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Biomimetic Chemistry on Tandem Protein/Lipid Damages under Reductive Radical Stress

Sílvia Atrian[a], Krzysztof Bobrowski[b], Mercè Capdevila[c], Chryssostomos Chatgilialoglu*[de], Carla Ferreri[d], Chantal Houée-Levin[e], Anna Maria Salzano[f], Andrea Scaloni[f], and Armida Torreggiani[d]

Abstract: The study of radical stress in the biological environment needs a comprehensive vision of all possible reactive species and their mechanisms. Among them, reductive stress is evaluated for its selective target of sulfur-containing compounds. The selective attack of reducing species like $H^{\bullet}$ atoms or e_{aq}^{-}/H^{+} to sulfur-containing amino acid residues has been proved in different substrates, peptides and proteins. The transformations include methionine to α-aminobutyric acid and cysteine/cystine residues to alanine, as recognized in several sequences so far, such as RNase A, lysozyme, Met-enkephalin, amyloid β-peptide and metallothioneins. The amino acid desulfurization is accompanied by the formation of low-molecular-weight sulfur-centered radicals that may cause geometrical *cis–trans* isomerization of unsaturated fatty acid residues in lipid bilayer. Thus, tandem protein/lipid damage is accomplished. Progress in research has given us a more comprehensive overview of the protein modifications and their roles, and the chemical biology approach will make its vital contribution to the study of free radical reactions, linking chemistry to biology and medicine.

Keywords: Lipids · Peptides · Radicals · Radiolysis · Reductive stress

1. Introduction

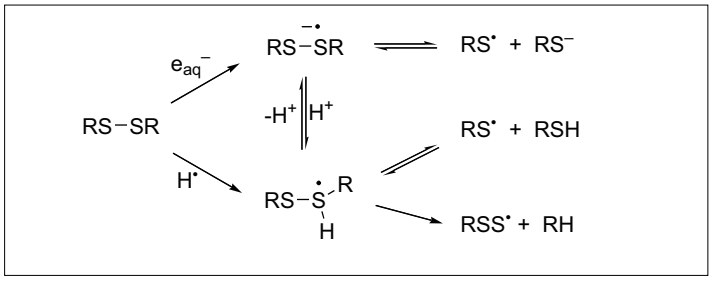
In living aerobic cells, free radicals are continuously generated by physiological processes, such as mitochondrial respiration and pathological conditions. By escaping the cell's antioxidant defences, free radicals can affect the structural and functional integrity of bio-molecules such as lipids, proteins and nucleic acids, leading to their modifications. These modifications are nowadays recognized to be involved in diseases such as cancer, as well as in the ageing process.[1] Exposure of proteins to free radicals may cause structural modifications of primary, secondary and tertiary assembly; consequently, the activity of enzymes, receptors and membrane transporters can be greatly affected.[2] The reaction of a protein with an attacking radical results in the generation of a radical center on the protein, which can be located in several different positions. In fact, due to the varied nature of the amino acid side chains, a multitude of possible sites are available for the attack, in addition to attack on the backbone. The nature of radicals formed on proteins depends on reactivity and redox properties of the attacking radicals, *i.e.* electrophilic radicals such as $^{\bullet}OH$ preferentially oxidize electron-rich sites. Also the presence of functional groups, which can stabilize the resulting radicals, can markedly affect the selectivity of the attack on side chains. The fate of radical species derived from protein damage has been subject of many investigations, although a clear picture of the degradation paths and their influence on the disease aetiology is far from being achieved. The most studied intermediates known to cause protein damage are reactive oxygen species (ROS) and in particular $^{\bullet}OH$ radicals,[2,3] whereas reductive stress has been less widely investigated.

The interaction of reducing reactive species derived from the ionizing irradiation of water, namely hydrated electrons (e_{aq}^{-}) and $H^{\bullet}$ atoms, with disulfides in aqueous solution is well understood.[4] Scheme 1 shows the disulfide radical anion ($RSSR^{\bullet-}$), derived from the direct electron attachment, to be in equilibrium with its protonated form, the sulfuranyl radical, which is also obtained by $H^{\bullet}$ atom attack to the sulfide moiety. Both reactive species dissociate reversibly into two entities, $RS^{\bullet}$ and RSH (or RS^{-}). EPR studies on the solid-state radiolysis of lysozyme[5a] and insulin[5b] showed that the electron adduct also affords irreversibly perthiyl radical (Scheme 1). This path may play an important role in the damage of proteins since $RS^{\bullet}$/RSH (or $RS^{\bullet}/RS^{-}$) do not diffuse apart.

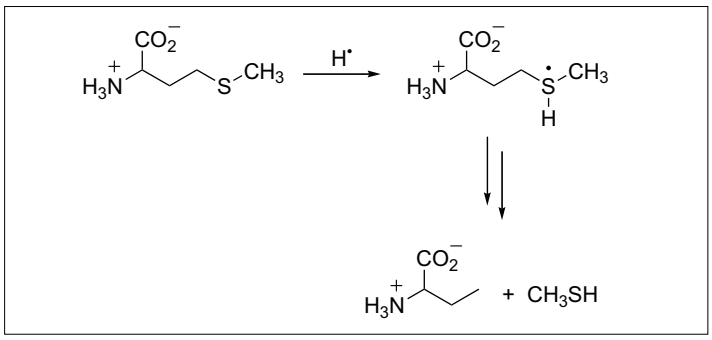
The reaction of $H^{\bullet}$ atoms with sulfur-containing amino acid residues is not limited to disulfide moieties. The reductive attack of $H^{\bullet}$ atoms on methionine (Met) is known to cause degradation as shown in Scheme 2, with formation of α-aminobutyric acid (Aba) and CH_3SH.[6]

To our knowledge, the involvement of the $H^{\bullet}$ atom in biology is restricted to its role as an intermediate in the ionizing radiation of water, whereas data are not available

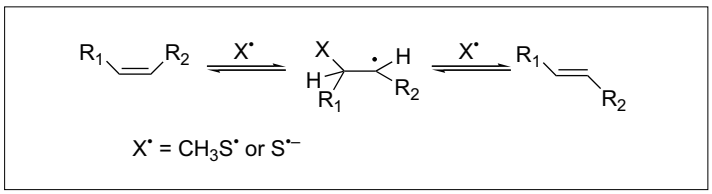
*Correspondence: Dr. Chryssostomos Chatgilialoglu[d]
Tel. : +39 051 639 8309
Fax : +39 051 639 8309
E-mail: chrys@isof.cnr.it
[a]Departament de Genètica, Facultat de Biologia
Universitat de Barcelona and IBUB
Av. Diagonal 645, 08028 Barcelona, Spain
[b]Institute of Nuclear Chemistry and Technology
Dorodna 16, 03-195 Warsaw, Poland
[c]Departament de Química, Facultat de Ciències
Universitat Autònoma de Barcelona
08193 Barcelona, Spain
[d]ISOF, Consiglio Nazionale delle Ricerche
Via P. Gobetti 101, 40129 Bologna, Italy
[e]Laboratoire de Chimie Physique, UMR 8000
Université Paris-Sud, 91405 Orsay cedex, France
[f]Proteomics & Mass Spectrometry Laboratory
ISPAAM, Consiglio Nazionale delle Ricerche
via Argine 1085, 80147 Napoli, Italy



Scheme 1.



Scheme 2.



Scheme 3.

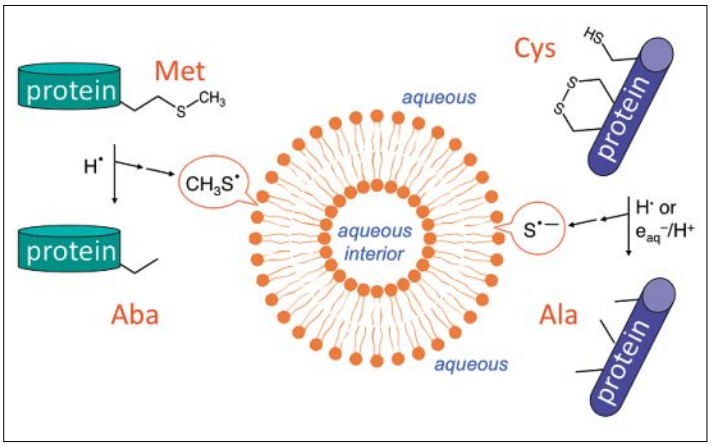


Fig. 1. Tandem protein–lipid damage under reductive radical stress. Sulfur-containing moieties like methionine (Met) or cysteine (Cys) are modified to α-aminobutyric acid (Aba) and alanine (Ala), respectively, by attack of H• or/and e_{aq}^- coupled H^+. Contemporaneously, the formation of diffusible sulfur-centered radicals such as $CH_3S^•$ or $S^{-•}$ able to migrate in the lipid bilayer, can induce *cis-trans* isomerization of unsaturated fatty acid residues.

in the literature on its presence in biological systems. However, its role has perhaps been underestimated, since, in chemistry, electrons are known to be efficiently converted to H• atoms when they react with protons (H^+), dihydrogen phosphate anions ($H_2PO_4^-$), hydrogen sulfide (H_2S) or ammonium salts (RNH_3^+), all compounds that are present in biological compartments at non-negligible concentrations.[7] On this basis, it is evident that specific research has to be done in this field to connect the chemical reactivity to the biological environment, including the scenario of reductive radical stress *in vivo*.

Reducing species like H• and e_{aq}^- have been found to produce specific damage to protein substrates in model studies that consists of a desulfurization reaction involving sulfur-containing amino acid residues.[8–13] This reaction corresponds to a mutation of the natural sequence. In fact, methionine residues are transformed to α-aminobutyric acid (Aba), whereas cysteine residues are mutated to alanine (Ala). Another relevant characteristic of this damage is that the amino acid desulfurization is accompanied by the formation of low-molecular-weight sulfur-centered radicals. Biomimetic studies based on liposome compartmentalization showed that these radicals are small and diffusible and can easily reach the lipid bilayer causing further damage. This second damage occurs at the level of the fatty acid residues of membrane phospholipids, in particular of the fatty acid unsaturations, which are known to be susceptible of attack by sulfur-centered radical, catalyzing *cis-trans* double bond isomerization.[14] Starting from *cis* unsaturated lipids, which are the naturally occurring isomers of membrane fatty acids, the reaction of methanethiyl ($CH_3S^•$) or sulfhydryl ($S^{-•}$) radicals converts this geometry to the corresponding geometrical *trans* isomer (Scheme 3).[15] The whole process, starting from the formation of H• or e_{aq}^-/H^+ is schematically reported in Fig. 1.

The specific reaction of lipid isomerization will be discussed elsewhere in this issue of CHIMIA, whereas in this article more attention will be given to protein damage and the research methodologies used for envisaging tandem protein–lipid damage. However, here we wish to recall briefly that the damaging effects of thiyl radicals emerged in the last decade,[16] counteracting the long-standing and most well-known beneficial effects of thiols. The damage effect starts from the consideration that, in the so-called 'repair reaction', cysteine residues can efficiently stop the radical cascade, by donating a hydrogen atom and trapping the radical intermediate of the chain, such as in case of lipid peroxyl radicals. However, by donating a hydrogen atom, the thiol (RSH) is transformed into its corresponding thiyl radical (RS•), which can in turn attack a substrate. For example, it can abstract a hydrogen atom from activated positions of biological molecules (amino acids, carbohydrates, nucleosides).[17]

The study of this subject requires different aspects to be considered that start with chemical reactivity and bring to the conclusive effects in the biological environment. The adequate context for this research is offered by the approach of chemical biology, which furnishes a combination of different disciplines and expertise. In the following paragraphs some aspects of the research in this field will be briefly summarized:

i) chemical and mechanistic aspects, dealing with the reaction sites and the resulting modifications;
ii) analytical aspects dealing with the identification and characterization of the modified substrates;
iii) expected biological influence of these modifications *in vivo*.

2. Chemical and Mechanistic Aspects of Lipid–Protein Damage

Biomimetic models can be very useful to address lipid–protein damage. Liposome technology is useful to simulate the membrane assembly and the organization of a lipid bilayer in aqueous environment under physiological conditions, in the presence of various amino acid and protein substrates. Liposomes made of a phospholipid-contain-

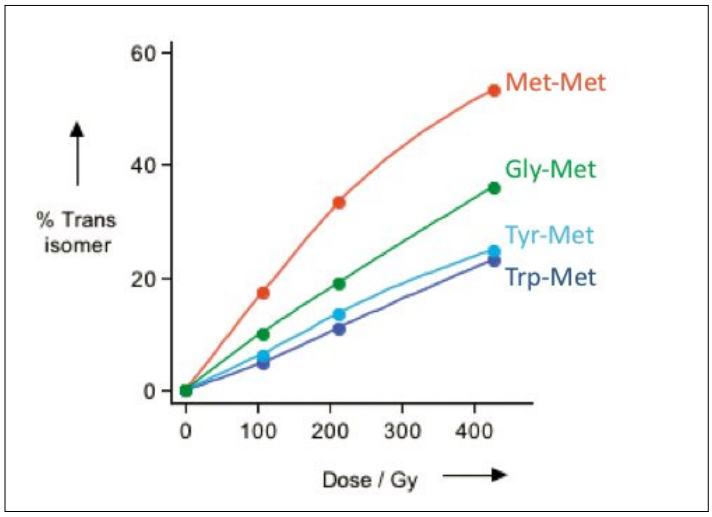


Fig. 2. Dose profile of the appearance of elaidate residues (% *trans* isomer) from γ irradiation of POPC vesicles containing 60 μM dipeptide and 0.2 M *t*-BuOH in 10 mM phosphate buffer at pH 7 and flushed with N_2

$$H_2O \rightsquigarrow e_{aq}^- (0.27), HO^\bullet (0.28), H^\bullet (0.062) \quad (1)$$

$$HO^\bullet/H^\bullet + t\text{-BuOH} \longrightarrow (CH_3)_2C(OH)CH_2^\bullet + H_2O/H_2 \quad (2)$$

$$e_{aq}^- + H_2PO_4^- \longrightarrow H^\bullet + HPO_4^{2-} \quad (3)$$

$$e_{aq}^- + N_2O + H_2O \longrightarrow N_2 + HO^- + HO^\bullet \quad (4)$$

ing unsaturated fatty acid moieties (such as oleic acid) have been used as an aqueous suspension in the presence of the desired amino acid (Met or Cys), peptide or protein. γ-Irradiation can produce selectively the reducing species, as explained below.[7]

Radiolysis of neutral water leads to e_{aq}^-, $HO^\bullet$ and $H^\bullet$ as shown in reaction 1. The values in parentheses represent the radiation chemical yields (G) in units of μmol J^{-1}. The experimental conditions can be tuned such that three short-lived species can be controlled and selected in their reactivity. For example, in deareated solutions in the presence of 0.25 M *t*-BuOH, $HO^\bullet$ radicals are scavenged efficiently whereas $H^\bullet$ atoms react only slowly with the alcohol (reaction 2, $k = 6.0 \times 10^8$ and 1.7×10^5 M^{-1} s^{-1}, respectively). The presence of phosphate buffer in the medium at physiological pH is also useful because a part of the solvated electrons will react with $H_2PO_4^-$ leading to $H^\bullet$ atoms (reaction 3). By saturating with N_2O, e_{aq}^- are efficiently converted into $HO^\bullet$ radicals (reaction 4). In N_2O-saturated aqueous solutions containing *t*-BuOH as $^\bullet OH$ scavenger, $H^\bullet$ atoms are the only reducing radical species.

As an example Fig. 2 shows the percentage of *trans* isomer formed as a function of irradiation doses using 10 mM phosphate buffer suspensions, 0.25 M *t*-BuOH and 60 μM of a dipeptide, deaerated by flushing N_2 at pH 7.[12] Under these conditions, reaction 3 plays an important role in converting e_{aq}^- to $H^\bullet$. In all cases the formation of the *trans* isomer increased nearly linearly with the dose exposure. The reaction of $H^\bullet$ atoms with methionine in dipeptides is analogous to the one shown in Scheme 2. By replacing Gly-Met with Met-Met, the dose profile of *trans* isomer formations doubled, indicating that the concentration of thiyl radicals is now also nearly doubled. On the other hand, in the cases of Tyr-Met or Trp-Met the percentage of *trans* isomer formation diminished due to the effective competition of primary radicals (mainly solvated electrons) with the aromatic moieties of Tyr and Trp that results in a lower $G(CH_3S^\bullet)$.

Another interesting case has been found with a pentapeptide, Met-enkephalin (Tyr-Gly-Gly-Phe-Met), which is known as an endogenous mediator interacting with opioid receptors, therefore with several biological activities in the field of pain control. The reactivity of this substrate under reductive conditions was to generate the corresponding desulfurated sequence, with the Aba instead of the Met residue, which is going to be studied for its biological effects compared to the natural sequence.[12] It is worth mentioning that pulse radiolysis experiments indicated that the Met residue is the main target. In Met-enkephalin the attack of the hydrogen atom occurs to about 50% on Met with formation of methanethiyl radical. The remaining percentage is divided roughly evenly between Tyr and Phe. With Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) the site of attack is limited to Tyr and Phe.

As protein models, lysozyme from chicken egg white (Lyso) and bovine pancreatic ribonuclease A (RNase A) were first used, since they are two enzymes with well-known structural features both containing Met residues (two and four, respectively) and disulfide bridges, whereas free thiol moieties are not present.[8–10] The proteins have been studied under several different conditions and tandem protein–lipid damage always occurred, thus confirming the interest in such degradations. Some insights of the structural changes detected in RNase A will be given in the next section of analytical techniques.

Successively, other proteins have been tested. The sequence of amyloid β-peptide of 40 amino acids, where only the Met residue is present as an S-containing amino acid, was studied, also for its connections with the alteration reported in Alzheimer's disease. In this case, reductive stress was able to produce the selective desulfurization of this sequence, and the identification of the resulting Aba-modified peptide has been possible.[11]

Lately, metallothioneins (MTs), small proteins extremely rich in cysteines, have been analyzed. This high-Cys content, up to 30% of their amino acids, confers them an exceptional capacity to coordinate heavy metals through the formation of metal–thiolate bonds. The presence of labile sulfide anions as non-proteic ligands was reported in the metal–MT complexes.[18] Therefore, the presence of both sulfur-containing amino acids and sulfide anions in the same molecular aggregate makes this system a very interesting case to determine the main targets of the reductive stress. Interestingly, a differentiation of reactivity can be assessed, since the reductive conditions seem to select the methionine and cysteine residues.[19]

In all these experiments the peptide/protein damage was found to be coupled with the unsaturated lipid isomerization and formation of *trans* fatty acids in the vesicles (*cf.* Figs 1 and 2). Indeed, liposomes containing double bonds are a very sensitive reporter system of the formation of diffusible thiyl radicals, since *trans* isomers are formed through the catalytic activity of S-centered radicals as isomerizing agents. This system can be proposed as a first screening of the protein/peptide potential damage at the level of S-containing residues, in order to proceed with analytical identification of the exposed sites of radical attack along the sequence.

3. Methods for Identification and Characterization of Radical-modified Protein Substrates

Radical damage is certainly part of the metabolic degradation that leads to cell death, and in this context the consequences of the reductive radical damage have not

been clearly defined. An important part of this research deals with the construction of molecular libraries of modified substrates. By using biomimetic models, the system represents a simplification of the cell environment that allows the reaction products to be isolated and identified, and provide a key for understanding radical stress *in vivo* by the use of the molecular libraries.

In examining tandem protein–lipid damage, an array of techniques is necessary, either for the lipid analysis (see above) or for the characterization of amino acid and protein modifications. Protein analysis needs the combination of various techniques, especially if the conformational aspects have to be addressed. Some of them are described here below.

3.1. *Raman Spectroscopy*

Investigation of protein structure by Raman spectroscopy has been practiced for more than three decades, during which specific band assignments, signatures of secondary structure and Raman markers of side-chain environments have been established.[20] The assignments are usually based on model compounds (*e.g.* amino acids or short peptides) and may be universal for the whole class of molecules (*e.g.* amide bands of proteins are assigned to polypeptide backbone modes). The peptide group of polypeptides and proteins gives rise to many representative Raman bands (amide I-VII); some of them are particularly useful. The best studied Raman band of proteins is the amide I band appearing between 1600 and 1700 cm^{-1}, which arises from the stretch vibration of the peptide C=O group. Because the C=O group is involved in different secondary structure elements *via* hydrogen bonding to the peptide NH group, the experimentally observed amide I band envelopes a multitude of single bands with different wavenumbers which can be resolved in many components attributable to different secondary structures. Thus, Raman spectroscopy can be used to quantify the contribution of distinct secondary structure motives to the overall protein structure. Both oxidative and reductive radical stress have been demonstrated to cause a similar trend of conformational changes in sulfur-containing proteins. Generally, a sensitive increase in the β-sheet percentage of proteins and a decrease in α-helix content have been observed. In addition, progressive unfolding of the helical structure due to the rupture of disulfide bridges and the loss of intra-molecular Tyr H-bonds has indicated a higher denaturizing ability of $H^{\bullet}$ than that of $OH^{\bullet}$ radicals.[21]

The analysis of the side-chain marker bands visible in Raman spectrum of proteins can give much information, for example on the side-chain environment of some amino acid residues (*i.e.* Tyr, Trp), on metal binding (*i.e.* His), hydrogen bonding and susceptibility of radical attack.[22] In addition, all the three states of Cys (free, coordinated to a metal, or oxidized with the setting up of a disulfide bridge (cystine)) can be evidenced by Raman spectroscopy in the spectral region typical of sulfur-containing moiety vibrations.[23a] Similarly, methionine sulfoxide in proteins can be visualized.[23b] The protein structure and amino acid content play a significant role in blocking the ready access of free radicals both to the sulfur-containing residues and the active site, strongly affecting both the radio-sensitivity of proteins and the potential of the tandem radical damage. For example, the lowest irradiation dose is enough to cause changes in Met residues of RNase A, whereas similar modifications are visible in lysozyme only at the highest dose.[9] However, Met and Cys have been generally shown to be among the most sensitive residues towards radical attack, as indicated by the splitting of the ~725 cm^{-1} band (~730 and 725 cm^{-1}), as well as in Cys bands (S–S: 500–540 cm^{-1}; M–S: <400 cm^{-1}) (Fig. 3, curve c). With respect to the Met radical-damage, the radical attack on these residues can take place both by $^{\bullet}OH$ radicals, leading to oxidation products or transfer of oxidizing equivalents to Tyr with phenoxyl radical formation,[24] and by $H^{\bullet}$ atoms, giving rise to generation of thiyl radicals ($CH_3S^{\bullet}$).

Tyr residues have also been shown to be sensitive towards radical attack. This conclusion has been taken from the analysis of the doublet at 850–830 cm^{-1} that depends on the state of the hydrogen bonding involving the OH group of Tyr (Fig. 3).[20] The $^{\bullet}OH$ species is generally considered to be the primary radical responsible for Tyr modifications, although $H^{\bullet}$ atoms can also attack these aromatic residues, giving rise to intra- and inter-molecular reactions. In addition to the direct radical attack on these residues, Tyr can be oxidized through intra-molecular electron transfer that can be initiated by other residues, such as Trp.[25] Proteins have been also irradiated in Ar-deaerated phosphate buffer solution. The presence of phosphate buffer, as the medium at physiological pH, helps to keep the native conformation of a protein. Moreover, it is also useful to increase the reducing species concentration. In fact, under these experimental conditions the reducing species present are two, hydrated electron and $H^{\bullet}$ atom (reaction 3). Unfortunately, the presence of the phosphate buffer in the samples makes a deep analysis of some spectral regions impossible, such as 1640–1670, 870–1100, and 500–570 cm^{-1}. Also in these experimental conditions the spectral modification visible in the bands due to disulfide bonds and Met were very similar to those observed in aqueous solution, further confirming that these residues are the preferential site of a reductive radical attack (Figs 3c and 3d).

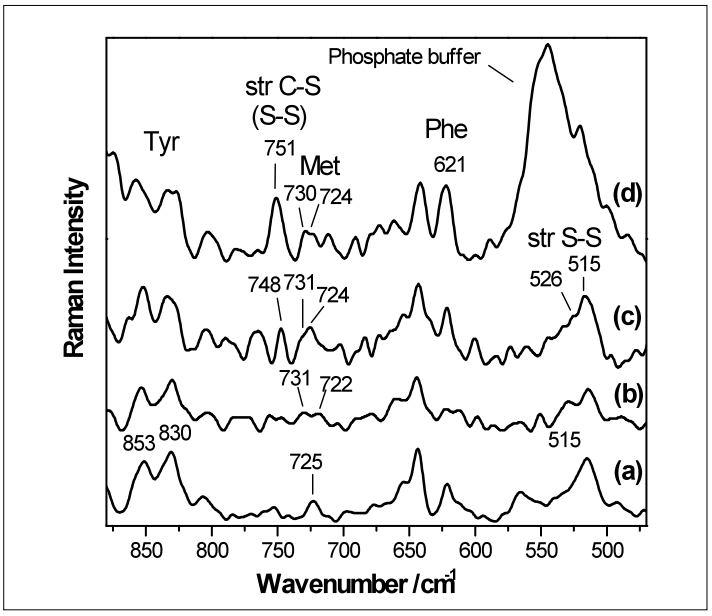


Fig. 3. The 880–480 cm^{-1} Raman region of native RNase A (a) and low dose-irradiated RNase A samples (23 Gy) obtained from: (b) N_2O-saturated RNase A solution ($OH^{\bullet}$, $H^{\bullet}$); (c) N_2O-saturated *t*-BuOH-containing RNase A solution ($H^{\bullet}$); (d) Ar-deaerated *t*-BuOH-containing RNase A buffer solution ($H^{\bullet}$, e_{aq}^{-}).

3.2. *Mass Spectrometry*

Mass spectrometry (MS) has been largely used in the past years for the study of free radical reactions affecting proteins. The wide use of MS methodologies is based on the observation that these reactions determine a specific molecular mass variation in the resulting protein products by causing a covalent modification of the amino acids, which is easily detectable by accurate mass measurements with respect to the untreated polypeptide material. In this respect, oxi-

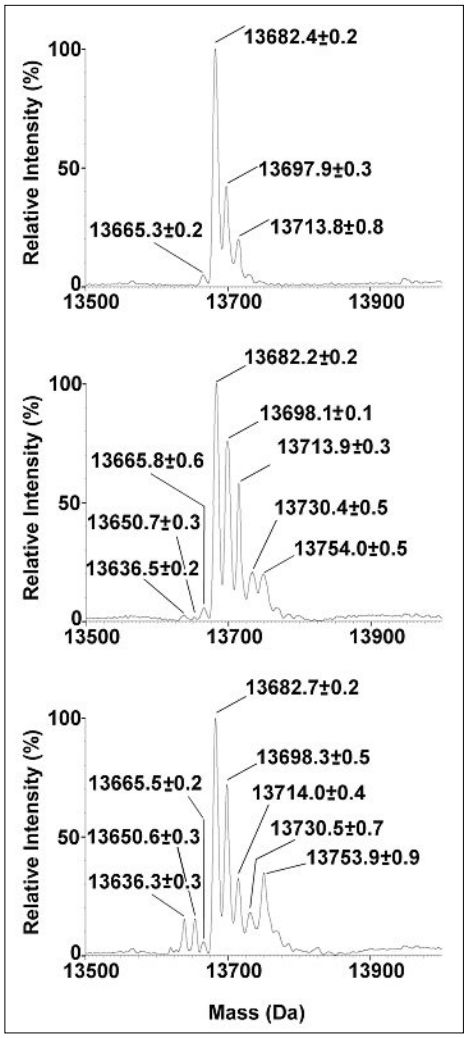


Fig. 4. ESI-Q-TOF-MS analysis of RNase A samples irradiated in 0.2 M *t*-BuOH with 0 (upper), 84 (middle) and 650 Gy (lower). Components A, B, C, D, E, F, G and H corresponded to native RNase A and protein species showing a Δm = +16, +32, −17, −46, −32, +48 and +72 Da, respectively, with respect to native RNase A.

dative modifications produced by ROS (reactive oxygen species) and RNS (reactive nitrogen species) have been widely investigated for a number of biological relevant protein targets in the course of both '*in vitro*' and '*in vivo*' studies.[26,27] In contrast, reductive modifications of proteins have not yet been extensively characterized.

Depending on the widely differing instrumentation in MS laboratories, bottom-up experiments are generally conducted,[28] performing analysis either on the intact protein products and on peptide digests. At first, the analysis of the intact irradiated protein sample is generally accomplished, trying to directly detect possible mass changes with respect to the starting material. Accurate mass spectrometric determinations are needed to detect the reductive modifications determining small changes in the protein molecular mass. High resolution mass spectrometers, such as electrospray-quadrupole-time of flight (ESI-Q-TOF) instruments, are widely used for this purpose. Specific mass differences can be tentatively ascribed to a certain protein modification on the basis of the exact molecular mass of the atoms introduced/removed. As an example, the mass spectra recorded for irradiated RNase A showed the presence of molecular species differing –46, –32 and +72 Da with respect to the untreated sample (Fig. 4).[13] On the basis of the recent findings on $H^{\bullet}$ reactivity towards sulfur-containing amino acids, the first two species were tentatively ascribed to the desulfurization of Met to Aba and of Cys to Ala, respectively. The species with Δm = +72 Da was tentatively associated with the addition of the *t*-butyl moiety from *t*-BuOH, used as an additive to the irradiation buffer. Since intact protein products originating from the same polypeptide species present very similar ionization properties, the quantification of their relative abundance as result of different experimental conditions (various buffers, additives, variable irradiation conditions/doses) can be obtained. In this sense, comparative quantitative experiments for modified products based on MS experiments provided useful information on the reactivity exerted by radical species towards proteins, helping to depict the molecular mechanism of these reactions.[29] Similarly, a different reactivity has been ascertained for RNase A after reductive irradiation in the presence of phosphate buffer. Under these conditions, desulfurized products were only detected at higher irradiation doses, while *t*-butyl adducts were always produced in lower amounts.

Furthermore, a punctual characterization of the reductive modifications occurring on the protein primary structure is necessary for a better comprehension of the molecular damage produced; to this purpose, an in-depth analysis at peptide/amino acid level has to be performed. Depending on the nature and the stability of the protein modification adducts, different MS approaches have been developed for the detection of specific modified peptides within peptide mixtures resulting from protein digestion with proteases or reagents with a high sequence specificity. Routine use of matrix-assisted laser-induced desorption ionization (MALDI-TOF) and ESI MS in the course of peptide mass fingerprinting experiments have allowed the identification of modified peptides in these protein digests, thus limiting the occurrence of modifications to specific regions within protein primary structure. Also in this case, the experiments are based on the comparison of the theoretically expected and experimentally determined molecular mass values of the peptide species.

High sequence coverage is a pre-requisite for the comprehensive detection of the protein modifications. To this purpose, experiments performed both with MALDI and ESI-based mass spectrometers are routinely performed, because some peptide species may only be revealed by a specific ionization technique, as result of the possible occurrence of ionization suppression phenomena.

Finally, the definitive assignment of the modification to specific amino acid residues is achieved by MS/MS experiments on peptide mixtures resulting from protein digestion, based on fragment fingerprinting upon collisional fragmentation approaches.[27] Based on the occurrence of specific variations of the mass signals within the fragmentation spectra of the modified peptides, these experiments assign the modification to a specific amino acid residue. As an example, the MS/MS-based structural characterization of the peptide digest from irradiation-induced reductive modification of RNase A demonstrated that only three out of eight Cys residues present within the whole protein primary structure were transformed into Ala (Fig. 5), thus proving that two unique disulphide bonds were selectively reduced during the reaction. These experiments also established that a unique Met residue, namely Met79, was reduced to Aba, while the remaining ones were oxidized with a variable degree, probably as a consequence of the sample manipulation.[13] Similarly, the nature of the Tyr residues affected by the addition of the *t*-butyl moiety from *t*-BuOH added as additive to irradiation buffer was also ascertained.

The MS-based data available in the literature on modified residues within the protein structure resulting from oxidative/reductive reactions exerted by reactive radical species well underline that the three-dimensional structure of the protein is an important factor, which may influence reactivity. To reach an in-depth knowledge of all parameters affecting these reactions, a critical estimation of all topological and physico-chemical characteristics of the protein under investigation has to be considered in advance and further evaluated in comparison with the data resulting from MS experiments.

Another example of less complexity is represented by the amyloid β-peptide (Aβ) that is also meaningful for the connection to Alzheimer's disease. The structure of 40 amino acids, which is the major form in the plasma, was studied in the natural and the reverted amino acid sequences, after exposure to gamma irradiation in aqueous solution. In reductive conditions, it was possible to demonstrate the specific attack to the Met35 residue, with formation of the corresponding mutated sequence, containing Aba instead of Met.[11]

When studying the effect of irradiation on metalloproteins,[20] using the example of the cadmium complexes formed by the

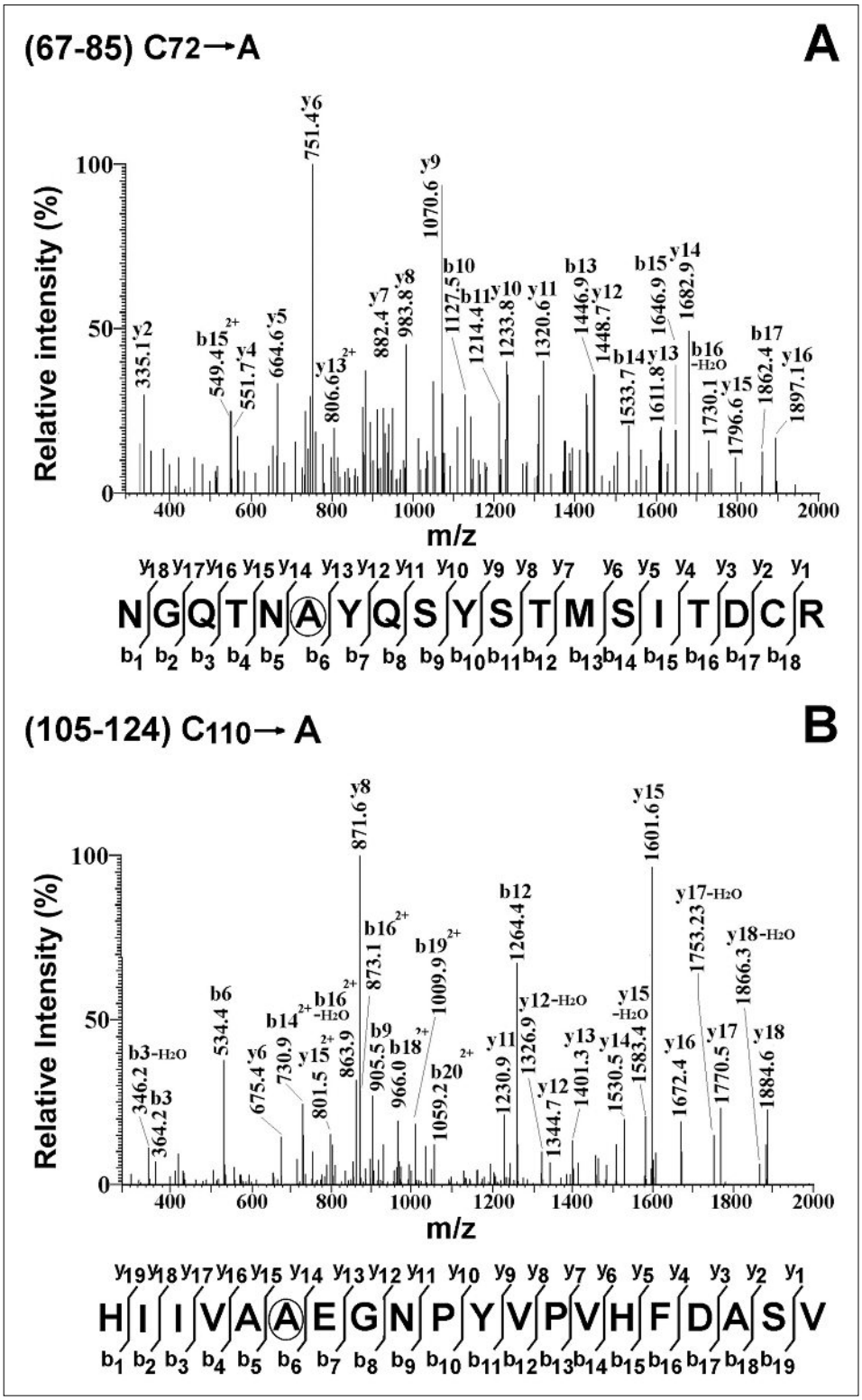


Fig. 5. MS/MS analysis of modified peptides in tryptic digest of RNase A irradiated in 0.2 M *t*-BuOH with 650 Gy. Fragmentation mass spectra of doubly charged ion at m/z 1099.5 and doubly charged ion at m/z 1068.5 are shown in panel A and B, respectively.

cork-oak metallothionein, ESI-MS analysis of pre- and post-irradiated samples showed that two kinds of alterations were generated: i) small variations of their mass that could be interpreted by desulfuration of some Cys or Met residues to Ala or Aba, respectively, and ii) massive molecular mass variations, compatible with the cleavage of the polypeptide backbone, so that the stronger the irradiation used, the shorter the generated peptide fragments. Surprisingly, in this case, the entity of the metal-MT complexes was maintained at pH 7 by the metal–thiolate bonds, and protein fragmentation was not observed unless the sample was subjected to acid pH (2.0), when cadmium ions are released from the protein. Therefore, in the case of metalloproteins, protein cleavage can be masked at pH 7.0 by the maintenance of metal–peptide bonds.

4. Expected Biological Consequences

The effect of radical stress on the cell metabolism and functions is a very active field of research connecting various disciplines in life sciences. Various examples are reported in the literature describing the effects of strong reductive stresses altering the cellular redox environment, such as the apoptosis during radiosensitizer use,[30] or the abnormal protein aggregation associated to cardiomyopathy.[31] The chemical mechanism involving sulfur-containing residues under reductive conditions discloses new scenarios that result in the loss of sulfur moieties, in particular due to a desulfurization reaction with formation of diffusible thiyl radical species. The harmfulness of sulfur-centered radicals has been demonstrated in the last decade and it is also discussed in the other papers of this CHIMIA issue. On the other hand, what are the possible consequences of amino acid mutation due to desulfurization? Cys to Ala desulfurization is an enzymatic process occurring during the production of elemental sulfur assisted by pyridoxal phosphate-dependent desulfurases NifSs,[32] but it is also known to be a chemical mutation, used formerly to study t-RNA amino acid triplet modifications.[33] Alanine is a genetically coded amino acid, therefore the mutation of Cys to Ala, which occurs by the radical process so far discussed, corresponds to a post-translational modification. Research on such mutations, which are also connected to free radical stress, will be important to contribute to a complete picture of the degeneration associated to diseases and aging. Analogously, the novel mutation occurring to Met residues as shown by reductive stress indicates other possible pathways of damaging a sequence, transforming a natural amino acid into a natural, non-genetically codified congener. α-Aminobutyric acid (Aba) corresponds to a homologation of the alkyl chains normally present in genetically codified amino acids, such as methyl (in Ala) and isopropyl (in Leu), with an ethyl unit. This modification can therefore be studied in order to understand the effects on the interaction with receptors, and more generally for its consequences on the structure–activity relationship based on alkyl substitutions.

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