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Tandem mass spectrometry and infrared spectroscopy as a help to identify peptide oxidized residues

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The final products obtained by oxidation of small model peptides containing the thioether function, either methionine or S-methyl cysteine, have been characterized by tandem mass spectrometry and IR Multiple Photon Dissociation (IRMPD) spectroscopy. The modified positions have been clearly identified by the CID-MS^2 fragmentation mass spectra with or without loss of sulfenic acid, as well as by the vibrational signature of the sulfoxide bond at around 1000 cm^{-1}. The oxidation of the thioether function did not lead to the same products in these model peptides. The sulfoxide and sulfone (to a lesser extent) have been clearly identified as final products of oxidation of S-Methyl-glutathione (GS-Me). Decarboxylation or hydrogen loss are major oxidation pathways in GS-Me, while they have not been observed in tryptophan-methionine and methionine-tryptophan (Trp-Met and Met-Trp). Interestingly, tryptophan is oxidized in the dipeptide Met-Trp, while that is not the case in the reverse sequence (Trp-Met).

1. Introduction

Oxidized proteins are often toxic if not destroyed or repaired[1]. The deleterious roles of oxidized proteins in various diseases such as neurodegenerative, auto-immune etc. ones, is known. Only the sulphur containing residues can be repaired after oxidation. Methionine oxidation can be reverted by enzymes that are devoted to the reduction of the well-known methionine sulfoxide (methionine sulfoxide reductases), which is linked to the fact that methionine sulfoxide modulates several functions in cells[2]. However if the oxidized form is not the sulfoxide, this residue cannot be repaired anymore. Surprisingly while the nature of the transients formed after one-electron oxidation of the thioether containing residues have been extensively studied (see [3] and references therein) the final products have not yet been characterized.

Nowadays the most powerful method of protein analysis is mass spectrometry (MS)[4,5]. If one wants to identify the modified residue(s), the method has to be combined with chromatography, enzymatic digestion etc. Thus the search for new oxidized forms is much easier with model peptides, for which analytical operations are simpler. For instance there is no need of subsequent biochemistry such as enzymatic digestion before mass spectrometry.

We are currently studying the oxidation of model peptides going from 2 to 5 residues and containing a thioether function, either methionine or S-Methyl-cysteine. The one-electron oxidation of such peptides has been studied for many years by pulse radiolysis, to identify the nature of the transients (see for example [3] and references therein).

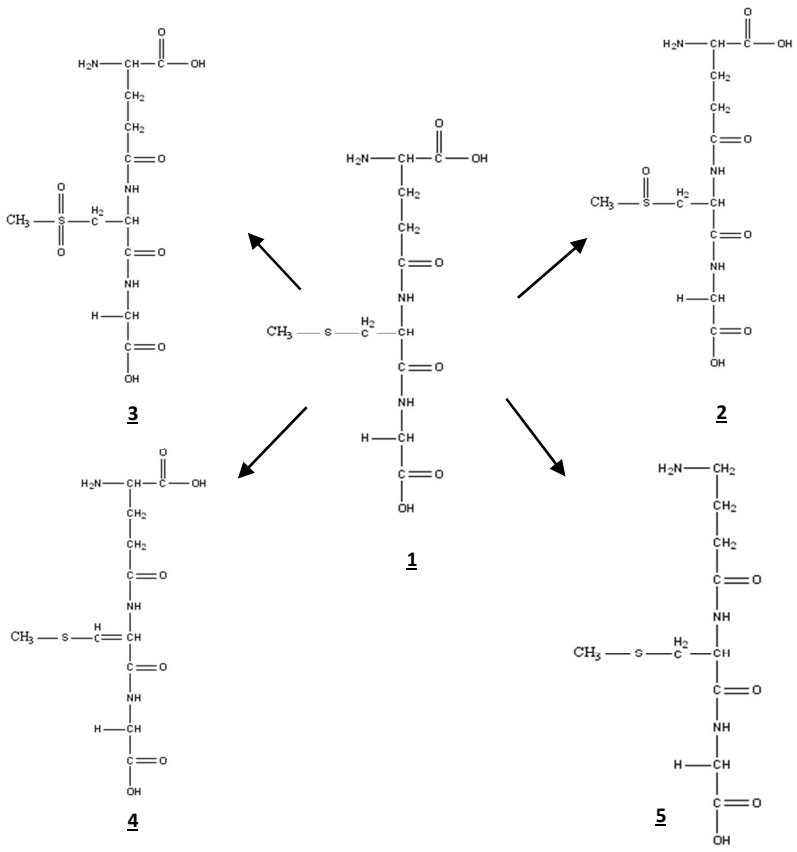
Our aim is to identify the final products of oxidation, still unknown, to study the relation between the sequence of peptides, the transients formed and the nature of final products and to find vibrational signatures which can become valuable tools in the structural characterization for larger peptides. The neighbouring group effects on the sulphur atom are known to be important for the transients but nothing is known about such effects on the final products. This aspect will be investigated as well.

InfraRed Multiple Photon Dissociation (IRMPD) spectroscopy combined with tandem mass spectrometry allowed us to provide a signature for the sulfoxide function in the gas phase using the band in the 1000 cm^{-1} energy region [6]. In methionine-containing dipeptides we have identified oxidized forms other than sulfoxide that appear after oxidation of some peptides only [7].

In this paper we focus on the oxidation of three peptides, S-Methyl-glutathione (**1** in Scheme 1), tryptophan-methionine

and methionine-tryptophan (Trp-Met and Met-Trp, Scheme 2).

S-Methyl-glutathione (GS-Me), a derivative of glutathione in which the cysteine (Cys) residue is replaced by a S-Methyl Cys, acts as an anti-oxidant by redox processes.



Scheme 1: Structure of GS-Me (**1**) and of its main oxidized forms (**2** to **5**).

It can be formed *in vivo* via conjugation by e.g. methyl halides such as methyl iodide even if its origin is still not understood [8]. This compound is also a model for antioxidants under assay. It also activates calcium-sensing receptor that modulates gene expression in human cells[9].

As for the relation between the nature of transients and the final compound, GS-Me is a tripeptide with a potentially large scope for neighboring group participation. There are many different functional groups within the same molecules, and in particular the sulfur-containing side chain is an interior residue. It represents a good model system for studying N-terminal decarboxylation induced through the initial reaction of $^{\bullet}$OH at the central S-Methyl-Cys residue. GS-Me can be employed as model for protein molecules where higher–order structures may bring functional groups into close proximity of the thioether one.

The sequences Met-Trp and Trp-Met are models of the active site of the TAT-RasGAP317–326 decapeptide (WMW) that has an antitumor activity through sensitizing cells to apoptosis[10, 11]. Any modification in this sequence would lead to tumour growth.

The one-electron oxidation of GS-Me has been studied by pulse radiolysis and flash photolysis, thus the first steps are known [12, 13]. Several free radicals are formed with various 2-centre 3-electron bonds between the sulphur radical cation and a nitrogen atom of the molecule, or the sulphur atom of another molecule of GS-Me. The mechanisms of oxidation of Met-Trp and Trp-Met have also been studied by pulse radiolysis [14, 15]. Few data indicate Intramolecular Electron Transfer (IET) between both residues in peptides. In Met-Trp the IET between $Met^{\bullet+}$ and Trp would proceed with a rate constant of ca. 10^7 s^{-1} [15]. Thermodynamic data [16, 17] suggest that one should not expect any oxidation product from methionine through the one-electron oxidation pathway. The final products were not identified for these three peptides. In this work we focus on them for a better understanding of the thioether function one-electron oxidation. All peptides were oxidized by gamma radiolysis in N_2O atmosphere with catalase and were analysed by IRMPD spectroscopy, which has been shown to be a useful tool for identification of modification in peptide residues in the gas phase [18,19,20,21].

2. Materials and methods

2.1 Materials

GS-Me, Met-Trp, Trp-Met and Trp were purchased from Sigma Aldrich and used without purification. All the solvents were of analytical grade. 1mM sample solutions were diluted in 50:50 water:methanol for recording the mass spectra to a final concentration of 100 µM.

2.2 Methods

2.2.1 Gamma radiolysis

γ-irradiations were carried out using the panoramic ^{60}Co γ-source IL60PL Cis-Bio International in the University Paris-Sud (Orsay, France). The dose rate was determined by Fricke dosimetry and kept constant at 30 Gy min^{-1}. Samples were purged gently under stirring while bubbling with N_2O for approximately 30 min before irradiation. Catalase (1µM) was present in the samples for irradiation, to remove hydrogen peroxide. Water was purified using a Millipore or Elga maxima system (resistivity 18.2 MΩ.cm).

Radical production and radiolysis. The well-known method of scavengers [22] allows a quantitative production of free radicals by stationary γ-radiolysis according to the following reactions. The chosen oxidant species was the $^{\bullet}$OH radical produced by radiolysis of N_2O-saturated aqueous solutions:

$H_2O \rightarrow {}^{\bullet}OH, H^{\bullet}, e^-_{aq}, H_2, H_2O_2, H^+, OH^-$ (1)

$e^-_{aq} + N_2O + H_2O \rightarrow {}^{\bullet}OH + OH^- + N_2$ (2)

Thus in N_2O-saturated aqueous solutions, radiolysis creates $^{\bullet}OH$ radicals with a radiation chemical yield (G) equal to 0.55 µmol J^{-1} (equations 1 and 2). H atoms are also created in much lower yield (0.05 µmol J^{-1}), which leads to desulfuration of Met.

2.2.2 MS operations

Ions were generated by electrospray ionisation (ESI) and the important ESI conditions were: a flow rate of 150 µL/h, a dry gas flow of 8 L/min, a nebulizer pressure of 1.5 bar, a spray voltage of -4500 V, and a drying gas temperature of 200 °C. Mass spectra have been recorded using a commercial 7 T FT-ICR mass spectrometer (Bruker, Apex Qe) [23]. After electrospray ionization of a solution of the protonated samples, ions are guided through a capillary and an ion funnel into a first hexapole ion trap, where they are collected for about 20 ms. One or multiple bunches of ions are then pulse-extracted into a 20 cm long RF quadrupole ion guide, where mass selection can be performed by resonant RF ejection of other species present. Ions are then collected in a 5 cm long hexapole cell, where they are collisionally cooled down using a flow of high-purity argon. A typical collection time for the current experiment in the hexapole collision cell varies from 500 ms to 1 s. Ions are then pulse-extracted towards the ICR cell, where they are irradiated by IR light for a fixed time period (typically 1s). For each wavelength, the mass spectrum is the Fourier transform of a time-domain transient averaged five times. The 63 mm long infinity ICR cell is placed inside the 155 mm diameter bore of a 7 T superconducting magnet (Magnex), and each trapping plate has a 6 mm diameter hole. Before entering the cell, the ions are decelerated by two ring electrodes of which the one closest to the trapping electrodes is segmented into two half-electrodes, allowing for a more efficient trapping by transferring longitudinal beam energy into transversal beam energy, the so-called "side-kick", or for a correction of transversal beam energies. Some 50 mm beyond the second trapping electrode, a hollow cathode is placed to be used for ECD experiments. A 3.45 mm diameter aperture in the cathode allows for the IR laser beam to enter the cell. The IR light then traverses the ICR cell along the axis of the bore of the magnet in a single pass configuration. The IR laser beam is focused using a 2 m focal mirror and has a nearly constant diameter throughout the cell region. IR spectroscopy in 800-2000 cm^{-1} wavenumber range was performed using the CLIO (Centre Laser IR d'Orsay) Free Electron Laser (FEL), which produces pulsed, tunable IR light covering the 100-2500 cm^{-1} wavenumber range. The CLIO IR-FEL is based on an electron linear accelerator [24], and an electron energy from 42 to 44.5 MeV have been used to perform our experiments. Typical average powers were about 1.5 W around 1000 cm^{-1} and 0.8 W near 2000 cm^{-1}. The IR-FEL output consists of 8 µs long macropulses fired at a repetition rate of 25 Hz, and each macropulse contains about 500 micropulses, each a few picoseconds long. The IR-induced ion fragmentation process has been efficiently assisted using an auxiliary CO_2 laser [25]. To record the CID-MS^2 fragmentation mass spectra, ions were first mass selected in the quadrupole and subjected to a typical collisional voltage of - 6V in the hexapole, acting as collisional cell. Fragments have been detected in the ICR cell. CID-MS^3 fragmentation mass spectra have been obtained by mass selecting one of the fragments present in the CID-MS^2 mass spectrum in the ICR cell and by introducing an inert gas in it.

2.2.3 IRMPD spectroscopy

IR multiple photon dissociation (IRMPD) is a multi-step resonant absorption process that is assumed to rely strongly on intramolecular vibrational energy redistribution (IVR) [26]. After several absorption steps, the ion has sufficient internal energy to dissociate. As a result, the IRMPD process, like multiple collision induced dissociation (CID), is assumed to be a slow heating process, and the fragment ions obtained by the two methods are the same. The IRMPD spectra in the 800-2000 cm^{-1} energy range have been obtained by monitoring the abundance of parent and fragment ions. If F is the sum of the abundances of the fragment ions produced by IRMPD and P the one of the parent ion, our IRMPD spectra correspond to the plot of –ln (P/(F+P)) as a function of the IR wavenumber.

3. Results and discussion

3.1 S-Methyl-glutathione

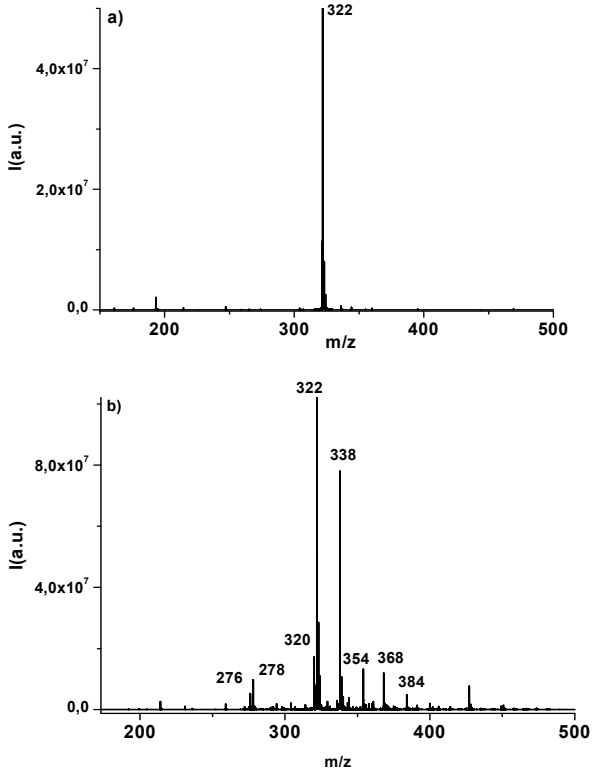


Figure 1: Mass spectrum of non-irradiated **(a)** and irradiated S-Methyl-glutathione **(b)** (irradiation dose 900 Gy).

Table 1. Most intense peaks observed in the mass spectrum of irradiated S-Methyl glutathione.

m/z, z=+1	Compound
384	$(GS\text{-}Me)H^+(O)_4\text{-}H_2$
368	$(GS\text{-}Me)H^+(O)_3\text{-}H_2$
354	$(GS\text{-}Me)H^+(O)_2$
338	$(GS\text{-}Me)H^+(O)$
322	$(GS\text{-}Me)H^+$
320	$(GS\text{-}Me)H^+\text{-}H_2$
278	$(GS\text{-}Me)H^+\text{-}CO_2$
276	$(GS\text{-}Me)H^+\text{-}H_2\text{-}CO_2$

The mass spectra of the non-irradiated and irradiated peptide in N_2O atmosphere with a 900 Gy irradiation dose are shown in Figure 1.

The most intense peaks observed in the mass spectrum of the irradiated peptide are gathered in Table 1 with their attributions.

The mass spectrum of the non-irradiated peptide shows almost only one peak at *m/z* 322, i.e. the mass of the protonated peptide.

The oxidation results mostly in addition of oxygen atoms (from one to four), loss of H_2 and decarboxylation. To characterize the final oxidation species, IRMPD spectroscopy and collision induced dissociation ($CID\text{-}MS^2$) of mass selected products have been performed. The $CID\text{-}MS^2$ fragmentation mass spectra are shown in the supplementary material (Figure 1SI to 5SI).

The identification of the main fragments for each oxidation product has been summarized in Table 1SI. The presence of the fragment in the $CID\text{-}MS^2$ fragmentation mass spectra corresponding to the loss of ammonia suggests that protonation of GS-Me occurs on the N-terminal amino group, as expected[27].

The IRMPD spectra of the main compounds were recorded in the 800-2000 cm^{-1} energy range and are reported in Figure 2.

The IRMPD spectrum of protonated GS-Me (*m/z* 322), reported in Figure 2a, shows several features at 1152, 1401, 1505, 1589 1664 and 1783 cm^{-1}. This spectrum is very similar to that already recorded by Gregori at al[27] for the protonated glutathione (GS), as expected because in GS-Me the cysteine is replaced by S-Methyl-cysteine.

By comparing the spectrum of protonated GS and GS-Me we can attribute the band at 1152 cm^{-1} to the bending of the C-OH groups of the Gly and Glu residues. The band at 1505 cm^{-1} may be attributed to the NH_3^+ umbrella bending mode and the Cys and Gly amide II modes.

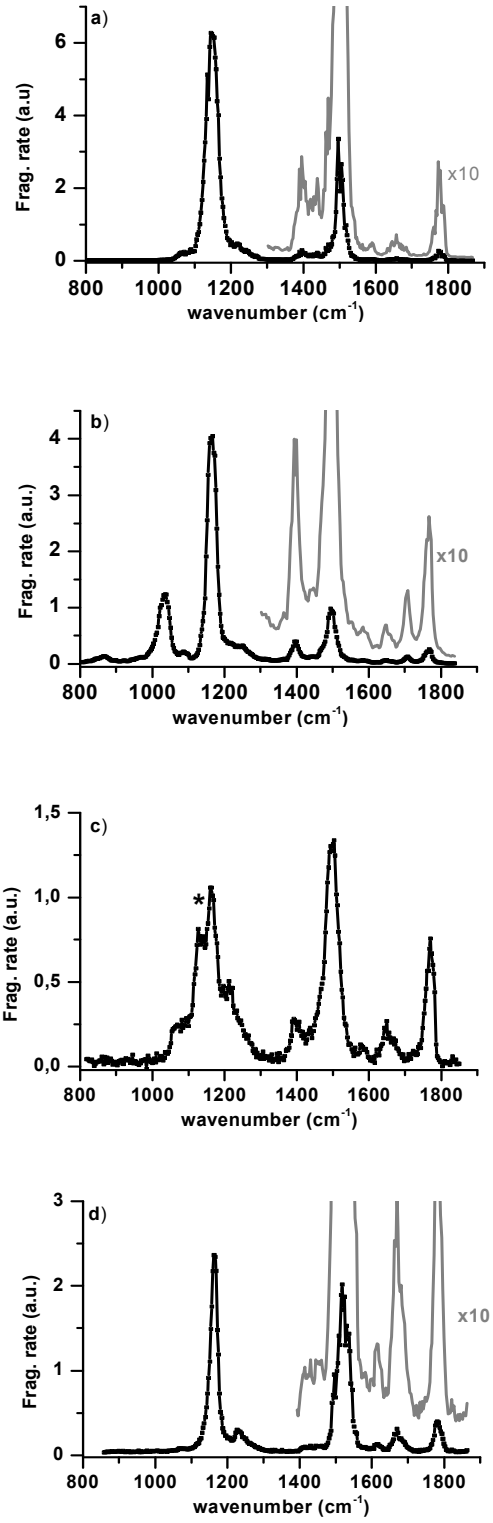


Figure 2: Experimental IRMPD spectra of **(a)** (GS-Me)H^+ **(b)** (GS-Me)OH^+, **(c)** (GS-Me)O_2H^+ and **(d)**, (GS-Me)H^+-CO_2 in the 800-2000 cm^{-1} energy range. The grey profiles in panels **(a)**, **(b)** and **(d)** report expanded spectra.

The small bands observed at 1589 and 1664 cm^{-1}, respectively, may be attributed to the NH bending mode and the Gly and Glu amide I vibrational modes, while the one at 1783 cm^{-1} may be attributed to the stretching modes of the C=O group of Gly and Glu.

The IRMPD spectrum of (GS-Me)OH^+ (*m/z* 338) is reported in Figure 2b. It exhibits a supplementary band at 1038 cm^{-1}, characteristic of the sulfoxide group, as already observed by the authors [6]. Hence it is attributed to **2** (Scheme 1). This band is not observed in the spectrum of the non-irradiated peptide and confirms that the oxygen is added to the sulphur atom.

As can be also inferred from Table 1SI, (GS-Me)OH^+ shows fragments at *m/z* 274 (which corresponds to the loss of sulfenic acid) and at *m/z* 209 (which corresponds to the addition of an oxygen atom on the sulphur of the y2 fragment) pointing out the formation of a sulfoxide. The Roepstorff-Fohlman nomenclature has been used to describe the fragments of GS-Me (Scheme 1 in SI). In the case of GS-Me the γ-Glu residue is the N-terminus of the peptide and the Gly residue is the C-terminus of the peptide. The y2 ions appear to extend from the C-terminus.

The IRMPD spectrum of (GS-Me)O_2H^+, reported in Figure 2c, does not have any band around 1000 cm^{-1}, indicating that this compound has no sulfoxide. The IRMPD spectrum of the commercial sulfone of methionine has already been recorded by the authors [7]. A sulfone has a characteristic band around 1108 cm^{-1}, corresponding to the stretching mode of the SO_2 group. By comparing the IRMPD spectra of (GS-Me)H^+ with the products (GS-Me)OH^+(O) and (GS-Me)O_2H^+, we can observe that only the IRMPD spectrum of the (GS-Me)O_2 H^+ shows a supplementary band at 1127 cm^{-1} (marked by an asterisk in spectrum 2c), that can be attributed to the sulfone[7] vibrational mode.

In conclusion the use of both CID-MS^2 fragmentation mass spectrum (Table 1SI) and IRMPD shows unambiguously that both oxygen and sulphur atoms are in the y2 fragment and that a sulfone is formed. (**3**, scheme 1).

For the first time the sulfone derivative has been clearly observed as a final product of oxidation by gamma radiolysis in the absence of oxygen. The biological role of sulfone is not yet understood. Better characterization might help to understand it.

The CID-MS^2 spectrum of (GS-Me)H^+-H_2 (*m/z* 320) exhibits a y2 fragment lacking 2H (Table 1SI), which shows that the loss of H_2 happened in the S-Me Cys residue. Hence we propose to assign it to **4** (scheme 1). The intensity of the signal was not enough to record its IRMPD spectrum.

The IRMPD spectrum of (GS-Me)H^+-CO_2 (*m/z* 278) is shown in Figure 2d. As may be inferred from Table 1, this peak corresponds to the loss of 44Da from the GS-Me and has been attributed to the decarboxylated tripeptide.

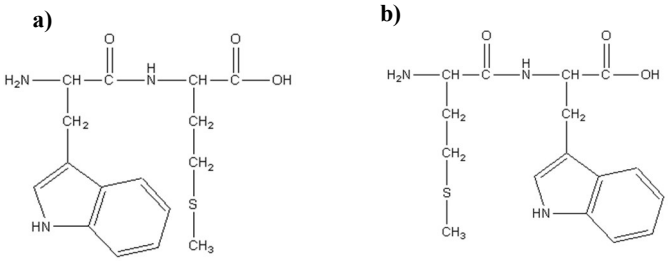
The C=O stretches of the carboxylate of Gly and Glu in glutathione are predicted at 1791 and 1807 cm^{-1}, respectively, according Gregori at alt.[27]. Unfortunately, these bands, as already observed for protonated GS, are not separated in our experimental conditions and we cannot really conclude from the comparison of the two spectra, as we previously did in the case of Lys-Met[7]. A mechanism leading to the decarboxylation of this residue, based on the identification of the transients in the oxidation of GS-Me by pulse radiolysis and flash photolysis, has been proposed [12, 13].

The CID-MS^2 spectrum helped in this case to localize the decarboxylation site (Table 1SI) and to shed light on the mechanism of formation of the final product of decarboxylation. The peak corresponding to the intact y2 is present in the CID-MS^2 fragmentation mass spectrum of (GS-Me)H^+-CO_2. This is in favour of a decarboxylation of the γ Glu residue (**5**, scheme 1).

Finally, products corresponding to additions of 3 and 4 oxygen atoms have also been observed (Table 1). They might correspond to the hydroxylation of the double bond of **4**. The intensity of the signals in the mass spectrum was not enough to record their IRMPD spectra.

3.2 Trp-Met and Met-Trp

The mass spectra of non-irradiated and irradiated Trp-Met and Met-Trp peptides are reported in Figures 6SI and 7SI, respectively. The most intense peaks observed in the mass spectra of the irradiated dipeptides are gathered in Table 2 with their attributions.



Scheme 2: Structures of dipeptides Trp-Met **(a)** and Met-Trp **(b)**.

The main peak observed in the mass spectrum of a non-irradiated sample of Trp-Met corresponds to the protonated dipeptide (*m/z* 336). The other peak at *m/z* 319 may be attributed to the decomposed peptide (loss of NH_3) or to impurities. The only oxidation product after irradiation at 800 Gy for the dipeptide Trp-Met has been observed at *m/z* 352. It corresponds to the addition of an oxygen atom.

Table 2. Most intense peaks observed in the mass spectra of irradiated Trp-Met and Met-Trp.

m/z, z=+1	Compound
352	$(Trp\text{-}Met)OH^+$
336	$(Trp\text{-}Met)H^+$
319	$(Trp\text{-}Met)H^+\text{-}NH_3$
392	Impurity
368	$(Met\text{-}Trp)O_2H^+$
364	impurity
352	$(Met\text{-}Trp)OH^+$
336	$(Met\text{-}Trp)H^+$

The IRMPD spectrum of $(Trp\text{-}Met)OH^+$ (*m/z* 352) has been recorded. It is reported in figure 3a and compared with that of $(Trp\text{-}Met)H^+$ (*m/z* 336). The most intense peaks observed in the CID-MS^2 fragmentation mass spectrum (reported in Figures 8-9SI) of $(Trp\text{-}Met)OH^+$ are gathered in Table 2SI with their attribution. The presence of the peak at *m/z* 335, corresponding to the loss of ammonia confirms again the protonation on the NH_2 group of the N-terminal amino acid. The product observed at m/z 352 corresponds to the formation of a sulfoxide, as may be confirmed by the presence of a band at 1028 cm^{-1} in its IRMPD spectrum. Again, this band is not present in the spectrum of the non-irradiated peptide and it is characteristic of the S=O vibrational signature. The other features observed in the IRMPD spectra are quite similar.

A fragment at *m/z* 288 in the CID-MS^2 fragmentation mass spectrum of oxidized irradiated dipeptide has been observed corresponding to the loss of sulfenic acid.

Both sets of data point out the oxidation of methionine to its sulfoxide. Interestingly, the oxidation of tryptophan did not yield a detectable product.

Noteworthy, there are much more peaks in the MS spectrum of Met-Trp irradiated with 900 Gy (Figure 7SI). Their masses correspond to additions of one or two oxygen atoms. An assignment of these products is reported in Table 3SI. They are depicted in scheme 3 (**7** to **10**).

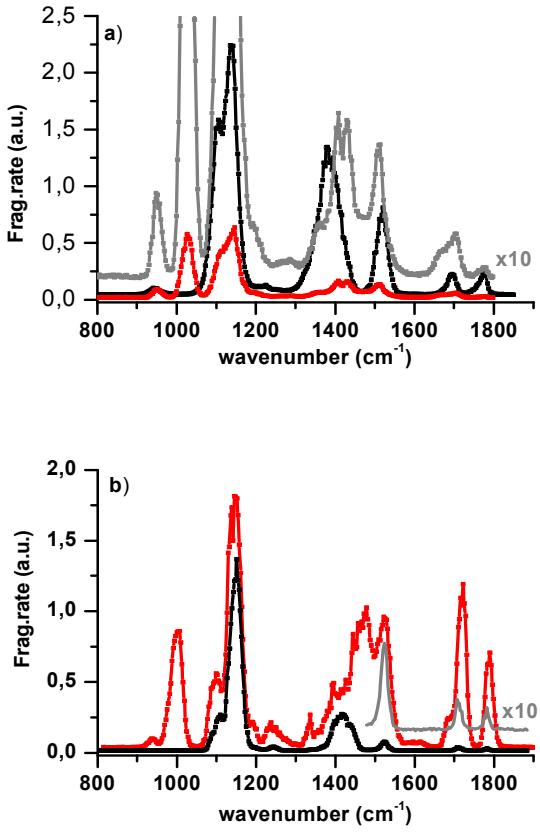
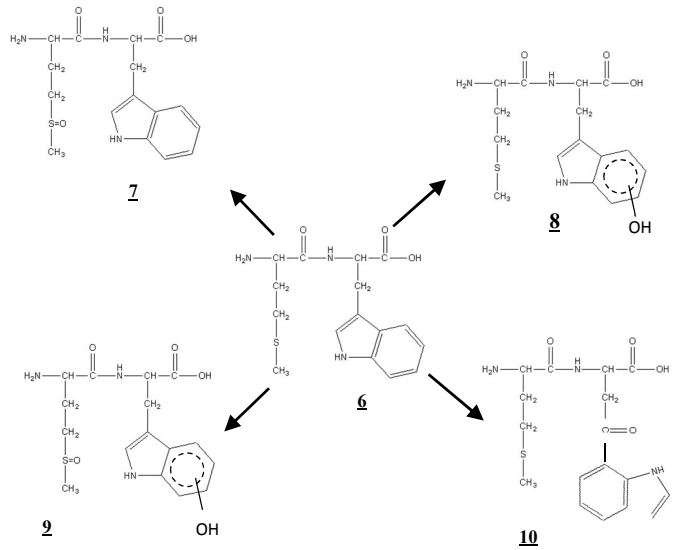


Figure 3: Experimental IRMPD spectra of non-irradiated (black) and irradiated (red) protonated Trp-Met **(a)** and Met-Tryp **(b).** The grey profiles report expanded spectra of **(a)** $(Trp\text{-}Met)OH^+$ and **(b)** $Met\text{-}TrypH^+$.



Scheme 3: The main oxidized forms (**7** to **10**) of Met-Trp (**6**).

The experimental IRMPD spectra of non-irradiated and irradiated protonated Met-Trp are reported in Figure 3b.

The CID-MS^2 fragmentation mass spectra of the main products of oxidation of Met-Trp are reported in Figures 10-12SI. The most intense peaks observed in the CID-MS^2 fragmentation mass spectra of the main products of irradiation of Met-Trp are gathered in Table 3SI with their attribution.

We can again observe the loss of ammonia in the CID-MS^2 fragmentation spectra of ions at m/z 336 and 352, confirming once more the protonation of the amino group of the N-terminal peptide. In the case of Met-Trp the addition of one oxygen atom occurs both on the sulphur atom (**7**, scheme 3) and on the tryptophan residue (**8**, scheme 3), as may be deduced from the interpretation of the IRMPD spectrum reported in Figure 3b and of the CID-MS^2 fragmentation mass spectrum of ion at *m/z* 352 (Table 3SI).

Several bands are observed in the IRMPD spectrum of protonated Met-Trp at 1094, 1139, 1406, 1510, 1691 and 1776 cm^{-1}. Two extra bands are observed in the IRMPD spectrum of (Met-Trp)OH^+ at 998 cm^{-1} and 1461 cm^{-1}, respectively. The former may be attributed to the stretching mode of the S=O group, the later to the bending mode of an aromatic COH group[30], indicating that the oxygen can be added both on the sulphur atom or on the aromatic ring. The bands at 1094, 1139, 1406, 1691 and 1776 cm^{-1}, respectively, are observed in both spectra and will not help the identification of the oxidation products. A tentatively assignment of these bands can be done based on the results obtained by Polfer [31] and Wu[32] on protonated tryptophan and other amino acids in the gas phase, studied by IRMPD spectroscopy and DFT calculations. The shoulder observed at 1094 cm^{-1} can be assigned to the bending mode of the NH group of the indole ring. Bands at 1139 cm^{-1} and 1406 cm^{-1}, respectively, may be attributed to the bending mode of the -OH group and the combination of the stretching mode of the C-OH group with the umbrella bending mode of the protonated amino group. The band at 1510 cm^{-1} may be assigned to the combination of the umbrella mode of the protonated amino group and the amide II vibrational mode. The band at 1695 cm^{-1} may be attributed to the amide I vibrational mode.

Finally the band at 1776 cm^{-1} is assigned to the stretching mode of the C=O group.

The presence of the fragment at *m/z* 288 in the CID-MS^2 fragmentation mass spectrum points out again to the formation of the sulfoxide (loss of sulfenic acid), while the loss of two water molecules and the presence of the fragment at m/z 221 (corresponding to the addition of one oxygen atom to the y fragment of the peptide) indicates the hydroxylation of tryptophan [5].

Thus, we exclude the formation of its isomer oxindolylalanine residue (Oia, structure b in scheme 2SI) in Met-Trp in our experimental conditions. The hydroxylation is a known process [33]. Many positions on both cycles can be concerned. The 5-hydroxy tryptophan would be the most abundant isomer. It has been identified by Todorovski et al[4] as induced by the addition of an oxygen atom in Trp containing peptides, as well as Oia. Interestingly, the addition of an oxygen atom on the dipeptide Trp-Met leads to the formation of a sulfoxide, while the addition of an oxygen atom on the reverse sequence (Met-Trp) can occur both on the Trp or on the Met residues.

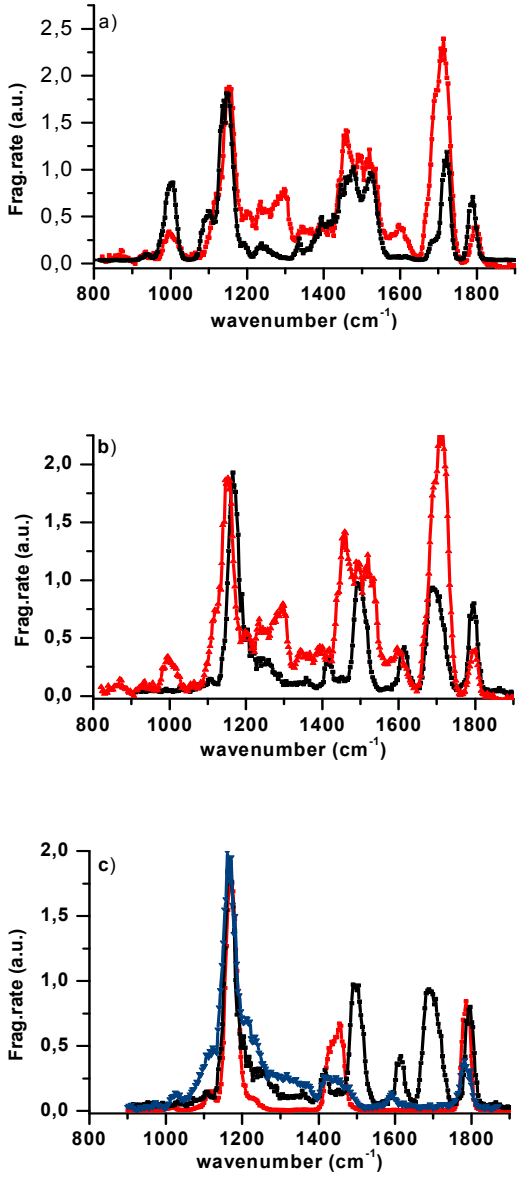


Figure 4: Experimental IRMPD spectra of protonated oxidation products **(a)** (Met-Trp)OH^+ in black (*m/z* 352) and (Met-Trp)O_2H^+ in red (*m/z* 368), **(b)** (Met-Trp)O_2H^+ in red (*m/z* 368) and (Trp)O_2H^+ in black (*m/z* 237) and **(c)** TrpH+ in red (*m/z* 205), (Trp)O_2H^+ in black and (Trp)OH^+ in bleu (*m/z* 221).

The product observed at *m/z* 368 may be assigned to the (Met-Trp)O_2H^+ peptide. The question is where the two oxygen atoms are located (products **9** and **10** in scheme 3).

The comparison between the IRMPD spectra of protonated (Met-Trp)OH^+ and (Met-Trp)O_2H^+ is reported in figure 4a and can help us to answer to this question. We can observe

that the peak at 998 cm^{-1} is present in both IRMPD spectra, confirming that a sulfoxide is formed in both cases.
A fragment at m/z 304 has been observed in the CID-MS^2 fragmentation mass spectrum of (Met-Trp)O_2H^+ (Table 3SI and figure 12SI). This fragment corresponds to the loss of the sulfenic acid and is diagnosed as coming from a sulfoxide. Both results suggest that a sulfone is not formed.
Moreover, the fragment at *m/z* 237 can be observed as well. It may be assigned to the addition of two oxygen atoms to the tryptophan residue leading to the formation of the well-known dihydroxytryptophan and/or the isomeric N-Formyl kynurenine (NKF [34] and references therein) (**10** in Scheme 3).
To shed some light on this point, bare tryptophan has been oxidized by gamma radiolysis in N_2O atmosphere with a 900Gy dose and analysed by IRMPD spectroscopy and mass spectrometry to characterise its oxidation final products.
The mass spectra of non-irradiated and irradiated Trp are reported in Figure 13SI. As we can observe from the mass spectra, the main products of oxidation correspond to the addition of one to four oxygen atoms to the tryptophan. We have focused our attention on the product observed at *m/z* 237, which may correspond to the protonated dihydroxy tryptophan and or/the isomeric NKF.
The IRMPD spectrum of (Met-Trp)O_2H^+ (*m/z* 237) obtained from the irradiated Trp has also been recorded for the first time and compared to the IRMPD spectrum of (Met-Trp)O_2H^+ (Figure 4b) and to the IRMPD spectra of $TrpH^+$ and $TrpOH^+$ (Figure 4c), respectively.
As can be observed from Figure 4, the IRMPD spectrum of (Met-Trp)O_2H^+ seems to be the superposition of the IRMPD spectra of protonated (Met-Trp)OH^+ (which presents the characteristic band of sulfoxide around 1000 cm^{-1}) and of (Trp)O_2H^+ (protonated NFK at *m/z* 237). According to calculations performed on protonated NFK at the B3LYP G-311+G** level of the theory using the Gaussian 09 program[35], three vibrational signatures of protonated NFK are identified at 1493, 1614 and 1689 cm^{-1}. They may be assigned to the NH bending mode of the amide group, the CO stretching mode of the ketone group and the CO stretching mode of the aldehyde, respectively. These bands have also been observed in the IRMPD spectrum of (Met-Trp)O_2H^+ but not in the IRMPD spectra of protonated Trp[32] or $TrpOH^+$ (Figure 4c).
The CID-MS^2 fragmentation mass spectrum of $TrpO_2H^+$ (*m/z* 237) has been recorded and compared to the CID-MS^3 fragmentation mass spectrum of the product at *m/z* 237 formed in the CID-MS^2 fragmentation mass spectrum of protonated (Met-Trp)O_2H^+ (Figure 5).
It is clear from Figure 5 that both products have very similar fragmentation mass spectra. The most intense peaks observed in the CID-MS^2 fragmentation mass spectra of ions at *m/z* 237 are gathered in Table 4SI with their attribution.
The fragments observed for NFK by Raven et al[34] are the same and confirm the presence of a NFK residue as oxidation product of Met-Trp. Moreover, the fragment at *m/z* 174 can be observed in Figure 5 and it is a useful peak to identify NFK modified peptides [4].

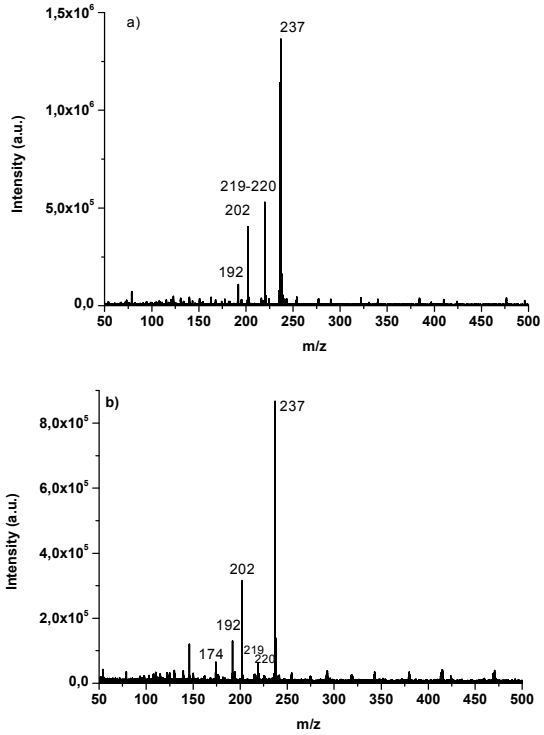


Figure 5: Comparison of the fragmentation mass spectra of $TrpOH^+$ (m/z 237), obtained as oxidation product of Trp **(a)** and as fragment of (Met-Trp)O_2H^+ **(b)**.

3.3 Discussion

In S-methyl glutathione, we have observed that the oxidation results mostly in addition of oxygen atoms to the sulphur (from one to four), loss of H_2 and decarboxylation. Loss of H_2 as well as addition of one or two oxygen atoms concerned only the thioether moiety and IRMPD helped to identify the sulfoxide and the sulfone functions. The role of the sulfone in oxidative processes is not yet well known. This is the reason why it is important to understand in which conditions it is formed.
As for the decarboxylation, the CID-MS^2 spectrum helped in this case to localize the decarboxylation site on the γ Glu residue (**5**, scheme 1). Our results suggest, as already observed by Bobrowski et alt. for the transient species[28], that two mechanisms are possible for decarboxylation during gamma-radiolysis for the stable products. The first one, already observed for dipeptides like Lys-Met and Val-Met[7], requires the presence of a C-terminal methionine and occurs particularly efficiently when both the sulfide function and the carboxylic group are located in the same C-terminal peptide unit. It is considered to proceed via an intramolecular mechanism based on an interaction between the oxidized sulfur function and the carboxylic group. The results observed for the final product of decarboxylation on GS-Me show that decarboxylation may occur not only from the C-terminal but also from the N-terminal carboxylic group. This

mechanism supposes the interaction between an •OH radical adduct, occurring in the first step of the oxidation, and a protonated amino group which is positioned α to a carboxylic group. Oxidative decarboxylation is an important reaction usually observed in the presence of metal ions as catalysts [29]. Such reactions can lead to the development of oxidative stress.

IRMPD and fragmentation mass spectra show that after γ radiolysis of a solution containing the dipeptide Met-Trp, the first oxygen atom can be added either to the sulphur or to the Trp residue. Moreover, the addition of two oxygen atoms does not lead to a sulfone, as observed for GS-Me, but to a mixture of sulfoxide plus hydroxylated Trp or to the formation of NFK (all of them having *m/z* 368).

Conversely, only methionine can be oxidized in Trp-Met and the addition of two oxygen atoms has not been observed in our experimental conditions.

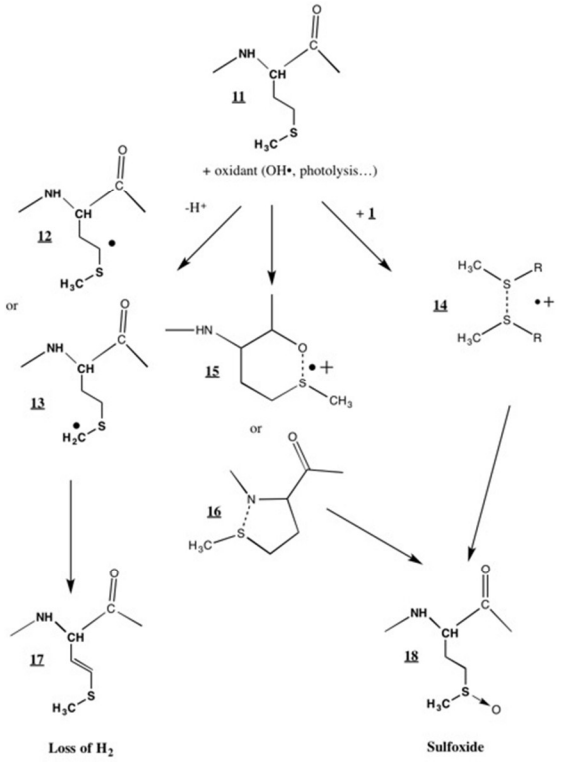
Some years ago, the Intramolecular Electron Transfer (IET) between the methionine radical and Trp in the dipeptide Met-Trp was investigated by pulse radiolysis (reaction 3) [15]. The oxidant was $Br_2^{\bullet-}$ and the Met radical cation was stabilized by a S∴Br 2-centre, 3 -electron bond.

$$\text{Met/S}\therefore\text{Br}\ldots\text{Trp} \rightarrow \text{Met}\ldots\ \text{Trp}^{\bullet+} + \text{Br}^- \qquad (3)$$

The IET rate constant of reaction (3) was equal to 10^7 s^{-1}, and the reaction appeared total at the microsecond timescale. The redox potential of Trp in various peptides has been reported to be rather constant and equal to 1.05 V vs. NHE[17]. That of methionine varies with the sequence, the geometry and the nature of the atom making the 2-centre, 3-electron bond[16]. Most often it is around 1.5 V *vs*. NHE, but it may be as low as 1 V [16]. Hence, one would expect little oxidation product from Met and only modifications of Trp. We observe the opposite: in both Met-Trp and Trp-Met, we find oxidized methionine. In addition, Trp is not oxidized in Trp-Met. Our results are in agreement with those reported about a model protein containing Met and Trp and other residues [36]. In this peptide, only Met was oxidized by a two-electron acceptor such as hydrogen peroxide, while the Fenton reaction (Fe^{2+} + H_2O_2) oxidized both residues.

A similar problem exists with tyrosine. The simultaneous presence of Tyr and Met in a peptide was investigated in several models. In Methionine enkephalin, for which the IET between the N-terminus Tyr and the C-terminus Met has been well documented[37,38], there was no methionine sulfoxide, as expected. With the dipeptides Met-Tyr and Tyr-Met, the IET between the Met radical and the Tyr residue resulted in the formation of the dityrosine dimer[7], but both peptides Tyr-Met and Met-Tyr underwent decarboxylation, which would be a process initiated by oxidation of Met. Thus, it seems that in many cases the IET, although thermodynamically favoured, can be reversed. Maybe the reactions at the microsecond/ millisecond timescales, observable by pulse radiolysis, are followed by slower rearrangements, perhaps due to displacements of redox equilibria.

A general mechanism for the one-electron oxidation of thioethers in peptides is provided in Scheme 4. The initial compound **11** can lead to several forms of free radicals, as documented in the literature [3]: i) carbon-centred ones (**12** and **13**); ii) sulphur-centred ones stabilized by a 2-centre, 3 electron bond with the sulphur atom of another non-oxidized molecule (**14**) or with an intramolecular nitrogen (**15**) or oxygen (**16**). The **12** species would lead to **17** containing a C=C double bond after free radical disproportionation **12** → **11** + **17.** As for the sulfoxide **18**, the state of knowledge indicates that it would come indifferently from the disproportionation plus hydration of **14**, **15** or **16** [39]. Finally, the sulfone is simply a subsequent oxidation of the sulfoxide. Its mechanism is still unclear.



Scheme 4: A general kinetic scheme for the oxidation of peptides containing methionine. The structures of the free radicals are taken from refs. 3, 12, 13, 14.

4. Conclusions

Oxidized proteins are toxic if not repaired or eliminated. Most of the biological roles of these oxidized forms is not known because they were not yet characterized. Their formation is a post-translational modification that can trigger various effects going from loss of enzymatic activity to impairments of cell growth, depending on which protein it takes place, the state of the cell, etc. All is not well understood concerning the final products observed in oxidative process, which justifies this work and eventual further ones. Among the misunderstood facts is the variation of the products with the nature of the peptides. One of the problems that one has to face when identifying the oxidation products coming from peptides or proteins is the fact that many of them have identical masses i.e. +16 coming from oxygen addition. The aim of this work was to use the conjunction of MS^n and IRMPD spectroscopy to unambiguously identify them. We were especially interested in thioether containing compounds for two reasons.

First, the final compounds coming from oxidation of methionine were not known despite the great importance of this residue. Second, in our previous work [6] we have shown that IRMPD provides a signature of the sulfoxide function

with its IR band around 1000 cm^{-1} in a non-congested region. This allowed us not only to identify other final oxidized forms of thioether functions, but also to revisit the mechanisms of IET in some peptides [7].

The oxidation of the thioether function did not lead to the same products in GS-Me and in dipeptides. We did not find decarboxylation nor hydrogen loss in Trp-Met or Met-Trp, while both processes were major pathways in GS-Me. The formation of these two species requires a transfer of the odd electron from the sulphur to the adjacent carbon, a process that was characterized very recently in photosensitized oxidation of peptides [39]. This IET seems not to take place in Trp-Met or Met-Trp.

The combination of tandem mass spectrometry with the identification of the y-series in the fragmentation mass spectra and the possibility to record infrared spectra of compounds in the gas phase not only allow a better characterization of oxidized protein residues, but also provide new tools to investigate important steps of protein oxidation, like IET.

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Notes and references

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