Romanian Reports in Physics, Vol. 59, No. 1, P. 93–99, 2007

RADIOLYTIC OXIDATION OF THE IMMUNOGENIC CONJUGATE TESTOSTERONE-3-CARBOXYMETHYLOXIM-BOVINE SERUM ALBUMIN AND RABBIT ANTITESTOSTERONE ANTISERUM*

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(Received October 10, 2005)

Abstract. The radiolytic oxidation of purified bovine serum albumin, rabbit antitestosterone antiserum and testosterone-3-carboxymethyloxim-bovine serum albumin is examined by employing either (*i*) a protein carbonyl assay or (*ii*) the relative tryptophan fluorescence. The experiments clearly evidence a linear dose dependence for the carbonyls and a multi-exponential dependence for the tryptophan fluorescence.

Key words: radiolytic oxidation, bovine serum albumin, immunogenic conjugate, carbonyl, tryptophan.

1. INTRODUCTION

The absorbed energy of ionising radiations can inactivate all biological compounds of biological interest: proteins, nucleic acids, lipids. A direct effect is observed when ionization affects the molecules themselves [1, 2]. When the molecules are irradiated in aqueous solutions, the indirect effect of the irradiation is mainly due to their interaction with reactive species (e.g., free radicals) produced by excitation or ionization of water. The irradiation of the proteins can lead to: destruction of the amino acid chains, production of new chemical groups, breaking of the peptide bonds, the formation of inter- or intramolecular cross-links, etc. By the action of ionising radiations peptides can be also formed from radiolytic

* Paper presented at the National Conference of Physics, 13–17 September, 2005, Bucharest, Romania.

oxidized proteins. The protein aggregation is also generated by oxygenated radicals formed by water radiolysis. These radicals could modify the structure of the proteins, finally leading to irreversible changes in their physico-chemical properties [3].

An increase of gamma-irradiation dose up to 5 kGy leads to protein fragmentation, followed by aggregation (creation of cross-links between molecules). In the radiolysis of water, hydrated electrons (e_{aq}), hydroxy (OH•), peroxy (HOO•), and H• are the main radicals produced. The hydroxy or peroxy radicals are the most effective in producing protein damages and contribute to their alteration. The oxygenated radicals can oxidize certain free amino groups of the protein amino acids (e.g., lysine, arginine, proline and histidine) and transform them into carbonyl groups.

The present work is an approach of the oxidation by gamma irradiation of bovine serum albumin (BSA) and of a set of immune components: immunogenic conjugate and antisteroid antiserum. As an indicator of the protein oxidation, the evaluation of the content of the carbonyl groups formed by gamma-irradiation was used [4]. Due to the fact that 2,4-dinitrophenylhydrazine (DNPH) reacts with carbonyl groups of the protein - resulting in the formation of a stable dinitrophenyl hydrazone - the total carbonyl content of a protein or mixture of proteins can be quantified by the ratio of the absorbance of the DNP group at 370 nm to that at 280 nm.

Tryptophan fluorescence modifications can be interpreted as a signal of protein alterations after irradiation. The observed fluorescence modification may be due to conformational changes, bond breaks or development of the fluorophores [5, 6]. It has been reported that, at a high level of gamma dose (0.5–5 kGy), the oxidation of tryptophan occurs. In examining the global effects of gamma irradiation on the immunogenic conjugate testosterone-3-carboxymethyloxim-bovine serum albumin (testosterone-3-CMO-BSA), the changes in the intrinsic fluorescence of its tryptophan residues, following gamma-irradiation, were measured.

2. MATERIAL AND METHODS

Pure BSA protein (>92%, Serva Feinbiochemica), testosterone-3-CMO-BSA and rabbit antitestosterone antiserum (RAA) were used in the study. All other chemicals used in this work were of analytical grade.

2.1. GAMMA-IRRADIATION

In this experiment, solutions of the immunogenic conjugate testosterone-3-CMO-BSA (50 mM phosphate buffer, pH 7.5) and antitestosterone antiserum were irradiated in 2 ml plastic tubes, with a ^{60}Co source (IRASM, IFIN-HH). The

aqueous solutions of BSA (phosphate buffer 10 mM, pH 7.0) were also irradiated. The samples were exposed to various gamma-irradiation doses, ranging from 0.1 to 1.5 kGy. All the irradiations were performed under air atmosphere, at room temperature.

2.2. PROCEDURE: SAMPLE PREPARATION AND CARBONYL GROUP ASSAY

In this procedure, the following reagents were used: HCl, 2M;

2,4-dinitrophenylhydrazine (Chemapol) 10 mM, in 2M HCl; 30% trichloracetic acid solution (Merck), 6M guanidine (Sigma), in 20 mM potassium phosphate pH 2.3 (adjust pH buffer with HCl solution to give pH 2.3).

For each irradiated sample a control sample was prepared, all samples being assayed in triplicate. 0.2 ml from each sample (irradiated and control) were transferred to two Eppendorf tubes and then 0.8 ml of DNPH and 2M HCl solution were added to the irradiated sample (*S*) and control sample (*C*), respectively. The tubes were dark preserved at room temperature for one hour, with vortexing every 15 minutes. After one hour, 0.5 ml trichloracetic acid 30% were added in each tube and mixed as above. All tubes were centrifuged at 11,188 g for 3 minutes. The supernatant was discarded and the precipitate resuspended for 15 minutes in 1 ml of (1:1) ethanol/ethyl acetate solution. After centrifugation at 11,188 g for 3 minutes and discarding the supernatant, the above step was repeated two more times. After the final wash, the precipitates were dissolved with 0.6 ml 6M guanidine hydrochloride solution at 37°C for 15 minutes. After dissolving the precipitate, the tubes were centrifuged at 11,188 g for 3 minutes, to remove any left over debris. For each sample, *S* and *C*, the optical density was measured against 6M guanidine hydrochloride solution, in the 200-500 nm spectral range. The absorption measurements were performed with a Varian Cary 100 spectrophotometer.

The carbonyl content was determined as follows:

– it was calculated the average OD for each sample for the 367-380 nm range

– it was substracted the average OD of *C* from the average OD of *S*; this represents the corrected absorbance (*CA*)

– the carbonyl concentration was computed by inserting *CA* in the following equation

$$\text{Carbonyl (mol/ml)} = [(CA)/(22{,}000*0.1)](600\ \mu l/200\ \mu l).$$

The extinction coefficient for DNPH at 370 nm is 22,000 $M^{-1}cm^{-1}$.

In order to determine the carbonyl content per mg of protein, the actual protein levels are found by measuring the absorbance at 280 nm in each sample control, *C*. The amount of protein is calculated from a BSA (dissolved in 6M guanidine hydrochloride) calibration curve (not shown here),

Protein concentration (mg/ml) = [(OD_{280}-yintercept)/slope](600 μl/200 μl).

Thus, the carbonyl content per mg of protein will be given by:

Carbonyl content (mol/mg) = (Protein carbonyl (mol/ml))/(Protein (mg/ml)).

2.3. FLUORESCENCE MEASUREMENTS

For fluorescence analysis, irradiated samples of the required dilutions of testosterone-3-CMO-BSA were excited at 270 nm and the fluorescence emission was measured at 350 nm using an Aminco Bauman spectrophotofluorometer.

3. RESULTS AND DISCUSSIONS

3.1. PURIFIED BSA

The damage produced by radiolytic oxidation, as a function of the irradiation dose, was determined by the carbonyl group assay. The results show the dependence on the quantitative composition of carbonyl species formed by protein irradiation. In a first attempt, pure BSA solutions of 1.25 and 2.5 mg/ml concentrations were irradiated with several selected doses, up to 400 Gy. The calculated values of the amount of carbonyl groups produced by irradiation of the BSA were plotted against the irradiation dose (Fig. 1).

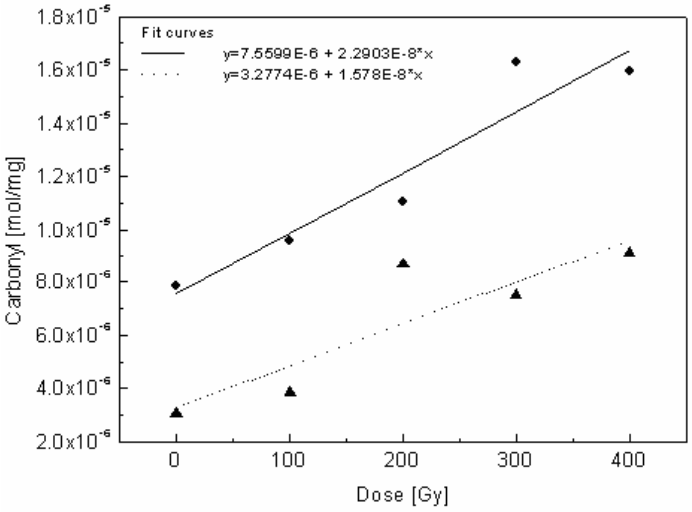

Fig. 1 – Dose dependence of the concentration of carbonyl groups formed as a result of the radiolytic oxidation of BSA in aqueous solution: 1.25 mg/ml BSA (▲) and 2.5 mg/ml BSA (●).

As one can see in Fig. 1, for both concentrations of the irradiated BSA, there is a linear growth with dose of the amount of carbonyl groups produced. One can notice that there is also a change (~30 %) in growth rate at various concentrations: obviously, the higher the protein concentration, the faster the process of carbonyl production.

3.2. RAA AND TESTOSTERONE-3-CMO-BSA

RAA was used in dilutions 1:10 and 1:100. In comparison with the doses used for BSA, the samples containing diluted antitestosterone antiserum were irradiated with much higher doses. After irradiation, the carbonyl group assay was employed and the optical densities, at 370 nm, were recorded for each sample. In Fig. 2 it is shown a plot of the measured values of the carbonyl specific optical density on the antiserum samples, as function of the irradiation dose.

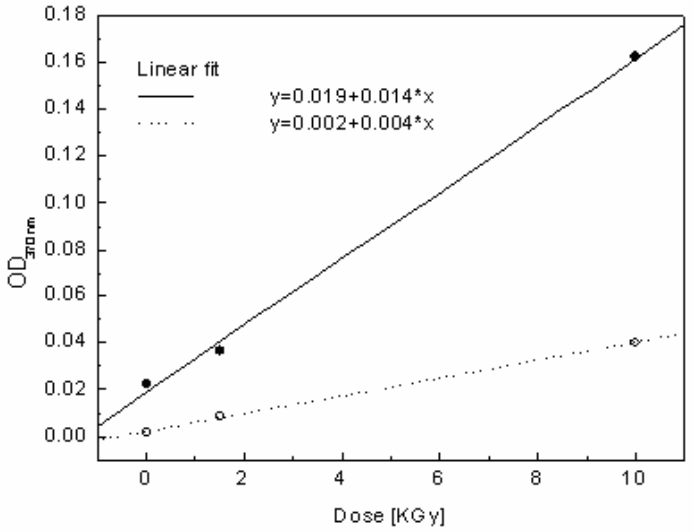

Fig. 2 – Dose dependence of the absorption at 370 nm of DNPH-carbonyl groups formed as a result of the radiolytic oxidation in the RAA samples: dilution 1/10 (●) and 1/100 (○) with 0.05 M phosphate buffer pH 7.5.

For both dilutions, there is a linear growth of absorption at 370 nm with the irradiation dose and, for a given dose, the yield of oxidation products decreases with increasing antiserum dilutions.

For the irradiated testosterone-3-CMO-BSA samples, another protein oxidative modification was followed by the relative tryptophan fluorescence. Commonly, in order to be put in evidence, this modification requires a broader range of doses. It must be noticed that both BSA and the testosterone-3-CMO-BSA

contain an identical amount of tryptophans in their structure. For this reason, there should not be differences in the extent of the modifications induced in each of them at a given dose. Thus, for the fluorometric method applied to irradiated testosterone-3-CMO-BSA, one can choose a broader range of doses, as compared with purified BSA. Aqueous solutions of testosterone-3-CMO-BSA were used in the concentrations of 1.25, 2.5 and 5 mg/ml. The relative variation of the emission fluorescence intensity versus the irradiation dose is shown in Fig. 3.

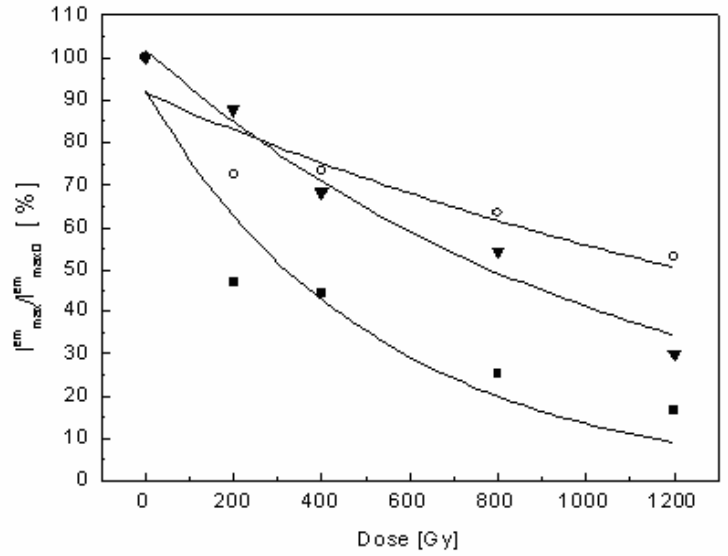

Fig. 3 – Relative fluorescence intensities and the corresponding fits as function of the irradiation dose, at three different testosterone-3-CMO-BSA concentrations: 1.25 mg/ml (■), 2.5 mg/ml (▼) and 5 mg/ml (○).

Radiolytic oxidation seems to provoke some damage to the exposed immunogenic conjugate. Differences in fluorescence intensity but not spectral distribution between control and irradiated samples indicate a radiation-induced decrease in tryptophan fluorescence. Essentially, there is a decrease in the fluorescence intensity with increasing dose of radiation. Within the dose range used in these experiments, the fluorescence decay may be explained by conformational changes experienced by the immunogenic conjugate [5].

4. CONCLUSIONS

The results of the presented experiments put clearly in evidence the modifications induced in protein solutions by gamma irradiation. The oxidation

and carboxyl formation in the native protein (BSA), as well as in testosterone-3-CMO-BSA and RAA, depend on the irradiation dose and the concentration of concerned proteins.

REFERENCES

1. M.J. Davies, S. Fu, R.T. Dean, *Protein hydroperoxides can give rise to reactive free radicals*, Biochem. J., **305**, 643–649 (1995).
2. M.J. Davies, S. Fu, H. Wang, R.T. Dean, *Stable markers of oxidant damage to proteins and their application in the study of human disease*, Free Radic. Biol. Med., **27**, 1151-1163 (1999).
3. E.L. Finley, J. Dillon, R.K. Crouch, K.L. Schey, *Radiolysis-induced oxidation of bovine alpha-crystallin*, Photochem Photobiol., **68**, 9–15 (1998).
4. E. Shacter, *Quantification and significance of protein oxidation in biological samples*, Drug Metab. Rev., **32**, 307–326 (2000).
5. E.L. Finley, J. Dillon, R.K. Crouch, K.L. Schey, *Identification of tryptophan oxidation products in bovine alpha-crystallin*, Protein Sci., **7**, 2391–2397 (1998).
6. E.R. Stadtman, *Oxidation of Free Amino Acids and Amino Acid Residues* in: *Proteins by Radiolysis and by Metal-Catalyzed Reactions*, Annual Review of Biochemistry, **62**, 797–821 (1993).