A Biomimetic Model of Tandem Radical Damage Involving Sulfur-Containing Proteins and Unsaturated Lipids

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Lipidomics research, which focuses on the global changes in lipid metabolites, has recently been concerned with the type and roles of unsaturated lipids in the biological environment. The structural change induced by their conversion from the naturally occurring *cis* fatty acid geometry to the more thermodynamically stable *trans* configuration can affect membrane arrangement as well as lipid metabolism.[1] In the biomimetic model of thiyl radical-catalyzed isomerization of *cis* phospholipids, it was shown that when thiyl radicals are generated in the aqueous compartment and are able to diffuse in the lipid bilayer, then the interaction with unsaturated fatty acyl chains efficiently produces *trans* double bonds.[2] These findings suggested that radical-based degradation of sulfur-containing amino acid residues that are known to release diffusible thiol molecules could be the primer for tandem radical damage involving protein and lipid domains. We modeled such damage using γ irradiation of lipid vesicle suspensions containing bovine pancreatic ribonuclease A (RNase A). The reaction of this protein with H$^{\bullet}$ atoms was studied, and the inactivation was connected to the specific damage of sulfur moieties with release of low-molecular-weight thiols.[3]

Liposomes were prepared by using dioleoyl phosphatidyl choline (DOPC) in the form of large unilamellar vesicles (LUVET) of 100 nm diameter.[4] The protein was added to the LUVET suspension and saturated with N_2O prior to irradiation at a dose rate of 14.5 Gy min^{-1}. 100 μL aliquots of the suspension were withdrawn at different irradiation times, over the interval of 2–70 min, for lipid isolation and derivatization to the corresponding fatty acid methyl esters.[5] This was followed by GC analysis to determine the *cis*/*trans* ratio. The solid circles in Figure 1 show the percentage of *trans* isomers (elaidate residues) formed as a function of irradiation dose. Control experiments in the absence of RNase A or by replacing RNase A with a protein without sulfur-containing amino acids, such as histone H1 type IIA from calf thymus, did not show any isomerization.

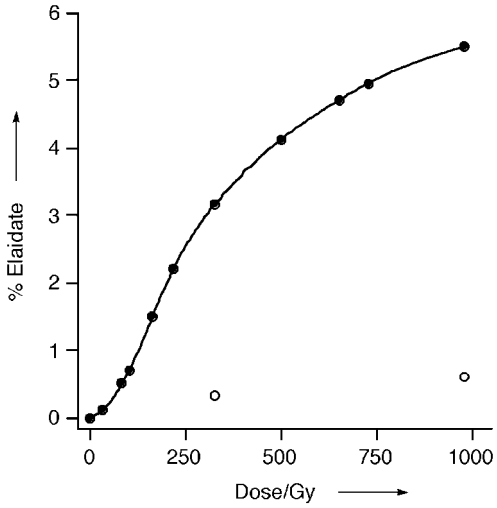

Figure 1. *Dose dependence of the appearance of elaidate residues in the isomerization of DOPC by γ irradiation of LUVET suspensions containing RNase A (●) or RNase T1 (○). The line is an interpolation of the data.*

In parallel, we followed changes in the enzyme activity,[6] as well as the transformation of the sulfur moieties by Raman spectroscopy, using lyophylized samples of aqueous solutions of native and irradiated RNase A. As expected from the radiation-induced inactivation,[3] a residual enzymatic activity of 67% was found after exposure to only 33.3 Gy, followed by a slower decrease of the activity, which reached 50% after 500 Gy. In the Raman spectra, the S–S and C–S stretching bands are visible in the 420–780 cm^{-1} spectral range. Native RNase A has four disulfide bridges that give rise to two different disulfide bands ($\nu_{S\text{-}S}$) at 514 and 535 cm^{-1}, and four methionine residues that exhibited a band ($\nu_{C\text{-}S}$) at 727 cm^{-1} (Figure 2A).[7] Exposure of the protein at low irradiation doses (23 or 61 Gy) produced small modifications in the spectral features of the $\nu_{S\text{-}S}$ bands, as well as of the $\nu_{C\text{-}S}$ peak at 652 cm^{-1} that originated from cystines, as shown in Figure 2B and C. On the other hand, 23 Gy of irradiation was enough to cause significant changes in the C–S bonds of methionines, as indicated by the splitting of the 727 cm^{-1} band into two components at 719 and 729 cm^{-1}; this suggests that a profound change in these sulfur-containing residues had taken place.[8,11]

The reaction sequence started from primary water radicals obtained by γ radiolysis of aqueous suspensions.

$$H_2O \xrightarrow{\gamma} e_{aq}^{-} + HO^{\bullet} + H^{\bullet} \quad (1)$$

The presence of N_2O efficiently transformed hydrated electrons into HO$^{\bullet}$ radicals.

$$e_{aq}^{-} + N_2O \xrightarrow{H^+} N_2 + HO^{\bullet} \quad (2)$$

Under these conditions, H$^{\bullet}$ and HO$^{\bullet}$ accounted for 10 and 90%, respectively, of the reactive species.[12]

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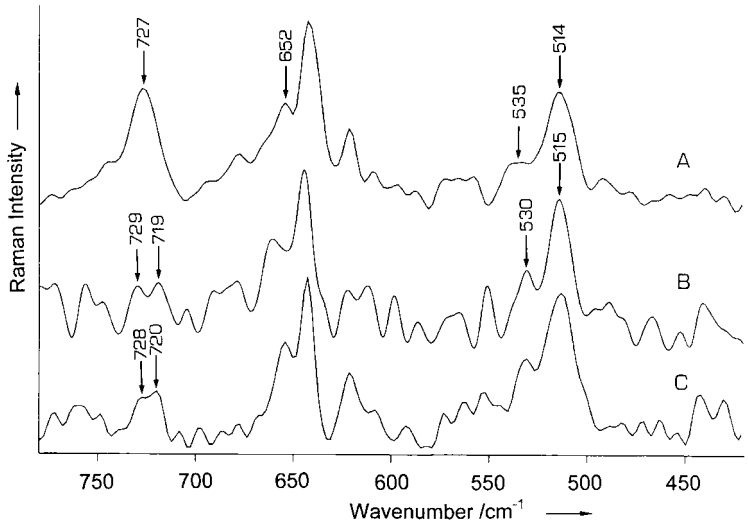
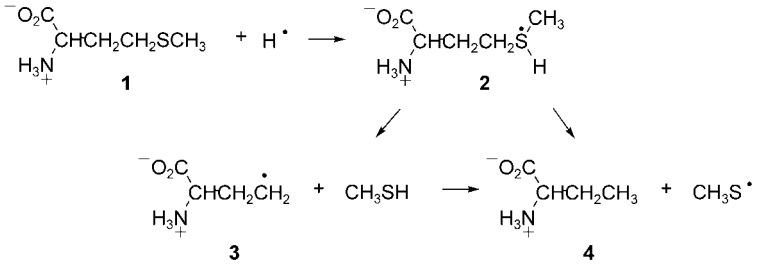


Figure 2. *Raman spectra of A) native RNase A and γ-irradiated RNase A with doses of B) 23 and C) 61 Gy.*

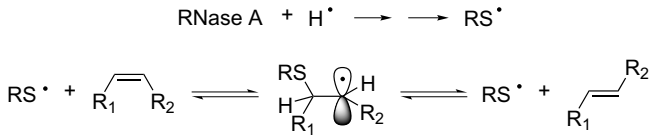
The interaction of HO• with RNase A can involve different sites, such as aromatic (tyrosine and phenylalanine) and sulfur-containing (cystine and methionine) residues, thus contributing to the enzyme inactivation.[13] On the other hand, the effectiveness of H• atoms for the inactivation of RNase A was reported together with their selective attack on cystine and methionine residues resulting in the formation of alanine and α-aminobutyric acid.[3] We suggest that the initial attack of H• on the disulfide and/or methionine residues afforded diffusible thiyl radicals in the aqueous compartment. By examining the initial time course of *trans* lipid formation in Figure 1, an induction period can be observed; this indicates that the formation of thiyl radicals is not a straightforward process. These thiyl radicals enter the bilayer, then reach and isomerize the lipid double bonds from *cis* to *trans* (Scheme 1).[14]

In order to gather some indications on the origin and nature of the isomerizing species, the monitoring of vesicle irradiation enclosing RNase T1, which is a ribonuclease enzyme containing only two disulfide bridges and no methionine residues, was also carried out. The open circles in Figure 1 show that formation of the *trans* isomer is only 0.6% after 1 kGy. Conformational differences between RNase A and T1 might explain the observed different behavior, or it can be suggested that the methionine moieties of RNase A[15] are the major source of diffusible thiyl radicals. In order to mimic this situation further, a N_2O-saturated DOPC suspension containing 60 μM methionine and 0.20 M *t*BuOH was exposed to 1 kGy irradiation. Under these conditions, HO• radicals are scavenged by *t*BuOH.[16a] It can also be calculated that ~25% of H• atoms escaped from *t*BuOH and reacted with methionine[16b] to afford 35% of *trans* isomer. Based on these observations and early work on the reaction of methionine (**1**) with H• atoms[19] in which the main product was α-aminobutyric acid (**4**), we propose that the H• atoms attack the thioether moiety of **1** to give the sulfuranyl radical **2**, which undergoes unimolecular cleavages to afford **3** and/or **4** and the thiyl radical, that is, the diffusible isomerizing radical species (Scheme 2).



Scheme 2. *Proposed mechanism for the formation of thiyl radicals from methionine under γ irradiation.*

In summary, the model system of DOPC vesicles containing RNase A offered the first evidence of tandem radical damage that is transferred from the protein to the lipid domain. Our model of the degradation of sulfur-containing amino acid residues in vesicles supports the hypothesis of an endogenous formation of *trans* lipids occurring during cellular radical stress, a pathway that is complementary to the dietary contribution. These findings are relevant to the study of in vivo *trans* lipid formation, since several reports in the literature of the last decade deal with the harmfulness of *trans* lipids taken from the diet.[20,21] Further work is in progress on the application of *trans* lipids as markers of radical damage to methionine-containing proteins; they can be envisaged as a sensitive analytical tool based on the amplification of the damaging potential of low-molecular-weight thiols through the catalytic cycle of *cis*–*trans* isomerization.



Scheme 1. *Thiyl radical-catalyzed isomerization of* cis *phospholipids.*

Experimental Section

LUVET were prepared as earlier described[2,14a] by using DOPC (1.9 mg, 0.0025 mmol) in water (950 μL) purified with a Millipore (Milli-Q) system. A protein solution (0.6 mg in 50 μL of Milli-Q water) was added to the LUVET suspension. The suspension was then transferred to a vial equipped with an open-top screw cap and a Teflon-faced septum, and saturated with N_2O prior to γ irradiation. Workup and analysis of the irradiated reaction mixture was carried out as previously reported.[2,14a]

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Keywords: isomerization · lipids · liposomes · protein damage · radicals · Raman spectroscopy

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[8] Significant changes also occurred in other characteristic regions of the Raman spectra upon irradiation (data not shown), for example, the doublet intensity ratio of the 830 and 850 cm^{-1} bands decreased substantially; this is associated with the tyrosines residues.[9] The conformational changes of RNase A were evaluated by using a method based on a combination of X-ray determined structures and Raman data.[10] After 23 Gy of irradiation, the α-helix percentage decreased from 18% to about 12% and the β-sheet percentage increased from about 48% to 54%. Increasing the dose to 61 Gy did not cause further conformational changes. It is worth noting that Met13, Met29, and Met30 are located in the α1 and α2 helices.

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[15] Three of the four methionines in RNase A are partially exposed on the surface.

[16] a) The rate constants of reaction of the $HO^{\bullet}$ radical with *t*BuOH[12] and methionine[17] are $6.0\times10^{8}\ M^{-1}s^{-1}$ and $2.3\times10^{10}\ M^{-1}s^{-1}$, respectively; b) The rate constants of reaction of the $H^{\bullet}$ atom with *t*BuOH[18] and methionine[17] are $1.1\times10^{6}\ M^{-1}s^{-1}$ and $\sim10^{9}\ M^{-1}s^{-1}$, respectively.

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