Radiation Physics and Chemistry xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Radiation Physics and Chemistry

journal homepage: www.elsevier.com/locate/radphyschem

Radicals initiated by gamma-rays in collagen and its main components

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ARTICLE INFO

Keywords:
Collagen
Gamma-rays
EPR spectroscopy
Radicals
Radiation sterilization

ABSTRACT

Radical products generated by gamma-rays were identified in collagen and in microcrystalline powders of glycyne, L-proline and L-hydroksyproline. Reagents irradiated at 77 K were studied by EPR spectroscopy in the range of 100–350 K using temperature control system. Two radical centers found in collagen were localized in proline ring at α and γ positions to the carbonyl group. There were neither terminal radicals generated by chain scission nor transient products disrupting hydrogen bonding system of irradiated collagen.

1. Introduction

Skin allographs are valuable biomaterial which might be used as a wound dressing in the case of skin burns, trophic ulcers or tumors. Cell-free allographs preserve native porous architecture and maintain the original chemical structure of the collagen matrix. In order to apply the material for biomedical purposes, sterilization is required as a preliminary treatment prior to further cell culture. It is generally accepted that exposure to ionizing radiation is the most certain and reliable process to eliminate pathogens in this type of materials. However, some reports in literature suggest that radiation sterilization of the collagen based materials results in their degradation followed by the deterioration of mechanical properties, increase in susceptibility to enzymatic digestion and to dissolving in neutral and acidic media. The adverse macroscopic consequences initiated by the generated radicals result from the modification of collagen structure as well as from the development of oxidative degradation (Headlam et al., 2000). Similar processes are created under native conditions in skin via metabolic pathways and as a result of external factors, i.e. exposure to ultraviolet radiation (e.g. Herrling et al., 2006).

Collagen is a simple protein, composed of three α-helixes wound together in a triple helix. It is the most abundant component of skin. When exposed to ionizing radiation to eliminate bioburdens, collagen might partially loss functionality due to radiation induced modification. Generally, radical processes might cause removal of some functional groups, e.g. amine groups, disruption of hydrogen bonding system, insertion of new, usually oxygen containing substituents, etc. The changes have influence on the spatial structure of the protein. The severe disruption results in loss of some important features what makes the studies on irradiated collagen vital not only from radiation sterilization point of view but also due to radiation protection and radiotherapy issues. This is the reason why radical processes in collagen are important both for basic science and for biomedical applications.

Chipara et al. (2003) investigated radical processes of collagen irradiated with protons at room temperature. The superposition of anisotropic singlet of peroxyl radical and quintet of alkyl radicals were confirmed by EPR spectroscopy. Identification of the second species proved that scission of macromolecules was an important consequence of irradiation. Spin trapping technique has been widely used in order to investigate short-lived radicals in collagen and its components in aqueous solutions (Suzuki et al., 1981; Matysik et al., 2002; Headlam et al., 2000). Irradiation of L-proline and 4-hydroxy-L-proline solutions triggers reactions leading to the opening of the pyrrolidine ring or to hydrogen abstraction from methylene groups. In aqueous solutions the indirect effect of ionizing radiation is observed thus the results can not be straightforwardly extrapolated to the anhydrous conditions.

The problem was also recognized in relation to UV irradiation which destabilizes collagen biomolecule initiating skin aging and wrinkling (Metreveli et al., 2005, 2006a, 2006b; Herrling et al., 2006). Many studies performed in aqueous media have shown that α-helix of a single chain is more sensitive to damage than the triple helical structure of the peptide. EPR studies of the collagen aqueous solutions frozen at 77 K, and then UV irradiated, has demonstrated septet of hyperfine splitting 1.12 mT assigned to the proline residue. At elevated temperature the intermediate has been converted to glycine radical. The species created by light in frozen aqueous solutions does not necessarily are formed in lyophilized collagen irradiated with gamma-rays.

Free radicals in amino acids occurring in the collagen and their derivatives were extensively studied in the past. The results suggest that the unpaired spin centers are usually localized at carbon atoms as

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http://dx.doi.org/10.1016/j.radphyschem.2017.03.034
Received 26 October 2016; Received in revised form 6 February 2017; Accepted 21 March 2017

a consequence of dehydrogenation, release of amino group or carbon dioxide (Aydin and Osmanoglu, 2011; Box et al., 1966, 1977; Strzelczak et al., 2009; Gramza et al., 1997).

Structure of amino acids significantly influences radical processes. Depending on thermodynamic features, the molecules can exist either in canonical or zwitterionic forms. The second structure promotes ionic reactions what might influence the final effect of exposure to gamma-rays. The stability of the glycine zwitterion conformers is lower than proline and depends on water contents (Yang et al., 2016). It was found that proline zwitterion is conformationally predominant if the molecule is hydrated by at least 5 water molecules which form inner shell interacting with amino and carboxylic functional groups. The same relationship was suggested by Kokpol et al. (1988) for glycine.

The studies reported were focused on the elucidation of radiation initiated damages in collagen and in amino acids occurring in the highest concentration in the protein, namely glycine (G), proline (P) and hydroksyproline (HyP). The Electron Paramagnetic Resonance (EPR) investigations were performed under cryogenic conditions and upon gradual warming of the samples to the selected temperatures in order to stabilize primary products, and then convert them into the secondary radicals. When interpreting collagen spectra recorded at various temperatures, the results obtained for amino acids making up the peptide chain were taken into account. The goal of the study was to compare the radicals formed in collagen with those formed in its main components, to confirm if radical centers of collagen are located at main chain or beyond backbone and if under experimental conditions identification of primary species is possible.

The studies were conducted in the air atmosphere in order to reproduce conditions usually prevailing at the time of radiation sterilization.

2. Experimental

The studies were performed using collagen extracted from calf skin and amino acids being the major residues of the protein. Collagen and glycine were purchased from Sigma Co. L-hydroxyproline was supplied by BDH Biochemical, L-proline by CALBIOCHEM, DL-proline by L. Light & Co. Ltd. The amino acids were in the form of polycrystalline powder, whereas collagen was a reagent prepared by a freeze-dried procedure.

The samples in sealed quartz tubes were exposed to gamma-rays in a BRIT Gamma Chamber 5000 at a dose rate of 4.1 kGy/h. The irradiation was performed at liquid nitrogen temperature with a dose of 5 kGy. Before EPR measurements the irradiated items were stored under cryogenic condition for several hours.

The EPR spectra have been recorded using Bruker EPR spectrometer system EMX^{plus} –A operating in X-band, equipped with a high sensitivity probehead and Bruker temperature control system EMX 41VT. The experimental signals were recorded using the following parameters: modulation amplitude 0.1 mT, sweep width 70.0 mT, resolution 7000 points and microwave power 0.107 mW. Number of scans was adjusted to the intensity of the spectra and was 4–8 for amino acids and 8–16 for collagen.

The spectra were recorded in the temperature range of 100–350 K using controlled warming procedure. Upon heating each sample was kept for 5 min at selected temperature and then the spectrum was recorded. In some cases indicated in figure after heating the samples were refroze and the EPR signals were additionally measured at 100 K. Some of the experimental signals were simulated by WINEPR SimFonia Bruker software.

3. Results and discussion

Glycine is one of the main components of collagen because the amino acid appears as every third residue in the structure of the peptide. Polycrystalline glycine in the zwitterionic form, when irradiated under cryogenic conditions, shows two lines at a distance of 50.4 mT attributed to the released hydrogen atoms (out of range in Fig. 1) and a doublet of hyperfine splitting (hfs) 2.57 mT and g=2.0038 corresponding probably to the radical anion G1 shown in Scheme 1. The spectrum demonstrates that unpaired spin interacts with one hydrogen of methylene group whereas contribution of the second one, located in the vicinity of nodal plane of carbonyl group, is within the limit of linewidth (Bennett and Gale, 1968; Tamura et al., 1966; Bennett et al., 1966). This primary intermediate presented in Scheme 1

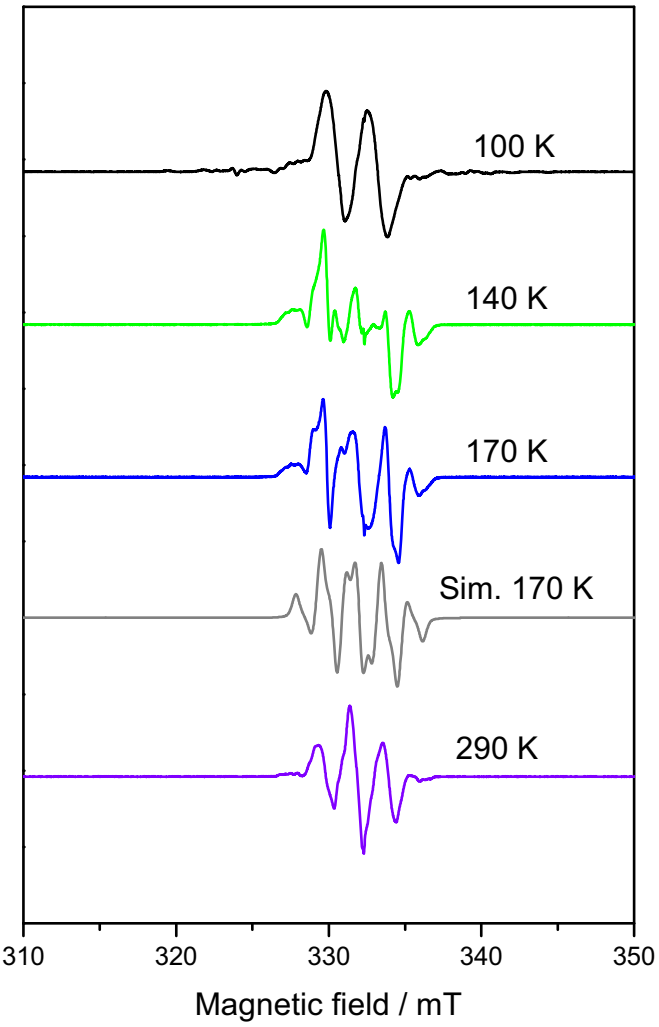
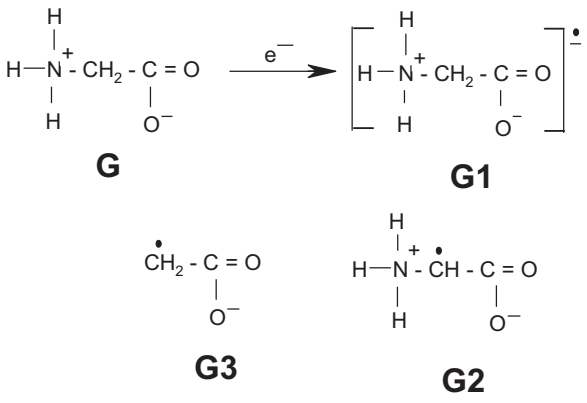


Fig. 1. EPR spectra of glycine gamma-irradiated with a dose of 5 kGy at 77 K and thermally annealed to indicated temperatures.



Scheme 1. Glycine radicals initiated by after ionizing radiation.