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Debora Scuderi[1,2], Jacqueline Bergès[3,4], Pedro de Oliveira[1,2], Chantal Houée-Levin[1,2*]

[1]Laboratoire de Chimie Physique, UMR 8000, Université Paris-Sud, 91405 Orsay Cedex, France.
[2] CNRS, UMR 8000, Bldg 350, 91405 Orsay Cedex, France.
[3] Laboratoire de Chimie Théorique, UMR 7616, Université Pierre et Marie Curie, 4 Place Jussieu, 75252 Paris Cedex 5, France
[4] CNRS, UMR 7616, 75252 Paris Cedex 5, France

Corresponding author:

Prof. Chantal Houée Levin

Chantal.houee@u-psud.fr

Laboratoire de Chimie Physique, CNRS, UMR 8000, Université Paris-Sud, F-91405 Orsay, France

Abstract

Methionine is an essential amino acid, unfortunately prone to oxidation. The mechanism of its oxidation by $^{\bullet}$OH radicals has been studied for more than 40 years and still remains misunderstood. We have reinvestigated the oxidation of this residue in model peptides, aiming at i) improving the identification of free radicals by the use of more modern quantum chemistry methods; ii) reinvestigating the one-electron reduction potentials as a function of the position in the sequence; iii) identifying the final compounds, which were still unknown; iv) reinvestigating the intramolecular electron transfer (IET) involving this residue.

Keywords:

Methionine oxidation, sulfoxide, pulse radiolysis, mass spectrometry, IRMPD, DFT computations, electrochemistry.

Introduction

It is well known that protein residues are all susceptible to be oxidized by the so-called "Reactive Oxygen Species" (ROS), i.e. hydrogen peroxide, oxygen free radicals and various oxygen-containing free species such as the NOx, hypochlorite etc [1]. Protein oxidation is a major event in all diseases and in ageing. Among the various residues, methionine (Met) plays a central role. It is easily oxidized by all ROS. The single product that was known is methionine sulfoxide (MetSO), which can even be oxidized to the sulfone. More importantly, methionine sulfoxide can be enzymatically reduced by the methionine sulfoxide reductases, hence the couple MetSO/Met is considered as a protection against ROS.

Despite this wealth of knowledge, many points remain to be elucidated. Oxidation by $^{\bullet}$OH radicals leads to methionine free radicals having an interesting 2-centre, 3-electron (2c-3e) bond because the methionine sulphur-centred radical cation is stabilized by donation of any free doublet from a vicinal atom. In peptides and proteins, it can be nitrogen, oxygen or another sulphur atom [2,3]. The identification of these free radicals was performed by their absorption spectra obtained in pulse radiolysis or in flash photolysis ([4,5] and references therein), by EPR and CIDNP [6-8]. The reduction potential of methionine has never been measured and one relies on that of small compounds like dimethyl sulphide, without knowing in what sense the neighbouring residues could modify it. As for the final compounds, very few investigations have been reported. In proteins only methionine sulfoxide has been detected. In small peptides, decarboxylation can occur when Met is the C-terminus [9]. However, several other processes could take place. Finally we have reinvestigated the problem of intramolecular electron transfer (IET) involving methionine in peptides.

We have addressed these four points by performing again calculations by quantum chemistry based upon recent knowledge about the adequacy and the precision of some methods and investigated the topology of these free radicals. We have made new attempts to measure the redox potentials of the Met•+/Met couple in some model peptides and shown that it does depend on the position in the polypeptidic chain. Finally, thanks to mass spectrometry coupled to Infrared Multiple Photon Spectroscopy in the gas phase (IRMPD) we have shown that compounds other than the sulfoxide are formed. In what follows, we summarize some of our most prominent results and we take the example of the peptides Met Val and Val Met to enlighten some of the problems encountered.

Materials and methods

PRODUCTS

Methionine, methionine sulfoxide and Catalase (Cat) (bovine liver, suspension in water) were obtained from Sigma (France) and used without purification. The peptides were purchased at Sigma Aldrich (L'isle d'Abeau, France) or at Bachem (Switzerland). They were used as received. The catalytic activity of Cat was checked using a solution 1 mM of H_2O_2. 1 µL of the suspension was added to the solutions before irradiation. Water was obtained either from an Elga Maxima or from a Millipore system (conductivity 10^{-18} Siemens).

GAMMA RADIOLYSIS

γ-irradiations were carried out using the panoramic ^{60}Co γ-source IL60PL Cis-Bio International (France) in the University Paris-Sud (Orsay, France). The dose rate was determined by Fricke dosimetry [10] and kept constant at around 30 Gy min^{-1}. Samples were gently purged while stirring under a nitrous oxide (N_2O) atmosphere for approximately 60 min before irradiation. N_2O was delivered by ALPHA GAZ. Its global purity is 99.998%.

All irradiations were performed at room temperature.

The well-known method of scavengers [10] allows a quantitative production of free radicals according to the following reactions. The chosen oxidant species was the $^{\bullet}OH$ radical produced by γ radiolysis of N_2O-saturated aqueous solutions:

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

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

The radiation chemical yield (G) is 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 lead to de-sulphuration of methionine [11]. The H_2O_2 yield is also lower (0.07 µmol J^{-1}). Catalase was added to remove it in order to prevent the two-electron oxidation of methionine [12].

ELECTROCHEMISTRY

Cyclic voltammetry was carried out with a three-electrode system connected to a EG&G PAR 273A potentiostat which was computer-controlled via the M270 software. The system consisted of a Teflon electrochemical cell containing a boron-doped diamond working electrode, a platinum gauze counter electrode and a saturated Calomel reference electrode (SCE). A sodium perchlorate (VWR, France) 0.1 M, pH = 2.0 buffer was prepared with pure

water obtained from a Milli-RiOs 8 unit followed by a Milli-Q academic purification set (water resistivity: 18.2 MΩ.cm). Solutions were made with the dipeptides Gly-Met, Met-Gly, Met-Met, Val-Met and Met-Val, Met enkephalin, from Bachem (Switzerland), and with the amino acid methionine (Met) from Sigma (France), used as received from the suppliers. Electrolysis was performed in 20 mM phosphate buffer, pH = 7.0, in a three electrode cell with a glassy carbon stick as the working electrode, whose potential was set at +1.5 V vs. SCE for 3 hours. For comparison, solutions having the same concentration were subject to the same procedures except for the applied potential difference. Blanks consisting of pure buffer were treated likewise. All solutions had a concentration of 1 mM in their solutes and were thoroughly de-oxygenated with argon prior to the electrochemistry experiments.

MS OPERATIONS

IR multiple photon dissociation (IRMPD) is a multi-step resonant absorption process relying on intramolecular vibrational energy redistribution (IVR) [13]. Infrared spectra are 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.

The IRMPD spectra in the 800-2000 cm^{-1} energy range have been obtained with the IR beam of the CLIO FEL coupled to the modified Paul ion trap or a FT-ICR mass spectrometer. Details on the performance of our modified Bruker Esquire 3000+ were already published [14]. Different electron energies were used (40-44 MeV). Typical average powers were about 1 W around 1000 cm^{-1} and 0.4 W near 2000 cm^{-1}.

COMPUTATIONAL METHODS

DFT methods and basis sets. To the best of our knowledge no DFT method has been specifically adapted to the calculations of redox potentials. In this work, we have chosen two DFT hybrid methods (BH&HLYP and PBE0) because of their different properties concerning free radicals.

We used the relatively small basis set 6-31G(d) (SB) and the greater basis set 6-311+G(2d,2p) (GB) considering the experimental and the theoretical difficulties linked to measurements of redox potentials.

QM/MM calculations. The computations were performed thanks to the Gaussian G09 package [15] using the QM/MM method (Oniom). In this procedure, the polypeptide was

divided into two layers which were treated with different levels of calculations. The Quantum Mechanics (QM) method chosen was the DFT method pbe0/6-31G(d). For the Molecular Mechanics (MM) method, we used the empirical potentials uff with the electrostatic charges embedded (Qeq).

Solvation. Solvation effects were accounted for with the COSMO option for the Polarised Continuum Model CPCM considering an aqueous environment. Some calculations were also performed with IEFPCM that gave identical results.

Redox potentials. The one-electron reduction potentials were calculated as described in [16]. For each dipeptide, the Gibbs energies ΔG_1 and ΔG_2 (in kJ mol^{-1}) for the molecule and the cation species, respectively, relative to the molecule of lowest energy (taken as reference, 0 kJ mol^{-1}) were calculated. $\Delta G_{(aq)S}$ is thus equal to $\Delta G_1 - \Delta G_2$. Calculations were performed with the Gaussian09 package [15].

Results and discussion

CAN WE MEASURE THE REDOX POTENTIAL OF METHIONINE RESIDUES BY CYCLIC VOLTAMMETRY?

All the compounds studied gave rise to irreversible responses in cyclic voltammetry carried out with a boron-doped diamond electrode. Determining the redox potential of an irreversible couple exhibiting just the oxidation wave is not trivial, even if it may be estimated under certain circumstances ([42] and references therein). We compare wave onset potential values (E_{onset}, the values at which the current starts increasing) and anodic peak potential values (E_{pa}) and mention some trends that stand out.

Oxidation of Met and Met-Met. The E_{onset} values coincide for both Met and Met-Met (E_{onset} = 1.27 V, Figure 1 and Table 1) [17]. The current magnitude for the Met-Met wave was roughly two-fold that of Met, as one would expect for a molecule having two $-SCH_3$ groups. The oxidation wave for Met-Met peaks 0.13 V beyond that of Met (E_{pa} = 1.65 V vs. E_{pa} = 1.52 V), an indication that the former may be slightly more difficult to oxidize than the latter.

Oxidation of Gly-Met and Met-Gly. Gly-Met, like the previous two compounds, starts being oxidized at E_{onset} = 1.27 V, and its less intense wave when compared to that of Met may be explained by a smaller diffusion coefficient (Figure 1). Met-Gly is more difficult to oxidize

that Gly-Met, which is confirmed by both the wave onset potentials (E_{onset} = 1.37 V vs. E_{onset} = 1.27 V) and the anodic peak potentials (E_{pa} = 1.75 V vs. E_{pa} = 1.57 V, Table 1). If the redox potential follows the same trend as the E_{pa} values, we may expect the redox potential of Met-Gly to be ca. 0.2 V higher than that of Gly-Met. Surprisingly, the anodic peak current, I_{pa}, is higher (≈50%) for Met-Gly than for Gly-Met, a result which is not easy to rationalize.

Oxidation of Met-Val and Val-Met. Like Gly-Met and Met-Gly, the behaviors of the dipeptides Val-Met and Met-Val are quite distinct. On the one hand, Val-Met has a E_{onset} very close to that of Met on its own, but the anodic peak is reached earlier, at E_{pa} = 1.48 V, meaning that the methionine residue in this dipeptide is easier to oxidize (Figure 1 and Table 1). Interestingly, its oxidation peak current i_{pa} is about 10% lower than that of Met (55.5 vs. 62.0 µA), which may be explained by a smaller diffusion coefficient for Val-Met when compared to Met, due to the larger size of the former. On the other hand, Met-Val starts being oxidized at a clearly higher wave onset potential, E_{onset} = 1.32 V, a process that is 0.05 V less favorable than for Val-Met. This trend had previously been observed for Gly-Met and Met-Gly [17] and suggests that when Met is present in dipeptides, the Met residue is more difficult to oxidize when it occupies the N-terminal position. Having said that, the shift in E_{pa} is less pronounced in the case of Met-Val and Val-Met (E_{pa} = 1.55 V vs. E_{pa} = 1.48 V) than in the case of Met-Gly and Gly-Met (E_{pa} = 1.75 V vs. E_{pa} = 1.57 V), but the fact that some peaks are poorly defined leaves some room for uncertainty regarding their accurate positions.

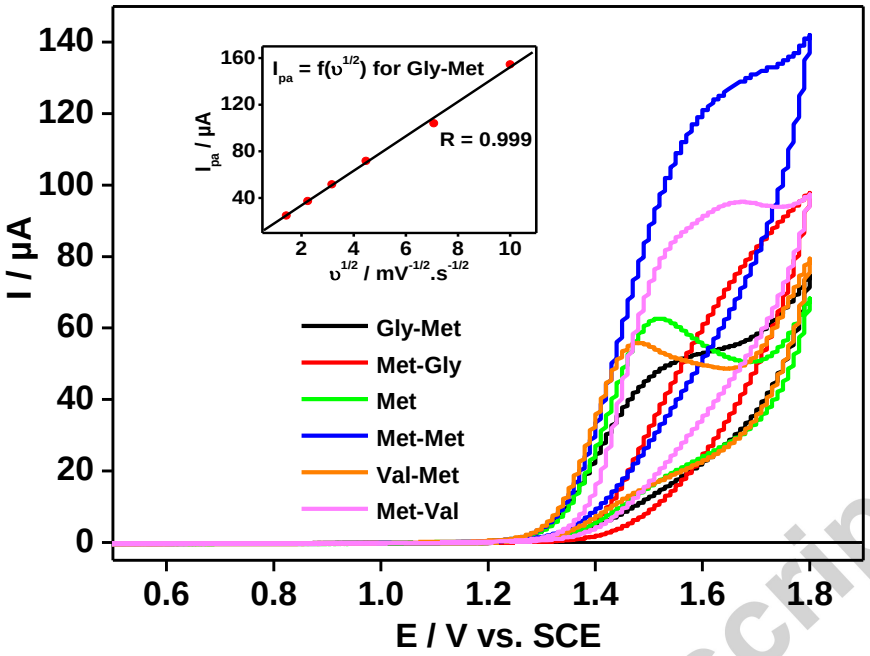


Figure 1: Cyclic voltammograms of 1 mM solutions of Gly-Met, Met-Gly, Met, Met-Met, Val-Met and Met-Val, in sodium perchlorate 0.1 M, pH = 2.0 buffer, at 10 mV/s. Inset: dependence of the anodic peak current, i_{pa}, on the square root of the scan rate, $\nu^{1/2}$, for a 1 mM solution of Gly-Met.

Compound	E_{onset} (V vs. SCE)	E_{pa} (V vs. SCE)
Met	1.27 (1.51)	1.52 (1.76)
Met-Met	1.27 (1.51)	1.65 (1.89)
Gly-Met	1.27 (1.51)	1.57 (1.81)
Met-Gly	1.37 (1.61)	1.75 (1.99)
Val-Met	1.27 (1.51)	1.48 (1.72)
Met-Val	1.32 (1.56)	1.55 (1.79)

Table 1. Oxidation wave onset potentials, E_{onset}, and peak potentials, E_{pa}, (V vs. SCE) for Met, Met-Met, Gly-Met, Met-Gly, Val-Met and Met-Val. The values in parenthesis are in V vs. NHE.

Surprisingly, the peak current for Met-Val is more than 30% higher compared to that of Met and there seems to be a second oxidation peak at E_{pa} = 1.67 V. These results are hard to rationalize, but possible explanations are the following: i) Met-Val adopts a conformation that renders its diffusion coefficient higher than that of Met, which would result in a higher current; ii) either two different conformation isomers of Met-Val which co-exist in solution give rise to two separate oxidation peaks (implying that they are more than 0.1 V apart), or a single Met-Val conformer undergoes an oxidation process in two consecutive steps. These results are by and large verified both at lower and at higher scan rates.

The inset in figure 1 shows the dependence of the anodic peak current, i_{pa}, on the square root of the scan rate, $v^{1/2}$, for the dipeptide Gly-Met. The linear dependence indicates that diffusion controls the mass transport to the electrode surface. A similar behavior is exhibited by all the compounds studied.

STRUCTURE OF THE METHIONINE FREE RADICALS

We chose the set of initial conformations so that various pseudo cycles from 5 to 9-membered cycles with SS, SN or SO bonds could be formed in the radical cations. We found inspiration in the protein conformations that provided us starting points (Figure 2). In order to take into account different situations of the small sequences in polypeptides or proteins, we considered both the zwitterionic forms (ZW) as models of C- or N-terminals and the 2-methylated species as models of protein sequences.

All structures were fully optimized in their molecular and in their radical cation states, to obtain adiabatic and not vertical redox potentials. Each located stationary point was checked by evaluating harmonic frequencies. We used the geometries obtained after these optimizations with BH&HLYP as benchmarks for the PBE0 calculations.

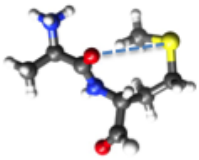
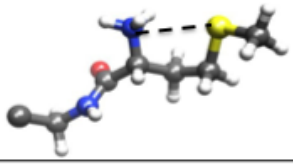
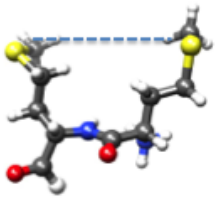
Conformation of methionine(s) putative free radical	Sequence	Structure
SO	Ala Met from human thioredoxin (PDB 1TOF)	
SN	Gly Met from Chymotrypsin (2YT6)	
SS	Met Met from Akt (PDB 3O96)	

Figure 2. Some protein sequences that may form methionine SX bonds upon oxidation. These sequences (and others of this kind) were taken as starting points for the calculations.

An examination of the DFT methods led to the conclusion that similar results were obtained both with BH&HLYP as with the CCSD(T) approach [18-20]. We used also the PBE0 model, based on the Perdew–Burke–Erzenrhof exchange-correlation functional [21,22] and we showed that the results were totally in agreement with those of BH&HLYP. In this paper we concentrated on results obtained in water.

The structure of the methionine free radical was thoroughly explored by DFT methods and by topological approaches [23,24]. We adopted the following nomenclature: for the SN radicals, S can be linked either to N of the amine function (SNam) or to N of the peptidic bond (SNpep). Also, the number of atoms in the pseudo cycle is included in the nomenclature (for instance SN5 for five atoms). Some stable structures are displayed in Figure 3.

In the SS radicals, the SS distance increased from R_{SS} = 2.79 Å for the simplest $Me_2S\therefore SMe_2^+$ up to R_{SS} = 2.94 Å for diSETAc cation ($C_2H_5(CH_3–CO)$-S∴S-(CO-$CH_3)C_2H_5^+$), and to R_{SS} = 3.01Å for the diSETA cation ($C_2H_5(CH_3–CO)CH_2$-S∴SCH_2-(CO-

$CH_3)C_2H_5$) [24,25]. In the Met-Met cation, the value is also relatively large (2.96 Å). It could result not only from the geometrical constraints but also from substituent effects.

The SN radicals were studied in the peptides Met-Met, Gly-Met and Met-Gly. In water, the zwitterions SNam are not stable. In all peptides, methylated or zwitterionic, among the SN5 radical cations the energy increased with the SN bond lengths [24]. The SN distance was shorter in the SN5am radicals (around 2.45 Å) than in SN5pep1 (2.57 Å). Intramolecular SNpep-type structures were also described by other authors following the oxidation of N-acetylmethioninamide [26] or of c-(Met-Gly) [27].

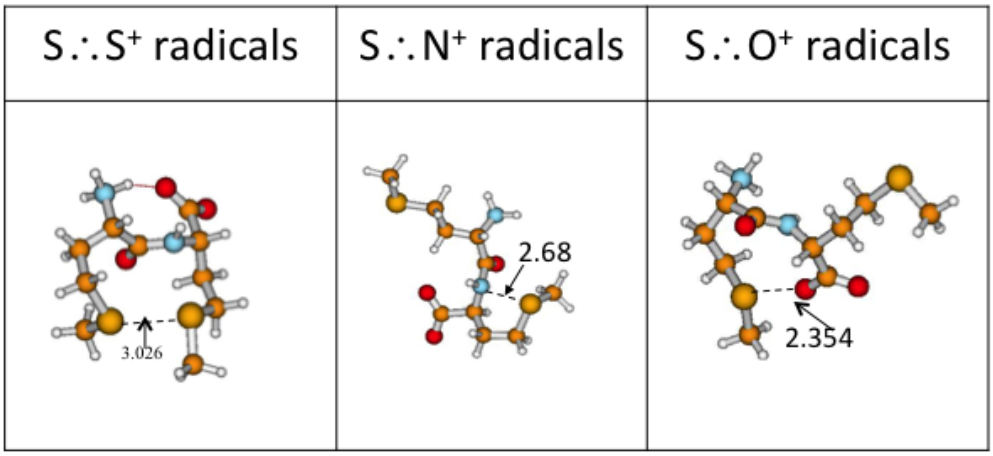


Figure 3. Some structures of stable free radicals formed from the dipeptide Met-Met in water. The distances are in Å.

SO radical cations were found in Met-Gly and Gly-Met. In Met-Met they were found in water only (in vacuum the species evolved towards a SN radical). Cycles involving 6, 7 or 9 atoms were obtained. Upon solvation, the SO distances in SOpep radicals increased (by 0.07-0.12 Å) [24]. Among the 17 structures that we have explored, the energies of the entities generally tended to increase with the SX bond length. It means that a parameter for the stability of the radical cation could be the overlap of the p lone pairs of the two heteroatoms involved in the 2c-3e bond. Only 7 optimized structures could be characterized. However, for Gly-Met we obtained several structures, and the most stable ones had a SO bond with the carboxylic/carboxylate group.

A topological analysis of the bond was also performed in these free radicals [24]. The main conclusions were: i) an amino nitrogen (respectively a peptidic oxygen) is more donating than a peptidic nitrogen (respectively a carboxylic oxygen), as expected from the highest

localization of the lone pair of the peptidic nitrogen (respectively of the carboxylic oxygen); ii) the 2c-3e interaction decreases along the series as follows: SN5am→SN8am→SN5pep1→SN6pep; iii) the S…O bond in radical cations of dipeptides is generally best described as an electrostatic interaction. In conclusion, the nature of the S∴X bond is closely related to the variation of the bond length.

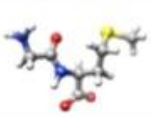
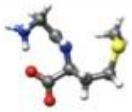
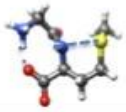
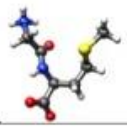
Gly Met	Molecule	Cation	E°vs NHE (V)	
	BH&HLYP		BH&HLYP	PBE0
SO7			1.61	$-CO_2$
SO7			1.59	1.47
SN5			1.19	1.28
SN5			1.06	$-CO_2$

Figure 4: Some redox couples from the dipeptide Gly-Met and the values of the redox potentials obtained with BH&HLYP and with PBE0 and 6/31G(d). –CO2: the free radical was not stable and underwent decarboxylation.

CALCULATIONS OF REDOX COUPLE POTENTIALS

Since the direct measurement of redox potentials by electrochemistry was not possible, we turned to calculations thereof. "Redox couples", made of a dipeptide and its radical cation, were constructed by optimizing first the structure of one of the entities of the couple (either the molecule or the radical) and then the other one starting from the previously optimized structure (Figure 4) [17]. We tried two methods (BH&HLYP and PBE0) and two basis sets (6-31G(d) and 6-311+G(2d,2p)).

Using the smallest basis set, a striking difference between both methods is that with BH&HLYP all radical cations were stable, whereas with PBE0 some Gly-Met and Met-Met radicals underwent decarboxylation. This is quite an interesting point since decarboxylation does occur in some peptides (vide infra). However, calculations with the largest basis set did not confirm the bond break. Using the largest basis set, the computations confirm the

experimentally observed tendency: the redox potentials are lower for Gly-Met (1.3-1.7 V) than for Met-Gly (1.7-2 V).

The redox potentials of the dipeptides are in the range expected for methionine amino acid. In some conformations it reached lower (1.3 V) or much higher values (2 V). Experimental results indicated such possible variations [28-30]. One might try to predict the values by looking at the atoms in the neighbourhood of the sulphur atom. Unfortunately, the nature of the atom involved in the 2c-3e bond does not help to rationalize the scale of redox potential. It is not so simple and one should be careful because the results might vary with the level of theory.

WHAT ARE THE FINAL PRODUCTS OF METHIONINE OXIDATION BY $^{\bullet}$OH RADICALS?

Large doubts remain about the final oxidized forms of the methionine residues. In studies of anaerobic one-electron oxidation of peptides or proteins, the sole final compound coming from the methionine residue is methionine sulfoxide MetSO [31-34].

Using authentic samples of methionine and its sulfoxide, we characterized the mass spectra with the fragmentation patterns coming from these two compounds and we observed the signature of the S=O bond clearly at around 1000 cm^{-1}. [35]. It allowed us to confirm that the sulfoxide was formed upon oxidation by $^{\bullet}$OH radicals and not only by H_2O_2.

Let us now consider the results about the oxidation of Met-Val and Val-Met both by radiolysis and by electrochemistry. The CID-MS^2 spectra of the dipeptides oxidized by electrolysis ($[(Val\text{-}MetSO)H]^+$ and $[(MetSO\text{-}Val)H]^+$) are reported in figure 5. Both mass spectra present the same fragmentation patterns observed for the samples oxidized by γ-radiolysis showing that the sulfoxide is also the main final product formed by electrolysis. The peaks with their interpretation are collected in Table 2 for both sulfoxides. The IRMPD spectra of the M+16 peak coming from the oxidation of Met-Val by radiolysis and by electrochemistry are shown in Figure 6. The good agreement between the two experimental spectra shows that the sulfoxides have similar structures when formed in both experimental conditions. Similar results have been obtained for $[(Val\text{-}MetSO)H]^+$. Some differences have been observed in the relative intensities showing that the sulfoxides formed by electrolysis are more fragile and are fragmented easier.

Compound	m/z, z=1

$(Val\text{-}MetSO)H^+$	265 (parent ion) 247(-H_2O from parent ion) 201(-CH_3SOH from parent ion) 166 ($MetSOH^+$, y''_1 fragment)
$(MetSO\text{-}Val)H^+$)	265 (parent ion) 247 (-H_2O from parent ion) 219 ((-H_2O -CO from parent ion) 201 (-CH_3SOH) 173 (-CH_3-CH_2SO-CH_3 from parent ion)

Table 2. Most intense peaks observed in the fragmentation mass spectra of the sulfoxides obtained from dipeptides oxidized by electrochemistry.

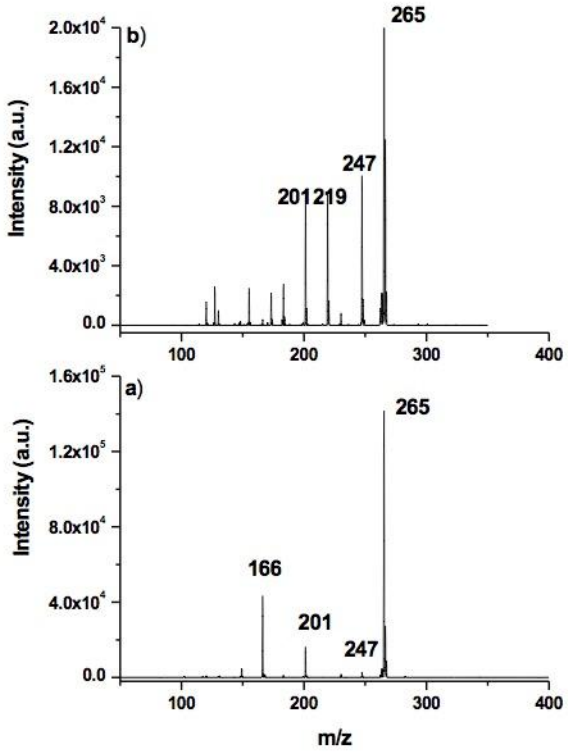


Figure 5: CID-MS^2 fragmentation mass spectra of protonated electrolyzed dipeptides (a) $[(Val\text{-}MetSO)H]^+$ and (b) $(MetSO\text{-}Val)H]^+$.

Similar studies were performed for various peptides, namely dipeptides containing Met (X-M and M-X, X = Val, Lys, Met and Tyr) and Met-enkephalin. They were oxidized by $^{\bullet}OH$

radicals in the absence of oxygen and in the presence of catalase to remove H_2O_2 [36]. The products detected after oxidation are gathered in Table 3.

The products corresponding to the addition of 16 Da to each peptide were observed for all the compounds that were investigated by mass spectrometry and IRMPD spectroscopy after γ irradiation with catalase. Thanks to the IRMPD, this mass increment was attributed to a sulfoxide, which exhibits a specific band at around 1000 cm^{-1}, clearly observed in all the cases except for the pentapeptide Met-enkephalin. Thus, we obtained a clear proof that methionine was not oxidized in this peptide.

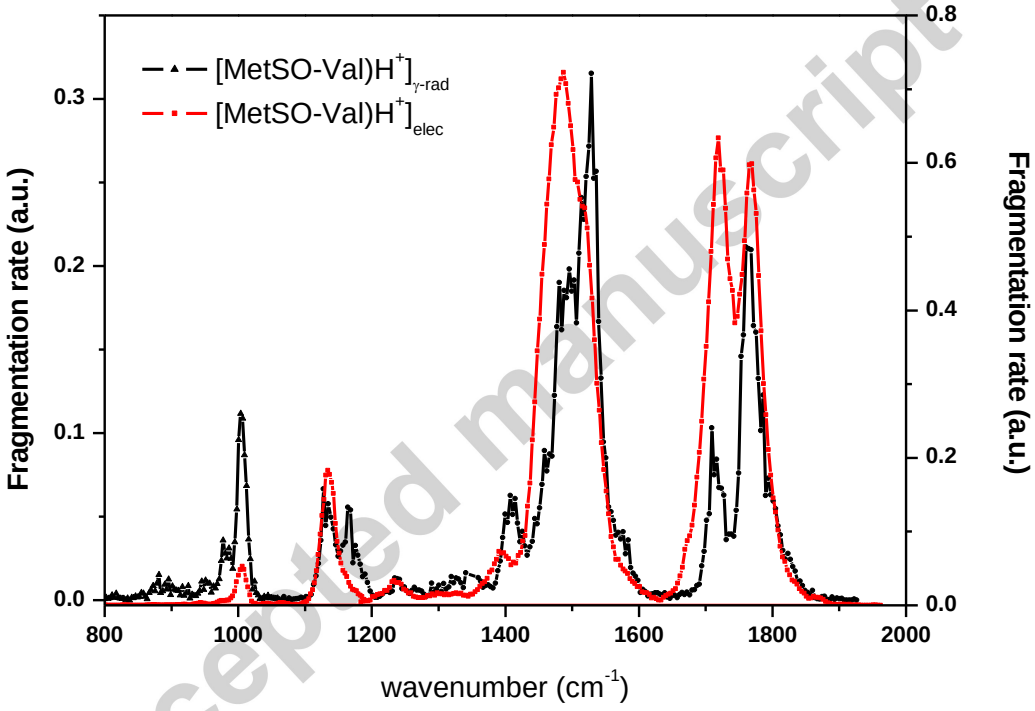


Figure 6: Comparison between the experimental IRMPD spectra of [(MetSO-Val)H^+] formed by γ-radiolysis (triangles) and electrolysis (squares) in the 800-2000 cm^{-1} energy range.

Peptide	Mass increment (+) or decrement (-) (Da)	Products
Lys-Met	+16	Lys-MetSO
	-44	Ly-Met -CO_2
	-19	(Lys-Met)H^+ -NH_3 -H_2
Met-Lys	+16	MetSO-Lys
Met-Tyr	+16	MetSO-Tyr
	-28 = -44 +16	(MetSO-Tyr) -CO_2
	+32	MetSO-L-DOPA*
Tyr-Met	+16	Tyr-MetSO
	-44	Tyr-Met -CO_2
	+32	L-DOPA-MetSO*
Met-Val	+16	MetSO-Val
	-2	Met(-H_2)-Val
Val-Met	+16	Val-MetSO
	-2	Val-Met(-H_2)
	-46	(Val-Met) -H_2 -CO_2
Met-enkephalin (Tyr-Gly-Gly-Phe-Met)	-1 (z=2)	Met-enkephalin dimer (dityrosines)
	+32	Met-enkephalin with DOPA
	+16	Met-enkephalin with PheOH
S-Me-Glutathione	+16	SO-Me-Glutathione
	+32	SO_2-Me-Glutathione
	-2	S-Me-Glutathione -H_2
	-44	S-Me-Glutathione -CO2

Table 3: the products detected after oxidation of Met-containing peptides by $^{\bullet}$OH radicals. MetSO: methionine sulfoxide; L-DOPA: dihydroxyphenylalanine coming from the oxidation of the tyrosine residue; Met(-H_2): methionine having lost 2 hydrogen atoms from its side chain; PheOH: substitution of –OH on the aromatic cycle of phenylalanine. * These compounds were formed after irradiation with higher doses (900 Gy).

In many dipeptides we have identified oxidized forms other than sulfoxide. In particular we focused on the oxidation of three peptides, S-methyl-glutathione (GS-Me), tryptophan-methionine and methionine-tryptophan (Trp-Met and Met-Trp) [37].

In GS-Me, we observed that the oxidation resulted mostly in the addition of oxygen atoms to the sulphur, 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.

As for the decarboxylation, the CID-MS^2 spectrum allowed in this case to localize the decarboxylation site on the Glu residue. Our results suggest that two decarboxylation mechanisms are possible during γ-radiolysis leading to stable products. The first one, already observed for dipeptides like Lys-Met and Val-Met, requires the presence of a C-terminal methionine and occurs particularly efficiently when both the sulphide 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 sulphur function and the carboxylic group. The results observed for the final product of decarboxylation with GS-Me show that the latter may occur not only from the C-terminal but also further up in the sequence. This mechanism supposes the interaction between an $^{\bullet}OH$ radical adduct, formed in the first step of the oxidation, and a protonated amino group in α position relative to a carboxylic group. Oxidative decarboxylation is an important reaction usually observed in the presence of metal ions as catalysts. Such reactions can lead to the development of oxidative stress.

WHAT ABOUT INTRAMOLECULAR ELECTRON TRANSFER INVOLVING METHIONINE?

Methionine oxidation has been shown to be the precursor of oxidation of other aromatic residues by IET. First we have reinvestigated this phenomenon on dipeptides. IRMPD and fragmentation mass spectra show that after γ radiolysis of a solution containing the dipeptide Met-Trp, the first oxygen atom could be added either to the sulphur or to the Trp residue. [37]. Similar results were obtained with Tyr instead of Trp. Only methionine could be oxidized in Trp-Met and the addition of two oxygen atoms has not been observed in our experimental conditions.

Amino acid or residue	E° (V vs. NHE)	Reference
Tyr	0.85 - 0.94	[38] and references therein
Trp	1.05	[39]
Met	1.2-1.8	[17]

Table 4: the one-electron redox potentials of Tyr, Trp and Met.

The redox potentials of the residues are gathered in Table 4. That of methionine varies with the sequence, the geometry and the nature of the atom making the 2c-3e bond [17]. Hence, one would expect fast intramolecular electron transfer (IET) between the Met radical and Trp (or Tyr) in these dipeptides, thanks to the redox potential difference between both residues and the very small distance between them. One would thus expect little oxidation products derived from the Met residue and mostly modifications on the Trp or the Tyr residues. We observe the opposite for Trp, as stated above. Our results are in agreement with those reported for a model protein containing Met and Trp among other residues [40]. Thus, it seems that in these 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.

We have investigated the case of Met-enkephalin, a pentapeptide, in depth. This peptide contains Tyr (N-terminus) and Met (C-terminus). Pulse radiolysis experiments suggested the existence of very fast IET between the methionine radical cation (C-terminus) and the tyrosine residue (N-terminus) [41]. Analysis of the final products led to the conclusion that the Met residue was not oxidized (Table 2), which is in total agreement with the former hypothesis [37]. We also investigated the oxidation of Met-enkephalin by electrochemistry. Interestingly, only the oxidation wave relative to Tyr was visible [42]. The experimental value of the anodic peak in Met-enk (E_{pa} = 1.05 V) was higher by more than 0.1V than the values obtained for Tyr, which may be rationalized by environmental effects.

We had previously performed preliminary theoretical approaches of the oxidation competition between Tyr and Met in isolated peptide models [43]. These first thermodynamic data indicated that $Tyr^{\bullet+}$ was more stable than $Met^{\bullet+}$. In order to test the thermodynamic feasibility of intramolecular Proton-Coupled Electron Transfer (PCET) in this peptide, we evaluated the redox potentials of each couple involved, i.e. $Met^{\bullet+}$/Met and $Tyr^{\bullet+}$/Tyr [42]. Because of the size of the peptide, we used QM/MM methods. The peptide underwent the QM/MM partitions depicted in Figure 6. Briefly, in the first one, Met was in the QM part of Met-enk. In the second one, it was the turn of Tyr to be in the QM part of Met-enk. The results are included in Figure 7. They confirm that the redox potential of Met is much higher than that of Tyr and that the IET is thermodynamically allowed.

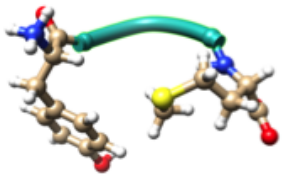
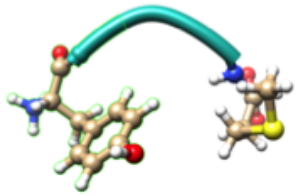
Structure of the peptide	E° (Tyr•+/Tyr) V vs. NHE	E°(Met•+/Met) V vs. NHE
	1.07	1.84
	1.15	1.93

Figure 7. The one-electron redox potentials of the Tyr and the Met residues for 2 conformers of Met-enkephalin. Calculations were performed using QM/MM methods (see the text).

There is a very good agreement between the measured and the computed values for the Tyr residue. The redox potential of Met is higher than that of Tyr by ca. 800 mV, in agreement with the very fast IET observed.

Recently, a study of the IET in one conformation of Met-enkephalin was performed by DFT and MD methods [44]. The most effective ET pathway connects an aromatic hydrogen atom on the tyrosine residue to a CH_2 group of the methionine residue. It is a "through-space" pathway according to the nomenclature, corresponding to a distance of 3.73 Å.

Conclusion

Since the beginning of the research about protein oxidation, many steps have been unveiled. It is known that protein oxidation can lead to toxic insoluble aggregates and that they cannot be repaired. Methionine oxidation is an important event. It plays roles in fibrillation of β-amyloid peptide and α-synuclein, and thus in Alzheimer's [45,46] and Parkinson's diseases [47,48]. Methionine sulfoxide is among the rare oxidized forms that can be repaired. Specific enzymes, known as methionine sulfoxide reductases, aim at reducing the sulfoxide groups.

Despite the pioneering work of Asmus and co-workers, the chemical mechanism of methionine oxidation is still not understood. It is complex and influenced by atoms and groups of atoms in the vicinity of the Met residue. Now we know that methionine sulfoxide is not the sole product, implying that one cannot expect the total repair of methionine oxidation. Indeed upon photolysis induced oxidation, 100% of the final compounds were adducts methionine-photosensitizer [49]. All studies by time-resolved methods indicate the involvement of sulphur-centred free radicals, but some final compounds derive from carbon-centred ones ([36,49]. So far, no study was able to account for a possible higher stability of carbon-centred radicals with respect to sulphur-centred ones. Finally, the IET events involving Met and aromatics do not always take place despite a very favourable thermodynamic control. There is probably a need for new ways of approaching the subject, both experimental and theoretical.

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Highlights

- Methionine oxidation has been reinvestigated by time-resolved, electrochemistry and mass spectrometry methods
- The final compounds have been identified
- Methionine oxidation can be repaired by intramolecular electron transfer in peptides