroxide at near physiological pH. For this we have subjected small Thr and Met containing model peptides to oxidation by hydrogen peroxide, catalysed by ferrous iron or ferrous EDTA, respectively. Thr and Met containing peptides were selected because we had already characterized a variety of potential parameters and neighbouring group effects which influence the oxidation of such Met-containing peptides by the free hydroxyl radical (see Scheme 1).[15]

Experimental

Materials

The peptides Thr-Met, Ala-Met, Thr-Leu and Met-Thr were obtained from Bachem Bioscience, Inc. (Philadelphia, PA). Met, Thr and Thr-NH_2 were obtained from Sigma Chemical Company (St. Louis, MO). The tripeptides Gly-Met-Thr and Gly-Thr-Met were synthesized by standard solid phase methods

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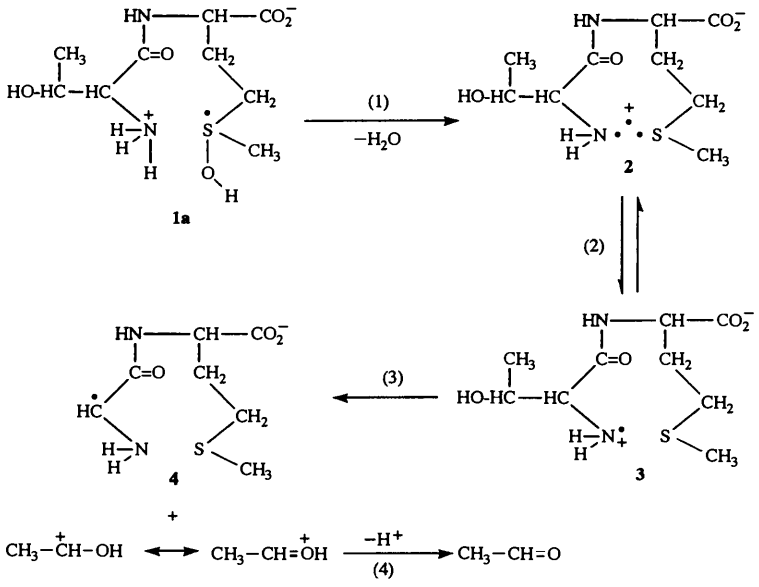


Scheme 1

employing FMOC-protected amino acids,[18] and sulfoxide standards were synthesized by the reaction of Met containing peptides with a slight excess of hydrogen peroxide (H_2O_2). All synthetic peptides were purified by HPLC and characterized by ^1H NMR and FAB mass spectrometry. The purity of all materials was controlled by HPLC. The other materials were of highest commercially available purity: Sigma provided ammonium ferrous sulfate [$(NH_4)_2Fe^{II}(SO_4)_2$], ferric chloride ($FeCl_3$), *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and methional ($CH_3SCH_2CH_2CHO$); Aldrich (Milwaukee, WI) provided ethylenediaminetetraacetate (EDTA) and naphthalene-2,3-dicarboxaldehyde (NDA; Fisher Scientific (St. Louis, MO) supplied sodium carbonate (Na_2CO_3), sodium phosphate, ammonium chloride (NH_4Cl), acetaldehyde (CH_3CHO), formaldehyde (H_2CO), acetone-[$(CH_3)_2CO$], 2-propanol and hydrogen peroxide (H_2O_2); bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce. The 2-propanol was freshly distilled before each experiment in order to minimize peroxide contamination.

Reactions

A standard oxidation reaction was carried out in a reaction volume of 1–2 ml of N_2-saturated aqueous solution, pH 7.5, containing 2.0×10^{-3} mol dm^{-3} carbonate buffer ($NaHCO_3$/Na_2CO_3), 5.0×10^{-4} mol dm^{-3} H_2O_2 and various concentrations of peptides, $(NH_4)_2Fe^{II}(SO_4)_2$ or $[Fe^{II}(EDTA)]^{2-}$ [from here on we will abbreviate $(NH_4)_2Fe^{II}(SO_4)_2$ with Fe^{II}]. A medium consisting of $NaHCO_3/Na_2CO_3$ was found suitable for the investigation of the oxidation reactions of amino acids catalysed by Fe^{II}.[19] In preliminary experiments we have carried out oxidation experiments at various concentrations of $NaHCO_3/Na_2CO_3$ between 0.5×10^{-3} mol dm^{-3} and 10×10^{-3} mol dm^{-3}. Maximum oxidation yields could be obtained at concentrations around 2.0×10^{-3} mol dm^{-3} $NaHCO_3$/Na_2CO_3. In contrast, only negligible oxidation yields, catalysed by Fe^{II}, were obtained with other buffers such as phosphate or HEPES, respectively. Any potential chelation by phosphate or HEPES would not be of such importance for the $[Fe^{II}(EDTA)]^{2-}$ catalysed oxidation. However, in order to keep the reaction conditions as constant as possible, we selected $NaHCO_3/Na_2CO_3$ as a buffer suitable for the catalysis by both types of ferrous iron. In the absence of EDTA, the actual form of catalytic iron present in our solutions cannot be specified since the only way to keep a significant concentration in solution will be by complexation with either the peptide, buffer or both. Nevertheless, the significant catalytic effect of both forms of ferrous iron, Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$, is evident from the fact that the ferrous iron catalysed reactions {*e.g.* at 5×10^{-4} mol dm^{-3} either Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$ and 5×10^{-4} mol dm^{-3} H_2O_2} are completed within 2 minutes after the initiation of the reactions (with high product yields), whereas no oxidation occurs during this period with 5×10^{-4} mol dm^{-3} H_2O_2 alone, and no oxidation at all occurs with either Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$ alone, respectively.

The reactions were performed in the following way: a stock solution of H_2O_2 was calibrated by two independent methods, (*i*) UV absorbance taking $\varepsilon_{240} = 39.4$ mol^{-1} dm^3 cm^{-1} [20] and (*ii*) titration with potassium permanganate.[21] Subsequently, stock solutions of (*i*) peptide in the absence or presence of EDTA in $NaHCO_3/Na_2CO_3$ solution of desired pH, (*ii*) $(NH_4)_2Fe^{II}(SO_4)_2$ in water and (*iii*) H_2O_2 in water, were separately deoxygenated by gently saturating them with N_2 (N_2-saturation of complexed $[Fe^{II}(EDTA)]^{2-}$ is less feasible due to the generally higher oxidation sensitivity of the complex). Then, an aliquot of Fe^{II} followed by an aliquot of H_2O_2 were added to the peptide-containing system. Immediately after the addition of Fe^{II} and H_2O_2, the N_2-flow for deoxygenation of the reaction mixture was stopped in order to avoid the loss of volatile products such as formaldehyde and acetaldehyde.

Product analysis

Peptides and sulfoxides. Peptides, methionine and their respective sulfoxides were quantified by reversed-phase HPLC, employing a Shimadzu instrument equipped with a SGE C18 column and a UV/VIS detector. Separation and elution was achieved with mixtures of 0.02% trifluoroacetic acid in water (mobile phase A) and 0.02% trifluoroacetic acid in water/acetonitrile (10:90, *v*/*v*) (mobile phase B). The separation of Thr-Met sulfoxide from Thr-Met and glyoxylylmethionine (for identification see Results) was achieved with isocratic elution employing a mobile phase consisting of 97.25% A and 2.75% B for 10 minutes. Subsequently, the absence of less polar reaction products was confirmed by running a gradient increasing the content of mobile phase B from 2.75% to 100% within 30 minutes.

Carbonyl products. The products formaldehyde, acetaldehyde, methional and acetone were quantified by reversed-phase HPLC after derivatization with 2,4-dinitrophenylhydrazine to yield the corresponding hydrazones, in a similar way to procedures described elsewhere.[15]

Ammonia. Ammonia was analysed in total reaction volumes

of 5 ml with an ammonia-sensitive electrode (Fisher Scientific) coupled to a voltmeter (Orion model 420 A). Calibrations were performed with NH_4Cl standards.

Thr-NH_2 and Thr. Thr-NH_2 and Thr were monitored after derivatization with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of sodium cyanide as described.[22] The resulting 1-cyanobenz[*f*]isoindole derivatives were analysed by reversed-phase chromatography employing fluorescence detection (excitation at $\lambda = 420$ nm, detection at $\lambda = 470$ nm). Calibration was achieved after derivatization of authentic standards of Thr-NH_2 and Thr.

Total yield of thiols and dimethyl disulfide. The total yields of thiols were quantified employing the procedure described by Ellman[23] and calibrated with authentic standards of cysteine. The reaction mixtures were analysed for thiols 2 minutes after the start of the reaction. At this time the ferrous iron-catalysed oxidations are completed (see above). On the other hand any further iron-catalysed oxidation of thiols to disulfides within 2 minutes is negligible, particularly in the presence of EDTA.[24] For confirmation, we also monitored the absence of even trace amounts of dimethyl disulfide in our systems, an oxidation product of methylmercaptane originating from *S*-dealkylation of an oxidized Met residue[25] (reversed-phase HPLC employing a SGE C_{18} column and UV-detection at $\lambda = 254$ nm; linear gradient from 2.75% B to 55% B within 30 minutes). The absence of dimethyl disulfide in our systems corroborates that no further oxidation of thiols had taken place at 2 minutes after initiation of the ferrous iron-catalysed oxidation.

Gas chromatography–mass spectrometry. The analysis of peptide derived products by gas chromatography–mass spectrometry (GC–MS) was performed according to a described procedure.[26,27] In brief, 30 minutes after the completion of a reaction, the reaction mixtures were lyophilized to dryness before they were subjected to acid hydrolysis in boiling 6N HCl. Subsequently, they were lyophilized and the dried samples reacted with BSTFA for trimethylsilylation. GC–MS analysis was done on a Girdel Model 30 GC connected to a Nermag Model R10-10 MS. Separation was achieved with He as carrier gas (inlet pressure: 4 psi) on a 15 m J&W DB1 column (i.d. = 0.25 mm), maintained at 150 °C for 5 minutes and subsequently heated to 300 °C with $\Delta T = 8$ °C/minute. The injection and detection port were maintained at 300 °C. The ionization voltage of the MS was 70 eV.

Results

A. Quantification of oxidizing species

The reaction of $[Fe^{II}(EDTA)]^{2-}$ with hydrogen peroxide proceeds *via* an initial addition of H_2O_2 to the ferrous centre yielding the highly oxidizing species **5a** ($k_5 = 7$–17.5×10^3 mol^{-1} dm^3 s^{-1}),[28,29] eventually followed by the elimination of either HO^- or H_2O [reactions (5)–(7)].[30]

$$[Fe^{II}(EDTA)]^{2-} + H_2O_2 \longrightarrow [Fe^{II}(EDTA)(H_2O_2)]^{2-}\ (\mathbf{5a}) \quad (5)$$

$$\mathbf{5a} \longrightarrow [Fe^{IV}(EDTA)(OH)]^{-}\ (\mathbf{5b}) + HO^- \quad (6)$$

$$\mathbf{5b} + OH^- \longrightarrow [Fe^{IV}(EDTA)O]^{2-}\ (\mathbf{5c}) + H_2O \quad (7)$$

Any of the intermediates **5a–5c** formally represents a hypervalent iron-oxo species which has been shown to react with various substrates by electron transfer or hydrogen abstraction.[29–33] For simplicity, the sum of species **5a**, **5b** and **5c** is from here on referred to as species **5** since at present it cannot be decided which of the structures actually prevails. Before the characterization of the potential products derived from the oxidation of peptides by **5**, it was necessary to standardize our oxidizing system with regard to the maximum amount of oxidizing species available through reactions (5)–(7) and/or subsequent processes (see below). The intermediate **5** can be intercepted by 2-propanol, $(CH_3)_2CHOH$.[29] The second column in Table 1 shows that the addition of 2-propanol to an oxidizing system consisting of a N_2-saturated aqueous solution of 2.0×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, pH 7.5, 5.0×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$ and 5.0×10^{-4} mol dm^{-3} H_2O_2 results in a maximum final yield of *ca.* 5.0×10^{-4} mol dm^{-3} acetone within experimental error limits for $[(CH_3)_2CHOH] \geqslant 5.0 \times 10^{-2}$ mol dm^{-3}. At $[(CH_3)_2CHOH] < 5.0 \times 10^{-2}$ mol dm^{-3} a gradual decrease of the acetone yields is observed. This can be attributed to competing reactions of **5** with hydrogen peroxide, a second equivalent of $[Fe^{II}(EDTA)]^{2-}$ or its own ligand, EDTA [reactions (8)–(10)].[30,31]

$$\mathbf{5} + H_2O_2 \longrightarrow 2H_2O + O_2^{\cdot-} + [Fe^{III}(EDTA)]^- \quad (8)$$

$$\mathbf{5} + [Fe^{II}(EDTA)]^{2-} \longrightarrow 2\,[Fe^{III}(EDTA)]^- + 2\,HO^- \quad (9)$$

$$\mathbf{5} \longrightarrow [Fe^{II}(EDTA^{\cdot})]^{2-} + \text{products} \quad (10)$$

In our systems the occurrence of reaction (10) is indicated through the formation of formaldehyde in the absence of 2-propanol (data not shown). Formaldehyde forms *via* decarboxylation and subsequent *N*-dealkylation of EDTA.[34] The important conclusion for our following experiments is that we can quantitatively convert H_2O_2 through the catalysis by $[Fe^{II}(EDTA)]^{2-}$ into an oxidizing species which efficiently oxidizes 2-propanol. No acetone formation was observed after 30 minutes reaction of 2-propanol with 5.0×10^{-4} mol dm^{-3} H_2O_2 in the absence or presence of 5.0×10^{-4} mol dm^{-3} $(NH_4)_2Fe(SO_4)_2$ and in the absence of EDTA.

The mechanism of acetone formation from 2-propanol may involve either the direct insertion of oxygen into the C_2–H bond followed by the elimination of water, or the abstraction of hydrogen from the C_2–H bond yielding (2-hydroxy)propan-2-yl radicals [reaction (11)]. The latter can subsequently reduce $[Fe^{III}(EDTA)]^-$ back to $[Fe^{II}(EDTA)]^{2-}$.

$$\mathbf{5} + (CH_3)_2CHOH \longrightarrow H_2O + HO^- + (CH_3)_2C^{\cdot}OH + [Fe^{III}(EDTA)]^- \quad (11)$$

Both routes are expected to yield a maximum of one equivalent of acetone per equivalence of hydrogen peroxide and $[Fe^{II}(EDTA)]^{2-}$. Based on a successful spin trapping of carbon-centred radicals during the reaction of **5** with alcohols,[32,33] the free radical mechanism [reactions (11)–(13)]

$$(CH_3)_2C^{\cdot}OH + [Fe^{III}(EDTA)]^- \longrightarrow (CH_3)_2C{=}O + H^+ + [Fe^{II}(EDTA)]^{2-} \quad (12)$$

$$(CH_3)_2C^{\cdot}OH + H_2O_2 \longrightarrow HO^{\cdot} + H_2O + (CH_3)_2C{=}O \quad (13)$$

appears to be more probable than other alternative routes such as direct oxygen insertion into the C_2–H bond.

Whenever both Thr-Met and 2-propanol are present in the reaction mixture, the relative yields of acetone and peptide oxidation products (representatively documented for acetaldehyde and sulfoxide, respectively) depend on the ratio of the initial concentrations of Thr-Met and 2-propanol (see Table 1, columns 3–6), indicating the competition of both substrates for the reactive oxygen species formed by the reaction of H_2O_2 with $[Fe^{II}(EDTA)]^{2-}$ (see Discussion).

B. Identification of products obtained from the anaerobic oxidation of Thr-Met

Peptides contain different reactive sites suitable for attack by reactive oxygen species. At pH 7.5, the primary sites of Thr-Met are expected to be the Met sulfur, the C_α–H bonds of the peptide backbone, the C_β–H bond of the Thr side chain, and a certain

Table 1 The effect of 2-propanol on selected products of the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ induced oxidation of Thr-Met. Reaction conditions: N_2-saturated 2×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, pH 7.5, 5×10^{-4} mol dm^{-3} H_2O_2, 5×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, with or without 1×10^{-3} mol dm^{-3} Thr-Met

	-Thr-Met	+1 × 10^{-3} mol dm^{-3} Thr-Met			
$(CH_3)_2CHOH$ 10^{-3} mol dm^{-3}	$(CH_3)_2C{=}O$ 10^{-6} mol dm^{-3}	$(CH_3)_2C{=}O$ 10^{-6} mol dm^{-3}	$CH_3CH{=}O$ 10^{-6} mol dm^{-3}	Thr-Met(SO) 10^{-6} mol dm^{-3}	-Thr-Met 10^{-6} mol dm^{-3}
0	0	0	65 ± 5	66 ± 12	354 ± 57
3	306 ± 41	144 ± 6	50 ± 11	60 ± 12	270 ± 42
5	360 ± 5	204 ± 9	32 ± 6	57 ± 13	185 ± 20
10	372 ± 19	275 ± 39	22 ± 1	41 ± 8	220 ± 14
25	459 ± 3	371 ± 43	14 ± 8	36 ± 9	175 ± 21
50	495 ± 5	433 ± 60	8 ± 6	28 ± 1	125 ± 35
100	487 ± 28	443 ± 82	9 ± 1	19 ± 3	70 ± 50

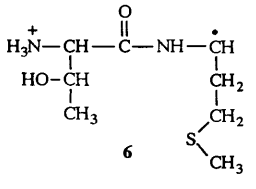
fraction of the deprotonated amino group (see Discussion). There are various potential products which can be used for a quantitative evaluation of the reaction mechanisms of peptide oxidation by the Fenton systems. Table 2 summarizes the quantitative analysis of key products obtained after oxidation of N_2-saturated aqueous solutions, pH 7.5, containing 2.0×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, 1.0×10^{-3} mol dm^{-3} Thr-Met, 5.0×10^{-4} mol dm^{-3} H_2O_2, and either added Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$, respectively.

For comparison, the first column contains product yields obtained through radiation chemically generated HO$^{\bullet}$ radicals. Several important features are apparent.

(*i*) There are significant differences between the two Fenton systems depending on the presence of EDTA. The $[Fe^{II}(EDTA)]^{2-}$-catalysed oxidation results in a product distribution which more closely resembles the one obtained through the free hydroxyl radical (though it is not identical) than the one derived from the Fe^{II}-catalysed oxidation. This becomes particularly evident from the comparison of the yields of sulfoxide, acetaldehyde and ammonia. Recently we demonstrated that sulfoxide is not a major product of the reaction of hydroxyl radicals with sulfides in the absence of oxygen.[35]

(*ii*) Both Fenton systems do not induce the hydrolytic cleavage of the Thr-Met peptide bond, in contrast to a mechanism proposed by Rana and Meares,[36] as evidenced by the lack of free Thr or Met among the reaction products.

(*iii*) A potential decarboxylation of the *C*-terminus *via* initially formed sulfur radical cations[12] or hydrogen abstraction from the Met C_α–H bond should yield radicals **6** or **7**, respectively. From the absence of methional ($CH_3SCH_2CH_2CHO$) and Thr-NH_2, respectively, we conclude that either intermediates **6** and/or **7** are not formed, or, if present, that they do not reduce ferric iron or H_2O_2, initiating the cleavage of their respective C–N bonds according to reactions (14)–(18) (Ox = Fe^{III} or H_2O_2; $Ox^{\bullet -}$ represents Fe^{II} or HO$^{\bullet}$ + HO$^-$; R = threonyl).



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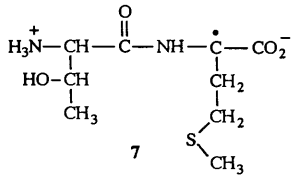

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6 + Ox ⟶ R–NH–CH$^{(+)}$–$CH_2CH_2SCH_3$ + $Ox^{\bullet -}$ (14)

R–NH–CH$^{(+)}$–$CH_2CH_2SCH_3$ + H_2O ⟶
H^+ + R–NH–CH(OH)–$CH_2CH_2SCH_3$ (15)

R–NH–CH(OH)–$CH_2CH_2SCH_3$ ⟶
R–NH_2 + O=CH–$CH_2CH_2SCH_3$ (16)

7 + Ox ⟶
R–NH–C$^{(+)}$(CO_2^-)$CH_2CH_2SCH_3$ + $Ox^{\bullet -}$ (17)

R–NH–C$^{(+)}$(CO_2^-)$CH_2CH_2SCH_3$ + H_2O ⟶
H^+ + R–NH_2 + O=C(CO_2^-)$CH_2CH_2SCH_3$ (18)

Instead, if formed, the potential intermediates **6** and **7** may dimerize with various carbon-centred radicals formed during the oxidation reactions (see below). At present we have not undertaken a detailed analysis of all potential dimerization products since these cannot be quantified due to the manifold of possible structures and the lack of authentic standards. A potential complication arises also from the fact that dimerization products can form between peptide and EDTA derived radicals of various types.

(*iv*) A quantitative material balance for ammonia formation has to take into account two distinct pathways. The formation of acetaldeyde *via* the free radical pathway of reactions (1)–(4) would yield radical **4** which may dimerize with other carbon-centred radicals or reduce ferric iron or H_2O_2. The latter redox processes should lead to the final elimination of ammonia [met = NH–CH(CO_2^-)$CH_2CH_2SCH_3$] [reactions (19) and (20)].

4 + Ox ⟶ H_2N–CH$^{(+)}$–CO–met + $Ox^{\bullet -}$ (19)

H_2N–CH$^{(+)}$–CO–met + H_2O ⟶
H^+ + NH_3 + O=CH–CO–met (20)

Some evidence for the formation of glyoxylylmethionine (O=CH–CO–met) in our experimental systems was obtained: HPLC analysis of a reaction mixture showed a product peak eluting before Thr-Met which was sensitive to reaction with 2,4-DNPH. Reversed-phase HPLC analysis (SGE C18 column; UV-detection at λ = 345 nm) of the reaction mixture which was incubated with 2,4-DNPH gave a new hydrazone peak. Analysis of this peak by negative FAB mass spectrometry (triethylamine matrix) revealed the presence of an ion with *m/e* = 385, *i.e.* a mass which would correspond to the molecular ion $[M]^-$ = 385 amu (rather than the more common $[M–H]^-$) of the 2,4-dinitrophenylhydrazone of O=CH–CO–met. Separately performed negative FAB mass spectrometric control experiments with synthetic 2,4-dinitrophenylhydrazones of acetaldehyde and butanal confirmed that indeed significant yields of $[M]^-$ were also obtained from these hydrazones, rendering some support for an assignment of *m/e* = 385 to $[M]^-$ of the 2,4-dinitrophenylhydrazone of O=CH–CO–met.

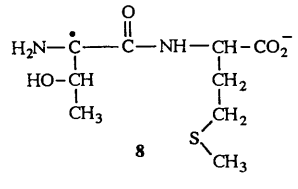

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Table 2 Oxidation of Thr-Met by free hydroxyl radicals and Fenton systems

Species	HO•[a], ref. 15 10^{-5} mol dm^{-3} min^{-1}	Fe^{II}/H_2O_2[b] 10^{-6} mol dm^{-3}	$[Fe^{II}(EDTA)]^{2-}/H_2O_2$[b] 10^{-6} mol dm^{-3}
-Thr-Met	2.1	232 ± 57	354 ± 57
Thr-Met(SO)	≤0.17	164 ± 15	66 ± 12
Thr-Met(SO_2)	n.d.	n.d.	<2[c]
CH_3CHO	1.2	6 ± 3	65 ± 5
NH_3	n.d.	19 ± 5	84 ± 5
Thr	n.d.	<2	<2
Thr-NH_2	n.d.	<2	<2
Methional	n.d.	<2	<2
Met	n.d.	<2	<2
CO_2	0.44	n.d.[d]	n.d.[d]
$(RSH)_{total}$	n.d.	<2	35 ± 5
$(CH_3S)_2$	n.d.	<2	<2
Material balance:[e]			
Σ_1	1.81 (86%)	189 (81%)	250 (71%)
Σ_2	—	183 (79%)	185 (52%)

[a] Yields are from γ-radiolysis; calculated on the basis of a dose rate of 32.96 Gy min^{-1} and G(HO•) = 6.0. [b] Reaction conditions: 2 × 10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, 5 × 10^{-4} mol dm^{-3} H_2O_2, 5 × 10^{-4} mol dm^{-3} Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$, 1 × 10^{-3} mol dm^{-3} Thr-Met, N_2. [c] NMR analysis of oxidized sample did not reveal any characteristic signal for the sulfone. [d] Not determined because of $NaHCO_3/Na_2CO_3$ as buffer medium. [e] For definition of Σ_1 and Σ_2 see text.

A second source of ammonia would be radical **8**. This species could initially form either *via* the deprotonation of a potential nitrogen radical cation intermediate **3** (Scheme 1) or *via* direct hydrogen abstraction from the Thr C_α–H bond of Thr-Met by **5**. Besides undergoing radical-radical recombination, radical **8** would possibly eliminate ammonia analogous to reactions (21) and (22) [R′ = $CH(OH)CH_3$].

$$\mathbf{8} + Ox \longrightarrow H_2N\text{–}CR'^{(+)}\text{–}CO\text{–}met + Ox^{\bullet -} \quad (21)$$

$$H_2N\text{–}CR'^{(+)}\text{–}CO\text{–}met + H_2O \longrightarrow H^+ + NH_3 + O{=}CR'\text{–}CO\text{–}met \quad (22)$$

As a consequence, ammonia and acetaldehyde may originate either both from one precursor **3** (elimination of CH_3CHO yields **4** which subsequently generates NH_3) (case 1) or independently from different precursors, *i.e.* acetaldehyde from **4** and ammonia from **8** (case 2). Therefore, we can only derive limiting product balances Σ for case 1 (Σ_1) and for case 2 (Σ_2). It is evident, however, that we can nevertheless account for the majority of primary products in all systems studied. The missing products in the $[Fe^{II}(EDTA)]^{2-}$-catalysed reactions are most probably accounted for by a manifold of potential symmetric and asymmetric radical–radical dimerization products from radicals **6**, **7** and **8**, respectively. Alternatively, dimerization may occur *via* symmetric and asymmetric disulfide formation, induced through ferric iron-mediated oxidation of thiol products. Free thiols were quantified by means of the Ellman-test (see earlier). The GC–MS analysis of oxidation reactions which had reacted for more than 30 minutes (*i.e.* way beyond the time necessary for the completion of the Fenton reaction, but time enough for some disulfide formation to take place) revealed the existence of some homocysteine-derived disulfides, indicated by a fragment with m/e = 278, characteristic for $[TMS\text{–}NH\text{–}CH(CO_2TMS)CH_2\text{–}CH_2S]^+$.[26,27] Control experiments confirmed that the latter was not formed during the mass spectrometric analysis of authentic Thr, Met and Thr-Met, respectively.

(*v*) As an additional product, particularly in the $[Fe^{II}(EDTA)]^{2-}$-catalysed reaction (*i.e.* a system which produces HO• radicals or HO•-like species; see below) we would also expect CO_2. In fact, the *C*-terminal decarboxylation of peptides containing *C*-terminal Met residues is one characteristic feature of the oxidation of such peptides by hydroxyl radicals.[12] For Thr-Met, *C*-terminal decarboxylation was found to occur with *ca.* 20–25% efficiency (Table 1).[12] We believe that *C*-terminal decarboxylation will also occur during the oxidation of Thr-Met with $[Fe^{II}(EDTA)]^{2-}/H_2O_2$. Quantification of CO_2 is, however, not possible in our systems due to the use of carbonate buffer and the competing oxidation of the EDTA ligand which yields CO_2 and formaldehyde (see earlier).[34]

Among the observed products, ammonia and acetaldehyde will be characteristic for the initial action of hydroxyl radicals[15] or hydroxyl radical-like species whereas sulfoxide formation will mostly reflect the initial action of a species different from hydroxyl radicals[35] (although we note that a hydroxy sulfuranyl radical may produce sulfoxide in the presence of Fe^{III}; see Discussion). The analysis of these three key products will be sufficient for the mechanistic evaluation of the occurrence of neighbouring group effects during the oxidation of the peptides. Below we shall describe the dependence of ammonia, acetaldehyde and sulfoxide on various parameters such as the peptide sequence, the concentrations of Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$, added Fe^{III} or $[Fe^{III}(EDTA)]^+$, EDTA and peptide, and pH.

C. Parameters influencing the product yields

1. The concentration of $[Fe^{II}(EDTA)]^{2-}$. Table 3 shows the respective yields of acetaldehyde, sulfoxide, and peptide consumption for Thr-Met, Thr-Leu and Ala-Met as a function of various concentrations of $[Fe^{II}(EDTA)]^{2-}$ under otherwise identical reaction conditions (N_2-saturated aqueous solutions, pH 7.5, 1.0 × 10^{-3} mol dm^{-3} peptide, 5.0 × 10^{-4} mol dm^{-3} H_2O_2, 2.0 × 10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$).

As expected from the primary sequence, no acetaldehyde is formed from Ala-Met. Within the concentration range (1.0–5.0) × 10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, the oxidation of Thr-Met affords significantly higher absolute yields of acetaldehyde as compared to the oxidation of Thr-Leu. However, the total consumption of Thr-Met is also significantly higher than that of Thr-Leu. For a mechanistic evaluation of our data it will be necessary to obtain estimates for the reactivity of the reactive species involved with the various functional groups of the peptides (see Discussion). An important observation is that acetaldehyde constitutes the major product (80%) of a very low (25 × 10^{-6} mol dm^{-3}) conversion of Thr-Leu at $[[Fe^{II}(EDTA)]^{2-}]$ = 3 × 10^{-4} mol dm^{-3} whereas it drops to a relative yield of only *ca.* 17% at $[[Fe^{II}(EDTA)]^{2-}]$ = 5 × 10^{-4} mol dm^{-3}. In contrast, the oxidation of Thr-Met shows constant

Table 3 Product yields as a function of the concentration of $[Fe^{II}(EDTA)]^{2-}$. Reaction conditions: 2×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, 5×10^{-4} mol dm^{-3} H_2O_2, 1×10^{-3} mol dm^{-3} peptide, N_2

Species/product	$[[Fe^{II}(EDTA)]^{2-}]$ 10^{-3} mol dm^{-3}	Yields (10^{-6} mol dm^{-3})		
		Thr-Met	Thr-Leu	Ala-Met
CH_3CHO	0	0.0	0.0	—
	0.1	26 ± 4	6.1 ± 2	—
	0.3	53 ± 6	20 ± 6	—
	0.5	65 ± 5	46 ± 5	—
	0.7	63 ± 5	42 ± 3	—
	1.0	51 ± 5	29 ± 3	—
Sulfoxide	0	0	—	0
	0.1	65 ± 3	—	30 ± 2
	0.3	68 ± 5	—	56 ± 7
	0.5	66 ± 12	—	30 ± 14
	0.7	47 ± 5	—	33 ± 1
	1.0	41 ± 1	—	33 ± 6
-Peptide	0	0	0	0
	0.1	167 ± 10	24 ± 1	310 ± 60
	0.3	358 ± 43	25 ± 4	424 ± 56
	0.5	354 ± 57	277 ± 22	290 ± 41
	0.7	345 ± 30	174 ± 37	310 ± 60
	1.0	266 ± 20	150 ± 50	300 ± 30

acetaldehyde yields of 16% ($[[Fe^{II}(EDTA)]^{2-}] = 1 \times 10^{-4}$ mol dm^{-3}), 15% ($[[Fe^{II}(EDTA)]^{2-}] = 3 \times 10^{-4}$ mol dm^{-3}) and 18% ($[[Fe^{II}(EDTA)]^{2-}] = 5 \times 10^{-4}$ mol dm^{-3}), respectively. This behaviour reflects the high intrinsic reactivity of the Met residue of Thr-Met towards the reactive species (see Discussion). Generally, an increase in the concentration of $[Fe^{II}(EDTA)]^{2-}$ between 0 and 5.0×10^{-4} mol dm^{-3} leads to an increase in the yields of both acetaldehyde and sulfoxide. A further increase of $[Fe^{II}(EDTA)]^{2-}$ to 1.0×10^{-3} mol dm^{-3} then results in a decrease of the peptide derived products, reflecting the competition between peptide oxidation and reaction (9).

2. The effect of pH. Table 4 displays the yields of acetaldehyde, sulfoxide and peptide consumption for Thr-Met, Thr-Leu and Ala-Met as a function of pH. At all pH values there are higher yields of acetaldehyde from Thr-Met than from Thr-Leu. This is most significant at pH 6.3 (reaction conditions: N_2-saturated aqueous solution, 2.0×10^{-3} mol dm^{-3} carbonate buffer, 1.0×10^{-3} mol dm^{-3} peptide, 5.0×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, 5.0×10^{-4} mol dm^{-3} H_2O_2). The sulfoxide yields, representatively examined for Thr-Met and Ala-Met, stay invariant over the pH region 6.3–9.1.

3. The effect of Thr-Met concentration. Table 5 displays the yields of two selected reaction products, Thr-Met(SO) and acetaldehyde obtained as a function of peptide concentration under otherwise similar conditions (N_2-saturated aqueous solution, 2.0×10^{-3} mol dm^{-3} carbonate buffer, pH 7.5, 5.0×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, 5.0×10^{-4} mol dm^{-3} H_2O_2). It is apparent that for 3.0×10^{-3} mol dm^{-3} Thr-Met the amount of consumed peptide approaches a value close to the concentration of applied hydrogen peroxide. The ratio of [acetaldehyde]/[Thr-Met(SO)] changes by a factor of *ca.* 2 in favour of the sulfoxide production over the employed range of peptide concentration. This finding has important mechanistic consequences (see Discussion).

4. The effect of combinations of $[Fe^{II}(EDTA)]^{2-}$ and $[Fe^{III}(EDTA)]^{-}$. Previously we had shown that the reaction of radiation chemically produced free hydroxyl radicals with aliphatic sulfides and Met-containing peptides only leads to negligible yields of sulfoxide in the absence of oxygen.[35,37] These sulfoxide yields could be enhanced *ca.* 2.8-fold by the presence of 2.0×10^{-4} mol dm^{-3} $[Fe^{III}(CN)_6]^{3-}$ (unpublished data) and 4.4-fold by the presence of 2.5×10^{-4} mol dm^{-3} O_2.[35] The higher yields in the presence of both oxidants were rationalized by their propensity to react with initially formed

Table 4 Effect of pH on the product yields. Reaction conditions: 2×10^{-3} mol dm^{-3} carbonate buffer, 5×10^{-4} mol dm^{-3} H_2O_2, 5×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, 1×10^{-3} mol dm^{-3} peptide, N_2

Species/product	pH	Yields (10^{-6} mol dm^{-3})		
		Thr-Met	Thr-Leu	Ala-Met
CH_3CHO	6.3	64 ± 5	24 ± 4	—
	7.5	65 ± 5	46 ± 5	—
	8.2	55 ± 4	38 ± 3	—
	9.1	61 ± 4	43 ± 5	—
Sulfoxide	6.3	62 ± 7	—	28 ± 9
	7.5	66 ± 12	—	30 ± 14
	8.2	58 ± 5	—	27 ± 8
	9.1	63 ± 6	—	36 ± 8
-Peptide	6.3	356 ± 10	128 ± 50	379 ± 70
	7.5	354 ± 57	277 ± 22	290 ± 41
	8.2	387 ± 40	110 ± 10	292 ± 50
	9.1	362 ± 40	114 ± 7	377 ± 50

hydroxy sulfuranyl type radicals according to reactions (23) and (24).[35,38]

$$HO\text{–}S^{\bullet}< + [Fe^{III}(CN)_6]^{3-} \longrightarrow H^+ + O{=}S< + [Fe^{II}(CN)_6]^{4-} \quad (23)$$

$$HO\text{–}S^{\bullet}< + O_2 \longrightarrow H^+ + O{=}S< + O_2^{\bullet-} \quad (24)$$

By analogy to our radiation chemical studies of Thr-Met,[15] the formation of acetaldehyde from Thr-Met suggests that sulfuranyl radical-like intermediates might also play a role in the $[Fe^{II}(EDTA)]^{2-}$-catalysed Fenton reactions.

In order to examine this possibility we performed the oxidation of Thr-Met by a standard oxidation system (N_2-saturated aqueous solution, pH 7.5, 2.0×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, 5×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, 1.0×10^{-3} mol dm^{-3} Thr-Met, 5.0×10^{-4} mol dm^{-3} H_2O_2) in the additional presence of either 5.0×10^{-4} mol dm^{-3} EDTA (system a) or 5.0×10^{-4} mol dm^{-3} $[Fe^{III}(EDTA)]^{-}$ (system b). From the results summarized in Table 6, it appears that the final yield of Thr-Met sulfoxide could be enhanced by the additional presence of $[Fe^{III}(EDTA)]^{-}$, consistent with the possibility of reaction (25) [we note the lower yields of sulfoxide in system a

$$XO\text{–}S^{\bullet}< + [Fe^{III}(EDTA)]^{-} \longrightarrow X^+ + >S{=}O + [Fe^{II}(EDTA)]^{2-} \quad (25)$$

Table 5 Effect of Thr-Met concentration on the yields of Thr-Met(SO) and acetaldehyde. Reaction conditions: 2×10^{-3} mol dm^{-3} carbonate buffer, pH 7.5, 5×10^{-4} mol dm^{-3} H_2O_2, 5×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$

[Thr-Met] 10^{-3} mol dm^{-3}	[Thr-Met(SO)] 10^{-6} mol dm^{-3}	[CH_3CHO] 10^{-6} mol dm^{-3}	[-Thr-Met] 10^{-6} mol dm^{-3}	[CH_3CHO]/ [Thr-Met(SO)]
0.5	41 ± 7	60 ± 5	185 ± 47	1.46
1.0	66 ± 12	65 ± 5	354 ± 57	0.98
1.5	80 ± 14	78 ± 6	410 ± 50	0.98
3.0	107 ± 15	84 ± 6	440 ± 50	0.79

Table 6 Effect of additional $[Fe^{III}(EDTA)]^-$ on the product yields. Reaction conditions: 2×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, pH 7.5, 5×10^{-4} mol dm^{-3} H_2O_2, 5×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$, 1×10^{-3} mol dm^{-3} Thr-Met, N_2

Species/product	System a: ($+5 \times 10^{-4}$ mol dm^{-3} EDTA)	System b: {$+5 \times 10^{-4}$ mol dm^{-3} $[Fe^{III}(EDTA)]^-$}
-Thr-Met	357 ± 50[a]	266 ± 40[a]
Thr-Met(SO)	40 ± 4	57 ± 5
CH_3CHO	50 ± 6	48 ± 1
NH_3	95 ± 14	81 ± 1

[a] Yields in 10^{-6} mol dm^{-3}.

Table 7 Comparison of the catalysis of $[Fe^{II}(EDTA)]^{2-}$ and $[Fe^{III}(EDTA)]^-$. Reaction conditions: 2×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, pH 7.5, 5×10^{-4} mol dm^{-3} H_2O_2, 1×10^{-3} mol dm^{-3} Thr-Met, N_2

Species/product	$[Fe^{II}(EDTA)]^{2-}$ 5×10^{-4} mol dm^{-3}	$[Fe^{III}(EDTA)]^-$ 5×10^{-4} mol dm^{-3}
-Thr-Met	354 ± 57[a]	160 ± 30[a]
Thr-Met(SO)	66 ± 12	0
CH_3CHO	65 ± 5	33 ± 5
NH_3	84 ± 5	119 ± 6
Methional	< 2	< 2

[a] Yields in 10^{-6} mol dm^{-3}.

(5.0×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$ + 5.0×10^{-4} mol dm^{-3} EDTA) as compared to the standard oxidation system (5.0×10^{-4} mol dm^{-3} $[Fe^{II}(EDTA)]^{2-}$) which is rationalized by the presence of excess EDTA which can compete for reactive oxygen species].

On the other hand, the results in Table 7 demonstrate that control reactions, performed with $[Fe^{III}(EDTA)]^-$ alone instead of $[Fe^{II}(EDTA)]^{2-}$, did not show any significant formation of Thr-Met sulfoxide. In the latter system significant yields of acetaldehyde and ammonia were generated which would account for either 75–95% of the consumption of Thr-Met (see limiting cases of material balances earlier). These overall differences between the $[Fe^{II}(EDTA)]^{2-}$ and $[Fe^{III}(EDTA)]^-$-catalysed oxidations should reflect the formation of different reactive intermediates in both systems.

5. Effect of peptide sequences. The influence of the relative location of Thr and Met in small model peptide sequences on the relative yields of acetaldehyde is demonstrated in Table 8.

Significant yields of acetaldehyde are particularly produced from Thr-Met in the presence of EDTA, and in negligible yields from Thr (although Thr oxidation generates high amounts of ammonia; data not shown). Thus, efficient acetaldehyde formation requires an *N*-terminal Thr residue and masking of the *C*-terminal carboxylate group of Thr, *e.g.* through peptide bond formation (compare also Thr-Leu with Thr).

Discussion

In order to evaluate if neighbouring group effects such as shown in reaction sequence 1–4 operate in the metal-catalysed oxidation of our model peptides by hydrogen peroxide, we have first to evaluate the potential nature of the primary oxidizing species for quantification of its reactivity with the various functional groups of the peptides.

Table 8 Formation of acetaldehyde as a function of peptide sequence. Reaction conditions: 2×10^{-3} mol dm^{-3} $NaHCO_3/Na_2CO_3$, pH 7.5, 5×10^{-4} mol dm^{-3} H_2O_2, 5×10^{-4} mol dm^{-3} Fe^{II} or $[Fe^{II}(EDTA)]^{2-}$, 1×10^{-3} mol dm^{-3} peptide, N_2

Peptide	Acetaldehyde, 10^{-6} mol dm^{-3}	
	Fe^{II}/H_2O_2	$[Fe^{II}(EDTA)]^{2-}/H_2O_2$
Thr-Met	6 ± 3	65 ± 5
Met-Thr	0	0
Gly-Thr-Met	0	0
Gly-Met-Thr	0	0
Met	0	0
Thr	3 ± 1	6 ± 2

The primary oxidizing species

Both forms of ferrous iron catalyse the hydrogen peroxide-induced oxidation of Met-containing peptides. However, product analysis suggests the operation of two different pathways. The results of the $[Fe^{II}(EDTA)]^{2-}$-catalysed pathway are consistent with the involvement of at least a fraction of either free HO$^\bullet$ or a HO$^\bullet$-like hypervalent iron-oxo species, *e.g.* **5** (see Tables 2 and 5 and below).

$$HO^\bullet + \text{2-propanol} \longrightarrow H_2O + (CH_3)_2C^\bullet OH \quad (26)$$

$$HO^\bullet + \text{Thr-Met} \longrightarrow \text{acetaldehyde} + \text{products} \quad (27)$$

$$HO^\bullet + [Fe^{II}(EDTA)]^{2-} \longrightarrow \text{products} \quad (28)$$

$$HO^\bullet + [Fe^{III}(EDTA)]^- \longrightarrow \text{products} \quad (29)$$

Table 1 displays the yields of seleceted products obtained as a function of the concentration of 2-propanol. The data demonstrate that 2-propanol can efficiently compete for the reactive species, Ox, which causes acetaldehyde formation in the $[Fe^{II}(EDTA)]^{2-}$-catalysed system. From competition kinetics we can derive an estimate‡ for the relative rate constants k_{ox} for the reactions of the reactive oxygen species Ox with Thr-Met ($k_{ox,TM}$) and 2-propanol ($k_{ox,P}$), respectively [eqn. (I)] ($k_{ox,Fe^{III}}$ = rate constant for the reaction of Ox with

$$[CH_3CHO]_o/[CH_3CHO] = 1 + (k_{ox,P}[\text{2-propanol}] + k_{ox,Fe^{II}}[[Fe^{II}(EDTA)]^{2-}] + k_{ox,Fe^{III}}[[Fe^{III}(EDTA)]^-])/(k_{ox,TM}[\text{Thr-Met}]) \quad (I)$$

‡ We excluded the data point for 3×10^{-3} mol dm^{-3} 2-propanol for eqn. (III) and the data points for (3–5) $\times 10^{-3}$ mol dm^{-3} 2-propanol for eqn. (IV) because the error limits do not allow a satisfactory calculation. Furthermore, for an estimation of the competition according to pseudo-first order kinetics we used a constant average concentration for Thr-Met of 0.9×10^{-3} mol dm^{-3} rather than 1.0×10^{-3} mol dm^{-3}, in order to account for some loss of Thr-Met during the reaction.

$[Fe^{III}(EDTA)]^-$; $k_{ox,Fe^{II}}$ = rate constant for the reaction of Ox with $[Fe^{II}(EDTA)]^{2-}$; yields of acetaldehyde in the absence, $[CH_3CHO]_o$, and in the presence, $[CH_3CHO]$, of 2-propanol).

Since at the start of the reaction $[Fe^{III}(EDTA)]^- = 0$ and at the end of the reaction $[Fe^{II}(EDTA)]^{2-} = 0$, we approximate an average rate constant of $k_{ox,Fe^{II/III}} = 0.5(k_{ox,Fe^{II}} + k_{ox,Fe^{III}})$, and derive eqn. (II).

$$[CH_3CHO]_o/[CH_3CHO] = 1 + (k_{ox,P}[2\text{-propanol}] + k_{ox,Fe^{II/III}}[Fe^n(EDTA)^{m-}]_{total})/(k_{ox,TM}[Thr\text{-}Met]) \quad (II)$$

Under the assumption that Ox = HO$^\bullet$, we obtain that $k_{ox,Fe^{II/III}} = k_{HO^\bullet,Fe^{II/III}} = 0.5(k_{28} + k_{29}) = 0.5$ (5.0×10^9 mol^{-1} dm^3 s^{-1} + 1.1×10^9 mol^{-1} dm^3 s^{-1}) = 3.05×10^9 mol^{-1} dm^3 s^{-1}.[39] If this assumption were valid then the data of Table 1, computed according to eqn. (III), should predict that $k_{ox,P}/k_{ox,TM} = k_{26}/k_{27}$.

$$[CH_3CHO]_o/[CH_3CHO] = 1.17 + (k_{ox,P}[2\text{-propanol}])/(k_{ox,TM}[Thr\text{-}Met]) \quad (III)$$

Experimentally, we obtain $k_{ox,P}/k_{ox,TM} = 0.14 \pm 0.02$ which is reasonably close to $k_{26}/k_{27} = 1.9 \times 10^9$ mol^{-1} dm^3 s^{-1}/ 9.8×10^9 mol^{-1} dm^3 s^{-1} = 0.19.[39] The value of $k_{27} = 9.8 \times 10^9$ mol^{-1} dm^3 s^{-1} was obtained as the sum of the rate constants for the reaction of HO$^\bullet$ with Thr, Met, *N*-terminal protonated Gly-Gly (representative for C_α-H), and *N*-terminal deprotonated Gly-Gly (representative for the deprotonated *N*-terminus; $k = 5 \times 10^9$ mol^{-1} dm^3 s^{-1} [39]), and assuming an average fraction of 15% of Thr-Met being present in the *N*-terminal deprotonated state at pH 7.5 (see below).

From the kinetic data we cannot exclude the participation of free HO$^\bullet$ in the oxidation of our peptides by the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$-system. Neither can we state that the oxidation is carried out exclusively by an iron-oxo species such as **5**. As will be shown below, fractions of both species may actually be involved in the oxidation process. In contrast, the Fe^{II}/H_2O_2-system does not appear to provide HO$^\bullet$ radicals for peptide oxidation (compare product distribution in Table 2). The latter finding is consistent with a recent report of Wink *et al.*, based on stopped-flow kinetic investigations of the oxidation of *N*-nitrosodimethylamine and $Ru(bpy)_3^{2+}$, respectively.[40]

The reactive sites of the peptides

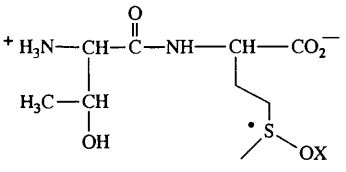
We could demonstrate (see above) that the relative reactivities of the oxidizing species with Thr-Met and 2-propanol, respectively, are close to the expected relative reactivities of the HO$^\bullet$ radical. Thus, we may also estimate the relative reactivities of the oxidizing species with different functional groups of the peptides on the basis of rate constants measured for HO$^\bullet$. The value for oxidation of Gly-Gly ($k = 2.4 \times 10^8$ mol^{-1} dm^3 s^{-1})[39] may serve as a general rate constant for hydrogen abstraction from The C_α-H bond. With k(HO$^\bullet$ + Thr) = 5.1×10^8 mol^{-1} dm^3 s^{-1}, k(HO$^\bullet$ + Leu) = 1.7×10^9 mol^{-1} dm^3 s^{-1} and k(HO$^\bullet$ + Met) = 8.3×10^9 mol^{-1} dm^3 s^{-1}, we obtain that HO$^\bullet$ or an equivalent iron-oxo species would attack Thr-Met with *ca.* 93% efficiency at Met and only *ca.* 6% efficiency at the Thr moiety at pH $\ll p K_{a,NH_2}$.[39] In contrast, 21% of HO$^\bullet$ would attack the Thr moiety of Thr-Leu (and 69% of the Leu side chain). However, at pH 6.3 the oxidation of both peptides, Thr-Met and Thr-Leu, gives similar efficiencies of acetaldehyde formation at $[[Fe^{II}(EDTA)]^{2-}] = 5 \times 10^{-4}$ mol dm^{-3}, *e.g.* 18% from Thr-Met and 17% from Thr-Leu, respectively (see Table 4). In particular at pH 6.3 we can exclude any significant direct attack of reactive oxygen species on the free *N*-terminal amino group of both peptides (compare, *e.g.*, pK_a = 8.25 for Leu-Gly[41]). The acetaldehyde yields from Thr-Leu might be rationalized by a direct attack of a reactive oxygen species on the Thr side chain (21% attack *vs.* 17% efficiency of acetaldehyde formation) but this does not apply to Thr-Met (6% *vs.* 18%, respectively). At more physiological conditions of pH 7.5 we expect that as much as 15% of the dipeptides are present in the *N*-terminal deprotonated state. With $k = 5.0 \times 10^9$ mol^{-1} dm^3 s^{-1} [39] as a reference value for the reaction of HO$^\bullet$ with a deprotonated peptide *N*-terminus, we calculate that at pH 7.5 *ca.* 85% of our reactive oxygen species would still directly add to the Met sulfur of Thr-Met whereas 5.2% would react with the Thr side chain, and *ca.* 7.6% would directly react with the deprotonated *N*-terminus. The latter reaction could yield a nitrogen-centred radical cation (such as **3**) which subsequently fragments into acetaldehyde and **4**. However, even at pH 7.5 we find an overall acetaldehyde formation with an efficiency of 18%, *i.e.* more than twice as high as the fraction of reactive oxygen species directly reacting with the deprotonated *N*-terminus. For Thr-Leu, we estimate that *ca.* 23% of the reactive oxygen species should react with the deprotonated *N*-terminus at pH 7.5, a value close to the experimentally observed efficiency of acetaldehyde formation of 19%.

The mechanism

We suggest that, in particular at pH 6.3, the formation of acetaldehyde from Thr-Met by the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ system may include a mechanism analogous to reactions (1)–(4). The difference between 7.6% direct attack of the reactive oxygen species on the *N*-terminus of Thr-Met and 18% efficiency of acetaldeyde formation would suggest that reactions (1)–(4) could also be of importance at pH 7.5. In accordance, we propose that neighbouring group interactions can play a role in the metal-catalysed oxidation of peptides. A hydroxy sulfuranyl radical (**1a**) can form with free HO$^\bullet$, generated, *e.g.* through reaction (30).

$$\mathbf{5a} \longrightarrow [Fe^{III}(EDTA)(HO^-)]^{2-} + HO^\bullet \quad (30)$$

Alternatively, we also suggest that sulfuranyl type radicals **1b** can form with hypervalent iron-oxo species, *e.g.* **5c**, and accordingly we have to include both intermediates **1a** and **1b** into our mechanistic scheme.



1a X = H
1b X = FeL_x

We note that, although potentially occurring, a mechanism like that shown in reactions (1)–(4) (starting from **1a** or **1b**, respectively) does not constitute a major pathway in the oxidation of our linear model peptides (showing an efficiency of 18%). However, this does not surprise us since such linear peptides can exist in a large number of possible conformations in solution, and not necessarily all of them may favour sulfur–nitrogen interaction. Moreover, competing reactions, such as the reaction of a sulfuranyl radical with ferric iron or hydrogen peroxide, may occur (see below) whereas such processes were avoided during the reaction of radiation chemically produced hydroxyl radicals with Thr-Met.[15] Further studies are now in progress with model peptides of defined secondary structure in order to characterize the influence of conformation on neighbouring group effects.

Additional pathways of acetaldehyde formation have to be taken into account: (*i*) the one-electron oxidation of a deprotonated *N*-terminus (to yield **3**) and (*ii*) a direct reaction

of a reactive oxygen species with the Thr moiety except at its *N*-terminus. The latter should be important for the Thr-Leu system at pH 6.3 and may include the formation of alkoxyl radicals which subsequently undergo α,β-fragmentation to yield **4** and CH_3CHO.

In the absence of EDTA we observe the predominant formation of Thr-Met sulfoxide. Here, we suggest that the metal merely catalyses oxygen transfer from hydrogen peroxide to the methionine sulfur and that no radical reactions are involved.

The intermediacy of sulfuranyl radicals

Further evidence for the intermediacy of sulfuranyl radicals during the oxidation of Thr-Met by the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ system is derived from the sulfoxide yields of the experimental systems a and b, presented in Table 6. The efficiency of sulfoxide formation of system a is 11% whereas that of system b amounts to 21%, in agreement with a potential reaction of a sulfuranyl radical **1a**/**1b** with excess $[Fe^{III}(EDTA)]^-$ according to reaction (25). This finding may provide some additional information as to whether free HO• radicals are involved in the overall oxidation process. If the sulfuranyl radical intermediate of Thr-Met oxidation were structure **1b** (*i.e.* containing an iron-oxo ligand) we would not expect that any excess ferric EDTA would efficiently further promote sulfoxide formation in a bimolecular process according to reaction (25) since sufficient iron of a redox state ≥3 would already be present within the intermediate **1b**. Thus any enhancement of sulfoxide formation by excess $[Fe^{III}(EDTA)]^-$ may reflect the intermediacy of a fraction of HO• and structure **1a**. On the other hand, the fact that much larger yields of sulfoxide are already formed by oxidation of Thr-Met through $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ in the absence of initially added excess $[Fe^{III}(EDTA)]^-$, as compared to free HO• (compare 18.6% *vs.* 6%; Table 2), may indicate that also significant amounts of **1b** participate in the oxidation process. A suggested combination of the involvement of both **1a** and **1b** in the formation of acetaldehyde from Thr-Met analogous to reactions (1)–(4) would be in agreement with spin trapping results which concluded that the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ system generates both free HO• and other HO•-like reactive intermediates, most probably **5**.[32,33] This suggestion derives further support from a recent study of Luzzatto *et al.*,[42] proposing that the $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ system produces significant yields of free HO•, although data were only collected for pH < 5.5.

A fine line needs to be drawn between the experiments performed in the initial absence and presence of added $[Fe^{III}(EDTA)]^-$. The initial presence of $[Fe^{III}(EDTA)]^-$ promotes the conversion of hydroxy sulfuranyl radicals into sulfoxide [reaction (25)]. In the absence of $[Fe^{III}(EDTA)]^-$, these hydroxy sulfuranyl radicals would predominantly yield products different from sulfoxide, and sulfoxide would, therefore, originate predominantly from reactive oxygen species different from hydroxyl radicals. Support for these mechanistic details comes from competition kinetics comparing the sulfoxide yields in the absence and presence of various concentrations of 2-propanol, displayed in Table 1. Computation of the sulfoxide yields according to eqn. (IV), where

$$[\text{Thr-Met(SO)}]_o/[\text{Thr-Met(SO)}] = 1.17 + (k_{ox.P}[\text{2-propanol}])/(k_{ox.TM}[\text{Thr-Met}]) \quad \text{(IV)}$$

$[\text{Thr-Met(SO)}]_o$ and [Thr-Met(SO)] refer to the sulfoxide yields in the absence and presence of 2-propanol, respectively, yields $k_{ox.P}/k_{ox.TM} = 0.022 \pm 0.002$ for [2-propanol] ≥ 25×10^{-3} mol dm^{-3}. This value is different by a factor of 8.6 from $k_{26}/k_{27} = 0.19$, clearly indicating that the species producing sulfoxide is not the hydroxyl radical.

A second line of evidence for the intermediacy of sulfuranyl radicals can be extraced from Table 5. It appears that the ratio of [acetaldehyde]/[Thr-Met(SO)] decreases with increasing peptide concentration. An increase of Thr-Met concentration between 0.5 and 2.0×10^{-3} mol dm^{-3} promotes the displacement of hydroxide from hydroxy sulfuranyl radicals according to reaction (31), as characterized by pulse radiolysis of Thr-Met.[15]

$$\text{HO–S}^{\bullet}< + \text{S}< \longrightarrow \text{HO}^- + [>\text{S}.\therefore\text{S}<]^+ \quad \text{(31)}$$

Parallel to the increase of the three-electron-bonded dimeric sulfur radical cation $[>S.\therefore S<]^+$ there was a concomitant decrease of the acetaldehyde yields since acetaldehyde formation requires the interaction of the peptide *N*-terminus with the hydroxy sulfuranyl radical.[15] The decrease of the [acetaldehyde]/[Thr-Met(SO)] ratio with increasing peptide concentration can be rationalized by the decomposition of a sulfuranyl radical **1a** or **1b** analogous to reaction (31), and would, therefore, be in accord with the intermediacy of a sulfuranyl radical. This also means that any oxidation of the thioether moiety by $[Fe^{II}(EDTA)]^{2-}/H_2O_2$ *via* one-electron transfer, generating a sulfur radical cation $>S^{\bullet+}$, should yield significantly less acetaldehyde.

We have to comment on the unusually high efficiency of acetaldehyde formation from Thr-Leu under conditions of excess H_2O_2, *i.e.* at $[[Fe^{II}(EDTA)]^{2-}] = 3 \times 10^{-4}$ mol dm^{-3}, as compared to $[[Fe^{II}(EDTA)]^{2-}] = 5 \times 10^{-4}$ mol dm^{-3}. At $[[Fe^{II}(EDTA)]^{2-}] = 3 \times 10^{-4}$ mol dm^{-3}, only 3×10^{-4} mol dm^{-3} H_2O_2 are necessary for a complete formation of **5a** [reaction (5)]. Subsequently, **5a** (or its products **5b** and **5c**) can either oxidize Thr-Leu or react with a second equivalent of H_2O_2 [reaction (8)]. It has been argued that $k_8 \geqslant k_5$.[30] Since $k_{32} > k_{33}$ (see above[39]) we expect that in the Thr-Leu containing system a larger fraction of **5a** would react *via* reaction (8) than in the Thr-Met containing system.

$$\mathbf{5} + \text{Thr-Met} \longrightarrow \text{products} \quad \text{(32)}$$

$$\mathbf{5} + \text{Thr-Leu} \longrightarrow \text{products} \quad \text{(33)}$$

$$2\,O_2^{\bullet-} + 2\,H^+ \longrightarrow H_2O_2 + O_2 \quad \text{(34)}$$

Reaction (8) yields $[Fe^{III}(EDTA)]^-$ and superoxide. Superoxide would dismutate according to reaction (34), rather than attack peptide, to produce additional H_2O_2 which would then be available for $[Fe^{III}(EDTA)]^-$-catalysed oxidation of the peptide. In Table 7 we have shown that even with Thr-Met such a process would yield acetaldehyde but no sulfoxide. Thus, efficient acetaldehyde formation for Thr-Leu is expected under such conditions. At $[[Fe^{II}(EDTA)]^{2-}] = 5 \times 10^{-4}$ mol dm^{-3}, there is no excess of H_2O_2 over $[Fe^{II}(EDTA)]^{2-}$, and reaction (8) should largely be suppressed. Consequently, at $[[Fe^{II}(EDTA)]^{2-}] = 5 \times 10^{-4}$ mol dm^{-3}, we find that the Thr-Met and Thr-Leu systems behave similarly with respect to overall peptide oxidation and absolute acetaldehyde yields.

Significance for the investigation of metal-catalysed oxidation of polypeptides and proteins

Our results demonstrate that the metal-catalysed oxidation of small Thr- and Met-containing model peptides proceeds *via* different pathways depending on the relative locations of the amino acids (neighbouring groups) in the peptide sequence, and the presence of EDTA. This implies, that the oxidation of a complete protein, whether site-specific or not, may well be subject to such neighbouring group interactions. The relative extent of the oxidation of a specific amino acid within a particular protein will not necessarily only depend on its relative reactivity towards an oxidant, but also on the possibility of radical transformations involving other functional groups closely located around the initial target. Which type of

neighbouring group effect one could expect will largely depend on the nature of an initial reactive intermediate formed at a target amino acid, and its environment. Clearly, the reactions presented here, involving a methionine sulfur and an *N*-terminal Thr, are only one model system for one particular reaction. Nevertheless, the concept of neighbouring group participation during protein oxidation adds an additional factor to the parameters controlling protein oxidation among which so far mostly surface accessibility or metal-binding have been considered.

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