FLUORESCENCE RESONANCE ENERGY TRANSFER, TIME RESOLVED SPECTROSCOPY AND PRECISION CALORIMETRY FOR CHROMATIN STRUCTURE DETERMINATION[#]

LILIANA RADU, DOINA GĂZDARU**, V. PREOTEASA*, B. CONSTANTINESCU****

*Department of Molecular Genetics and Radiobiology, "V. Babeş" National Institute, 99–101, Splaiul Independenței, Bucharest, Romania, e-mail: liliana1radu@gmail.com
**Department of Biophysics, Faculty of Physics, Bucharest University, 405, Atomiştilor St, Bucharest-Măgurele, Romania
***Department of Applied Nuclear Physics, "H. Hulubei" National Institute of Physics and Nuclear Engineering,. 407, Atomiştilor St, Bucharest-Măgurele, Romania

Abstract. Information on the chromatin structure from a normal tissue and from a tumoral tissue and also on the chromatin structure modifications produced by fast neutrons was obtained using the fluorescence resonance energy transfer method, time resolved spectroscopy and precision calorimetry determinations.

Key words: chromatin, fluorescence resonance energy transfer, time resolved spectroscopy, precision calorimetry.

INTRODUCTION

Chromatin is a complex of deoxyribonucleic acid (DNA) and proteins, which exists in eukaryotic cell nuclei. The fundamental building block of chromatin organization is the nucleosome [6, 18], that consists of about 146 base pairs of DNA wrapped in 1.67 left-handed superhelical turns around the histone octamer, consisting of 2 copies each of the core histones H2A, H2B, H3 and H4.

Chromatin structure influences key processes, including the expression of genetic information and the transfer of gene expression status at cell division. When the way that DNA is wrapped around the histones changes, gene expression can change as well.

Our previous studies have been oriented on the analysis of chromatin structure from normal tissues and from tumoral tissues [10].

[#]Presented at The 12th National Conference on Biophysics, Iaşi, June 13-16, 2013.
Received: May 2013;
in final form May 2013.

ROMANIAN J. BIOPHYS., Vol. 23, Nos 1–2, P. 61–68, BUCHAREST, 2013

The effects on DNA of ionizing radiations [4, 12] and particularly of fast neutrons [13] have been reported. Ionizing radiations determine strand breaks, base and sugar damages in DNA. In aerated solution, these effects are mainly due to the attack by hydroxyl radicals resulting from the radiolysis of water. Fast neutrons action on DNA produces single strand breaks (SSB) and double strand breaks (DSB).

Because in cells DNA is complexed with proteins to form the chromatin, the study of the fast neutrons action on chromatin is important. The interest in fast neutrons for radiotherapy and radioprotection determines the research of the molecular basis of their action.

In the present paper, a study of the fast neutrons (10–100 Gy) on chromatin is reported. Two types of chromatin were analyzed: a chromatin extracted from a normal tissue – the liver of Wistar rats and a chromatin extracted from a tumoral tissue – Walker carcinosarcoma maintained on Wistar rats. Information on the chromatin structure were obtained using the fluorescence (Förster) resonance energy transfer (FRET) method, the time resolved spectroscopy and precision calorimetry determinations.

MATERIALS AND METHODS

The chromatin was extracted from livers of Wistar rats and from Walker carcinosarcoma maintained on Wistar rats, according to standard procedures [5]. Chromatin samples with 2.5×10^{-4} M DNA concentration in 10^{-2} M phosphate buffer, pH = 7, were used.

A U-120 classical variable energy cyclotron was used as an intense source of fast neutrons, produced by 13.5 MeV deuterons on a thick beryllium target. The mean dose linear energy in water at the point of interest was 87.7 keV/μm and the mean dose rate monitored with transmission chambers was 0.3 Gy/min. The utilized fast neutrons dose domain was 10–100 Gy.

The analysis of the fast neutrons effects on chromatin was performed by: (a) fluorescence (Förster) resonance energy transfer (FRET) method (the transfer of energy from a donor fluorophore, dansyl chloride, to an acceptor fluorophore, acridine orange, both coupled at chromatin), (b) time resolved fluorescence of the complexes of chromatin with the fluorescent ligand ethidium bromide (the determination of the excited state lifetimes and of the relative contributions of the excited state lifetime of bound and free ligand) and (c) precision calorimetry (the establishment of the enthalpy of complexation of ethidium bromide with chromatin).

(a) Fluorescence (Förster) resonance energy transfer is a mechanism describing energy transfer between two chromophores. A donor chromophore, initially in its electronic excited state, may transfer energy to an acceptor

chromophore (in proximity, typically less than 10 nm) through nonradiative dipole-dipole coupling [11]. In the FRET method, double fluorescent labeling of the chromatin was performed with dansyl chloride (excitation wavelength λ_{ex} = 323 nm, emission wavelength λ_{em} = 505 nm) for proteins and acridine orange (λ_{ex} = 505 nm, λ_{em} = 530 nm) for DNA. The dansyl chloride reacts, under mild alkaline conditions, with the α- and ε-amino groups of the proteins, the cysteine sulphydryl group, the histidine imidazole group and the tyrosine phenolic group. Acridine orange is intercalated between chromatin DNA base pairs. Between these pairs of fluorescent ligands an energy transfer takes place [9].

The Förster energy transfer efficiency [15] is:

$$E_{\mathrm{f}} = \left(\frac{I_{\mathrm{A}}^{\mathrm{D}}}{I_{\mathrm{A}}} - 1\right)\frac{\varepsilon_{\mathrm{A}}}{\varepsilon_{\mathrm{D}}} \tag{1}$$

where I_A and $I_A{}^D$ are the fluorescence intensities of the acceptor, in the absence and respectively in the presence of the donor and ε_A and ε_B are the molar extinction coefficients of the ligands at the excitation wavelength.

An Aminco Bowman SPE 500 fluorimeter was used.

The energy transfer efficiency of this process depends on the inverse sixth power of distance, *r*, between donor and acceptor, determined by the expression:

$$E_{\mathrm{f}} = \frac{r^{-6}}{r^{-6} + \mathrm{R_0}^{-6}} \tag{2}$$

where R_0 represents the Förster critical distance at which 50% of the excitation energy is transferred to the acceptor. In the case of chromatin coupled with dansyl chloride and acridine orange [9], it was calculated R_0 = 2.97 nm.

(b) In time resolved fluorescence, the ligand ethidium bromide, EtBr (λ_{ex} = 480 nm, λ_{em} = 600 nm), was used as a chromatin DNA intercalating agent in a concentration of $6.25 \cdot 10^{-5}$ M. The fluorescence response function [16] is:

$$I(t) = \mathrm{A} + \mathrm{B}_1 e^{t/\tau_1} + \mathrm{B}_2 e^{t/\tau_2} \tag{3}$$

where *I(t)* is the time dependent fluorescence intensity, A, B_1 and B_2 are constants and τ_1 and τ_2 are the lifetimes for the bound and free excited states of the ligand.

A time resolved spectrofluorimeter FL 900 CD, Edinburgh Instruments was used. The fluorescence profiles were recorded based on time correlated single photon counting method. A hydrogen flash lamp with a 1 ns FWHM at 39 kHz frequency was used as a light source with λ_{ex} = 480 nm. The fluorescence intensities were least-squares fitted to single or double exponential functions by the Marquardt deconvolution method [14]. The time resolved spectrofluorimeter produces a precise determination of the excited state lifetimes of the bound and free fluorescent ligand and establishes the relative contributions of the excited state lifetimes of bound and free ligand, respectively.

The relative contribution of the excited state lifetime of bound ethidium bromide *f*(%) at chromatin was calculated as [1]:

$$f(\%) = \frac{100B_1\tau_1}{B_1\tau_1 + B_2\tau_2} \quad (4)$$

c) In the precision calorimetric determinations, the same DNA chromatin ligand ethidium bromide was used. Ethidium bromide was utilized as powder, put in the special gold cell of the calorimeter. The heat (ΔQ) of EtBr dissolution and the apparent heat of EtBr coupled to chromatin samples were determined.

$$\Delta Q_{\text{real}} = \Delta Q_{\text{apparent}} - \Delta Q_{\text{dissolution}} \quad (5)$$

For constant pressure (p), dp = 0, the enthalpy variation is [2]:

$$\Delta H = (Q_{\text{real}})_p \quad (6)$$

The molar enthalpy (ΔH_m) was calculated, as $\Delta H_m = \Delta H/n$, with n – mols number.

The molar enthalpy of complexation of ethidium bromide with chromatin from liver and from Walker carcinosarcoma was determined. A precision calorimeter LKB 8700 was used.

RESULTS AND DISCUSSION

(a) In Figure 1 is represented the dependence of the energy transfer efficiency (E_f) between dansyl chloride and acridine orange coupled at liver and Walker chromatin on the fast neutrons dose.

The smaller E_f value for tumoral Walker chromatin than for normal liver chromatin indicates a laxer structure of tumoral chromatin.

The decrease of FRET efficiency with fast neutrons dose denotes a more unstable chromatin tertiary structure, which determines a reduced intensity of the energy transfer. The increase of the average distance between dansyl chloride and acridine orange on fast neutrons actions (Fig. 2) reflects the enhancement of the distance between chromatin proteins and DNA, which suggests a loosening of the chromatin structure. The interaction between DNA and chromatin proteins is more loose by the neutrons action in case of tumoral chromatin.

(b) Since in a free DNA, the EtBr binding stoichiometry is 1:4 (one EtBr molecule gets intercalated between 2 base pairs – 4 nucleotides), all EtBr molecules are bound to a free DNA, when the concentration of DNA is 2.5×10^{-4} M phosphate (P) and that of EtBr is 6.25×10^{-5} M. Determinations on free EtBr solution and on EtBr coupled to pure DNA were done as controls.

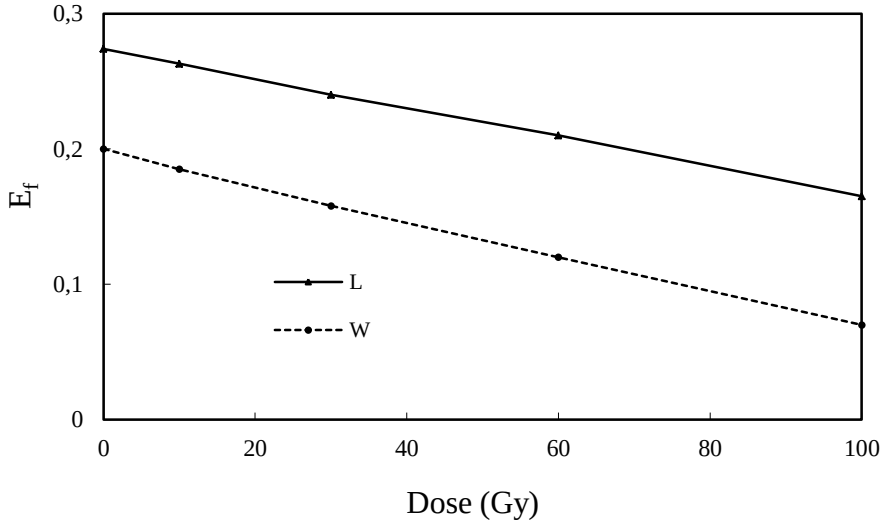

Fig. 1. The energy transfer efficiency between dansyl chloride and acridine orange coupled at chromatin (L-liver, W-Walker) *versus* fast neutrons dose.

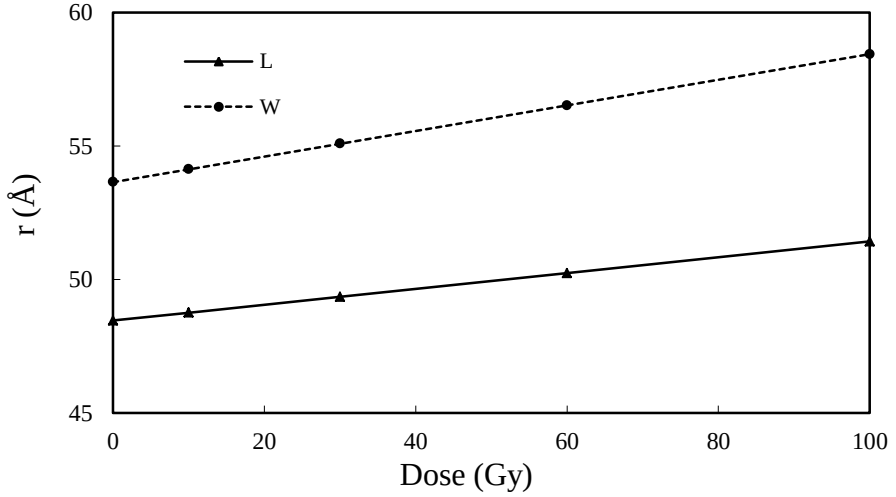

Fig. 2. The average distance between dansyl chloride and acridine orange coupled at chromatin (L-liver, W-Walker) *versus* fast neutrons dose.

In the case of EtBr binding to chromatin, in the same concentration conditions, only a part of the ligand is bound to chromatin DNA, because of the masking of DNA-binding sites by chromatin proteins. In the EtBr-chromatin

complex there is bound EtBr and also free EtBr. The obtained excited states lifetimes of the DNA ligand in EtBr-chromatin complex are: τ_1 = 24 ns for bound ligand and τ_2 = 2 ns for free ligand [8]. The obtained values for lifetimes of ligand excited states are close to those reported in [3].

Figure 3 represents the relative contribution of the excited state lifetime of bound ethidium bromide at chromatin *f*(%) *versus* fast neutrons dose, for liver and Walker chromatin.

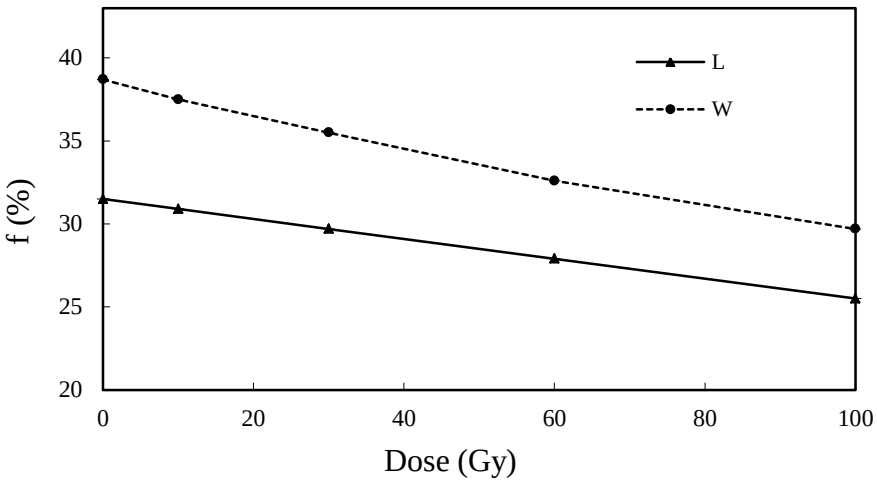

Fig. 3. The relative contribution of the excited state lifetime of bound ethidium bromide at chromatin (L-liver, W-Walker) *versus* fast neutrons dose.

As EtBr is intercalated only in intact regions of chromatin DNA, the decrease of *f*(%) on the fast neutrons dose domain 10–100 Gy indicates a reduction of chromatin DNA double strand structure, due to single and double strand breaks. At the same time, the presence of DNA breaks modifies the supercoiling of DNA and diminishes the amount of intercalated ethidium bromide. The more intense effect on chromatin DNA is produced in tumoral chromatin than in normal one.

(c) In precision calorimetry determinations, the molar enthalpy of ethidium bromide complexation to DNA was also measured. The obtained value: ΔH_m = –0.54 kcal.mol^{-1} is identical with the accepted value [7] for this coupling ΔH_m = –6.5 ± 0.5 kcal.mol^{-1}.

In Figure 4 is represented the molar enthalpy (ΔH_m) of complexation of ethidium bromide with chromatin samples *versus* neutrons dose**.** The obtained molar enthalpy values indicate the same action on chromatin DNA: the reduction of intact double strand structure.

The "energy landscape" is a mapping of all possible conformations of a molecular entity, or the spatial positions of interacting molecules in a system, and their corresponding energy levels [17].

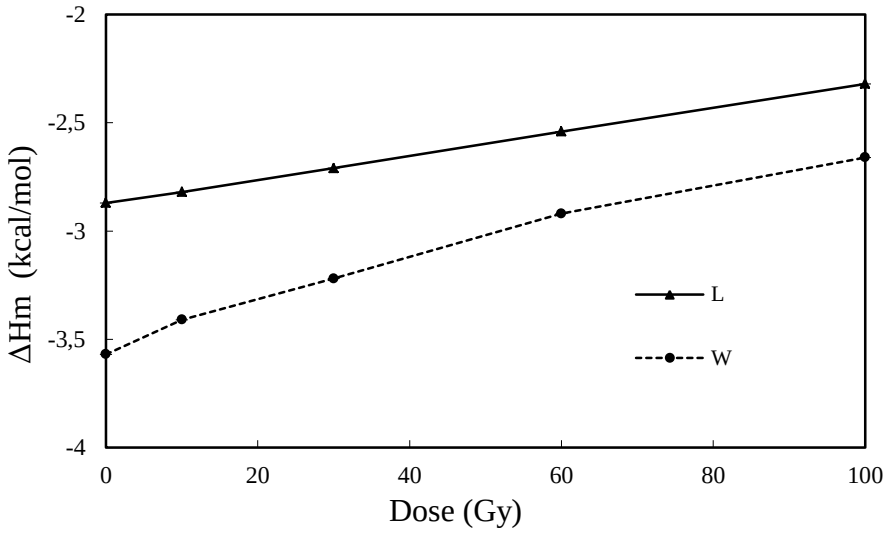

Fig. 4. The molar enthalpy of complexation of ethidium bromide at chromatin (L-liver, W-Walker) *versus* fast neutrons dose.

CONCLUSIONS

The normal chromatin has a more condensed structure than the tumoral chromatin, reflected by the values of the parameters analyzed: the energy transfer efficiency between dansyl chloride and acridine orange, the relative contribution of the excited state lifetime of bound ethidium bromide at chromatin and the enthalpy of complexation of ethidium bromide with chromatin.

The fast neutrons action on chromatin indicates the enhancement of the distance between chromatin proteins and DNA, which suggests a loosening of the chromatin structure. This distance variation is greater in Walker carcinosarcoma chromatin than in normal liver chromatin, due to a laxer structure of tumoral chromatin. Also, a reduction of chromatin DNA double-strand structure takes place. Single- and double-strand breaks, due to sugar and base modifications, are produced in chromatin DNA structure. The fast neutrons action on chromatin DNA is more efficient in tumoral chromatin than in normal one.

The knowledge of the normal and tumoral chromatin structure and of these chromatin structure modifications in the fast neutron actions is important in improving diagnostic and treatment in clinical applications.

Fluorescence resonance energy transfer, time resolved spectroscopy and precision calorimetry determinations can constitute additional methods to energy landscapes for biopolymers dynamic structure establishment.

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