EFFECTS OF ^{60}Co RADIATION ON BOTHROPSTOXIN-1 STRUCTURE.

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ABSTRACT

Gamma radiation is able to detoxify snake venoms and toxins without significantly affecting their immunogenic properties. This method has been successfully employed to attenuate toxins for antisera production without inducing toxic effects in animals undergoing immunization. However, the mechanism of attenuation is not fully understood and much work remains at the molecular level in order to further characterize the effects of radiation on these proteins. The present study was undertaken to evaluate structural modifications following irradiation of bothropstoxin-1 (bthTx-1), a myotoxin from *Bothrops jararacussu*. It is believed that the functional form of the toxin is a homodimer with the binding affinity provided by electrostatic and hydrophobic interactions. Purified BthTx-1 was irradiated with 500, 1000 and 2000 Gy of ^{60}Co gamma radiation. The irradiated and native toxins were compared by mass spectrometry, circular dichroism (CD) and tryptophan fluorescence quenching., results suggest that the monomer-monomer interactions are hydrophobic in nature. No significant differences were observed between the two forms of the toxin by CD spectral interpretation. However, significant losses of secondary structure could be observed when the native and irradiated BthTX-1 were compared after disulfide bond reduction. Fluorescence data indicates that the solvent accessibility of Trp 77 has been modified, which may explain the differences in quaternary structure.

INTRODUCTION

The irradiation of proteins in aqueous solution generally results in a loss or decrease of biological activity. This fact may be ascribed to the interaction of free radicals generated during water radiolysis with the protein, as well as to direct action of the ionizing radiation upon the protein. However, the immunogenic properties of proteins seem to be more resistant to radiation, and this feature has been successfully employed to attenuate snake venoms (1) and toxins (2) to immunize animals for antisera production. In previous works, we were able to detoxify snake venoms and purified toxins and these toxoids showed a high immunogenicity, inducing high titers of neutralizing antibodies (1, 2).

However the effects of radiation on protein solutions are not yet fully understood and much work remains to be done for a further characterization of irradiated macromolecules.

In the present work we used bothropstoxin-1 (BthTx-1) as a model to investigate the effects of ^{60}Co gamma rays on the structure of proteins using various biophysical techniques such as Circular Dichroism, Mass Spectrometry, and Fluorescence quenching. This toxin is a phospholipase A2 like molecule, with 4 alpha helixes and a beta-wing. Due to critical substitutions within the calcium binding loop, this toxin is unable to bind calcium and therefore has low, if any, catalytic activity. Although enzymaticaly inactive, BthTx-1 presents high myotoxic activity which is believed to be associated with an autocatalytical acylation of the toxin (3). According to crystallographic data (4) the toxin is a homodimer and the contact area between the subunits is very small. The interaction between the monomers involve a saline bridge between glutamic acid 12 of one monomer and lysine 80 of the other, 3 hydrogen bonds, and hydrophobic interactions between tryptophan 77 and valine 12 and their symmetrical equivalents.

MATERIAL AND METHODS

Bothropstoxin-1 Purification.

The myotoxin was purified using a single step cation exchange chromatography procedure, as previously described (5).

Irradiation,

A 2 mg/ml solution of BthTx-1 in 150 mM NaCl was irradiated with 500, 1000 and 2000 Gy, using a dose rate of 1600 Gy/hour, at room temperature and in the presence of atmospheric oxygen.

Mass Spectrometry,

1-LC-ESI-MS

Aliquots of native and irradiated BthTx-1 were injected onto a C8 capillary LC column (180 µm x 15 cm, 5 µm particle size) from LC Packings (San Francisco, CA). The samples were eluted with a gradient of 0 to 90% acetonitrile in 0.1% formic acid using a 140B dual syringe pump (ABI Biosystems; Foster City, CA) and a 2 µl/minute flow rate. Elution was monitored at 214 nm with a Kratos 783 UV-VIS detector (ABI Biosystems). The eluant passed into an electrospray ion source (Analytica of Branford; Brandford ,CT) fitted to a Finnigan MAT TSQ 700 mass spectrometer (San Jose, CA). ESI mass spectra of eluted peptides were obtained by scanning the instrument from 200 to 1900 m/z at a rate of 2 seconds per scan.

2-MALDI-TOF

1 µl of 2 mg/ml native or irradiated BthTx-1 in 150 mM NaCl were diluted in 1 µl of 10 mg/ml α-cyano-4-hydroxycinamic acid in acetonitrile with 0.1% trifluoroacetic acid and analyzed on a Voyager-DE (Perseptive Biosystems) mass spectrometer according to the following conditions:

Acceleration voltage: 20 kV
Pressure: 2.8 x 10^{-7} atmospheres
Laser wavelength: 337 nm
Pulse duration: 3 ns with 20 pulses/second.

Circular dichroism (CD),

All CD experiments were performed using a Jasco J 600 spectropolarimeter, equipped with a Peltier thermocontrol device. Far UV CD scans were measured from 280 to 190 nm at a scan rate of 20 nm/min, with a 2 sec response, in a 2 mm path cell, at a concentration of 200 µg/ml in 20 mM sodium phosphate pH 7. Spectra were obtained for irradiated and nonirradiated BthTx-1 under both reducing (2 mM beta mercaptoethanol) and non reducing conditions. Spectra were corrected for background by subtracting out a scan of buffer alone.

Thermal denaturations were performed on the irradiated and nonirradiated toxins using a 10 mm pathlength cell, from 20-90^{o} C, with a temperature gradient of 1 ^{o} C/min, and continuous monitoring at 222 nm. Samples conditions were 50 µg/ml in 20 mM sodium acetate, pH4. The Vant'Hoff enthalpy and the melting point of the samples were then calculated using a two state thermodynamic model based on the following equation:

$$\Delta G(T)= \Delta H_m ((1-T/T_m) + \Delta C_p(T-T_m-T \ln(T/T_m)) \quad (1)$$

where $\Delta G(T)$ represents the energy difference at the temperature T, T_m the melting point, ΔH_m the enthalpy variation at the melting point and ΔC_p the thermal capacity difference between the native and denatured states.

Solvent Mediated Fluorescence Quenching,

Aliquots of 50 µg/ml in 50 mM sodium acetate pH 5 of the native and irradiated samples were analyzed on a Perkin-Elmer 550 fluorimeter at 25^{o}C. The excitation wavelength was fixed at 295 nm and the emission was scanned from 300 to 400 nm with a 5 nm slit.

RESULTS

Unlike other toxins, the irradiated bothropstoxin samples did not show any insoluble protein. Reverse-phase chromatography/mass spectrometry analysis of the irradiated sample (Fig. 2) suggests that irradiation led to the formation of populations of modified proteins with different oxidation states as opposed to the non-irradiated toxin (Fig. 1) which shows a single protein peak. This observation is based on the complexity of the mass spectrum of the toxin following irradiation.

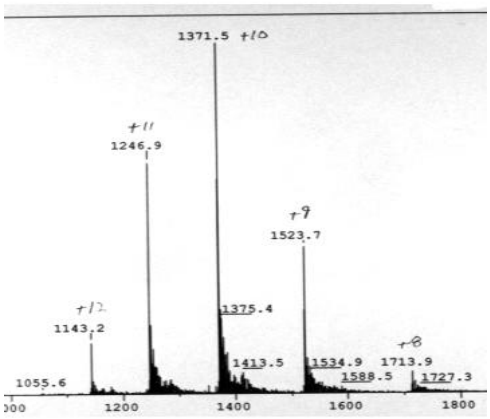

Figure 1: Electrospray Ionization Mass Spectrum of Native Bothropstoxin.

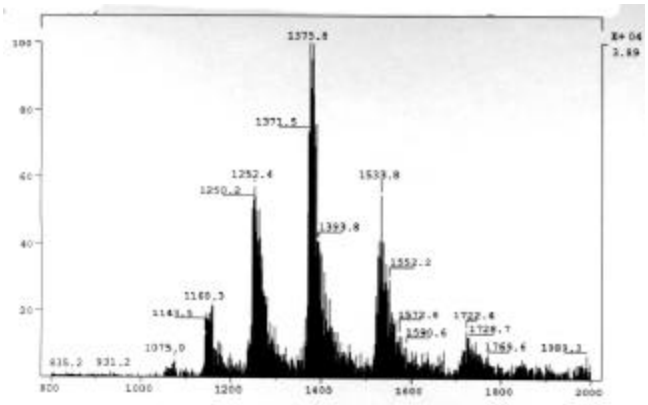

Figure 2: Electrospray Ionization Mass Spectrum of 2000 Gy Irradiated Bothropstoxin.

The MALDI-TOF spectra of the native toxin (Fig. 3) indicates the presence of BthTx-1 in the monomeric (m/z=13696.65, z=1,m/z=6850.78, z=2 and m/z=45677.09, z=3), dimeric (m/z=27388.58, z=1) trimeric (m/z=41157.65, z=1) and tetrameric (m/z=54833.92, z=1) forms. The polymeric forms of the toxin were not observed in any of the irradiated samples (Figs 4, 5 and 6)

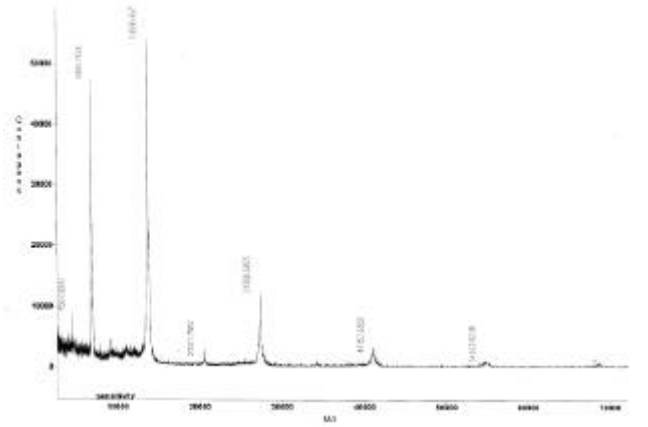

Figure 3: MALDI-TOF mass spectrum of native bothropstoxin.

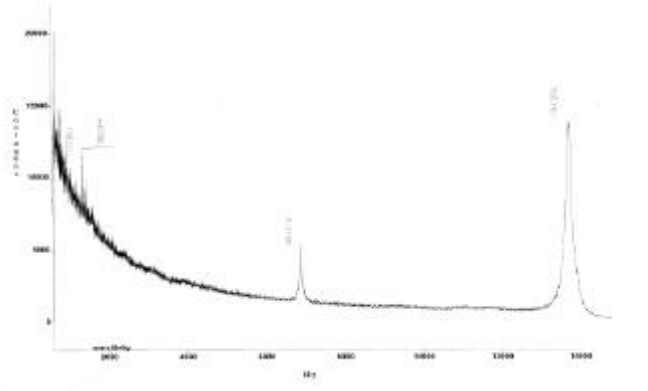

Figure 4: MALDI-TOF mass spectrum of 500 Gy bothropstoxin.

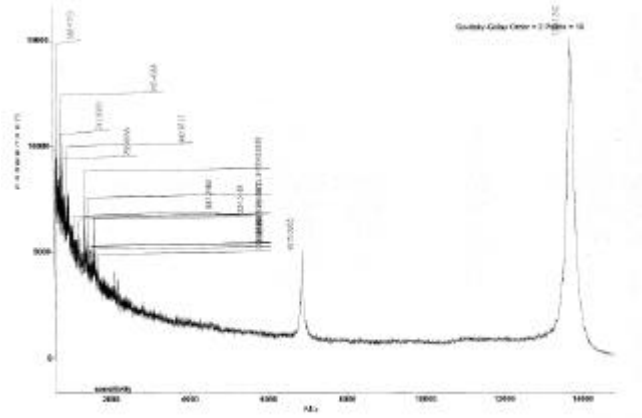

Figure 5: MALDI-TOF mass spectrum of 1000 Gy irradiated bothropstoxin.

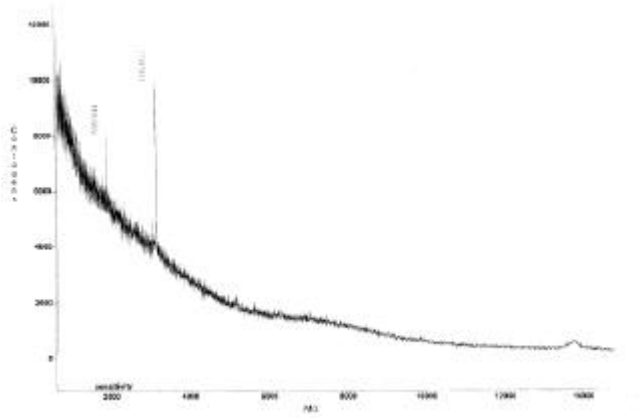

Figure 6: MALDI-TOF mass spectrum of 2000 Gy irradiated bothropstoxin.

. The CD spectral interpretation indicates that with doses above 500 Gy, only a slight decrease in the alpha-helix and beta-strand signatures occurs (Fig. 7).

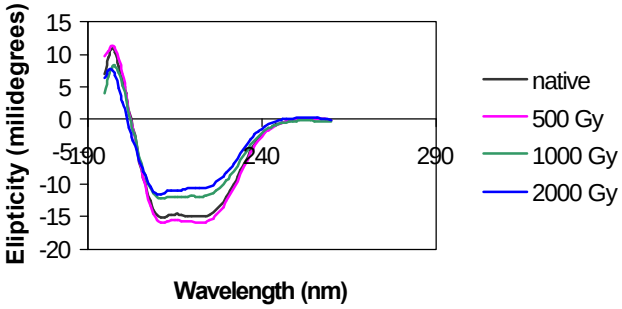

Figure 7 CD Spectra of Native and Irradiated bothropstoxin.

However, significant losses of secondary structure could be observed when the 2000 Gy irradiated BthTX-1 was compared before and after disulfide bond reduction (Fig. 8).

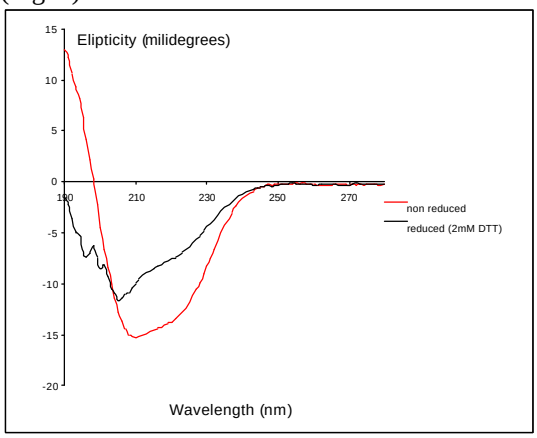

Figure 8: Effect of the Disulfide Bonds Reduction on the Secondary Structure of 2000 Gy Irradiated Bothropstoxin.

The thermal denaturation data indicate a dose-dependent decrease in the Vant'Hoff enthalpy (Table 1). We could also observe that the transition from the native to the denatured state started at lower temperatures for the irradiated samples (Figure 9).

TABLE 1. Melting Point and Vant'Hoff Enthalpy of the Native and irradiated Samples.

Sample	T melt (^{o}C)	ΔH (kcal/mol)
Native	65.6 ± 0,5	75
500 Gy	64,7 ± 0,5	60
1000 Gy	65.6 ± 0,5	43
2000 Gy	62,5 ± 0,5	32

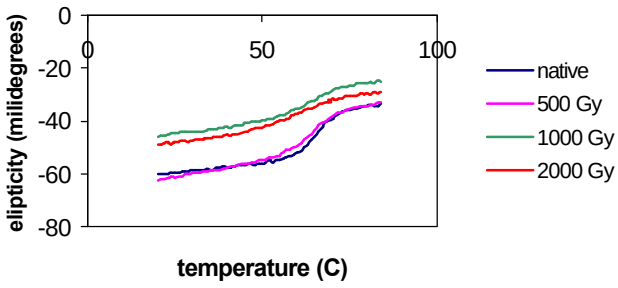

Figure 9: Thermal Denaturation of Native and Irradiated Bothropstoxin.

The fluorescence quenching experiments indicate changes in the exposure of the fluorophore (tryptophan 77) indicating modifications in the quaternary structure of the toxin dimer (Fig. 10).

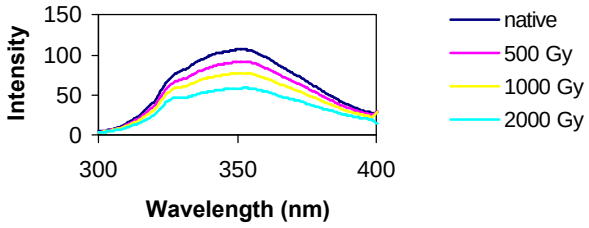

Figure 10: Solvent Mediated Fluorescence Quenching of Native and Irradiated bothropstoxin.

Discussion

Radiation has been successfully employed to attenuate the biological activity of a wide array of proteins. However, there is still a lot of work to be done to characterize the effects of ionizing radiation on biomolecules. In the present work, we used bothropstoxin-1 as a model to investigate the structural modifications that irradiated molecules undergo. The data collected indicate that the irradiation promoted oxidation of the molecule, as indicated by mass spectrometry. When analyzed by MALDI-TOF, multimeric forms of the native toxin can be detected, in opposition to the irradiated form of the protein, where only the monomeric form was detected. Perhaps, the increased entropy overcomes the electrostatic and hydrophobic interactions at the dimer interface following irradiation. There are also clear evidences of conformational changes with radiation doses above 500 Gy, as shown by circular dichroism. These modifications are even clearer when the reduced form of the 2000 Gy irradiated toxin is analyzed. Indeed, the CD spectrum of the irradiated and reduced protein shows a massive transition of the folded state to a random coil conformation, as indicated by the loss of signal at 222 nm. These data also indicate that, after irradiation, the secondary structure is stabilized by the seven intramolecular disulfide bonds present in the native toxin. The dose dependent decrease in the enthalpy, suggests a transition towards a lower energy conformation, while the increase in the transition range from the folded to the denatured state suggests the existence of molecules in different states of unfolding. Using solvent mediated quenching as a tool to investigate quaternary structure, we were able to observe changes in the solvent accessibility of the tryptophan 77, which is located at the dimer interface, suggesting modifications of the monomer-monomer interaction region. With these information, we conclude that the loss of biological activity of irradiated molecules may be ascribed to primary, secondary (and probably tertiary), as well as quaternary structure modifications, resulting from a progressive decrease in the enthalpy of the molecule, promoting irreversible unfolding.

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