Biophysical Aspects of Auger Processes, AAPM Symposium Series No. 8 (1992) 037-050
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INACTIVATION OF BACTERIOPHAGE T1 BY THE AUGER EFFECT FOLLOWING PHOSPHORUS RESONANCE ABSORPTION OF MONOENERGETIC SYNCHROTRON RADIATION

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ABSTRACT

Killing effect on bacteriophage T1 by the Auger cascade of phosphorus in DNA following K shell photoabsorption was studied with monoenergetic X rays obtained from synchrotron radiations. Phages embedded in nutrient

broth were irradiated under vacuum with X rays at the resonance peak (2153 eV), and below (2147 eV) and above (2159 eV) the peak. The corresponding mean lethal exposures (D_0) were 554, 332 and 434 kR, respectively. The Auger enhancements, as an energy dependent fractional increment of phage sensitivity, were 0.67 at 2153 eV and 0.28 at 2159 eV. Using the DNA absorption spectrum measured in this experiment, photoionization cross sections of Scofield (17), and the Auger yield after creation of a K shell vacancy, the number of phosphorus Auger cascades in one phage DNA at D_0 were calculated to be 0.00, 0.98 and 0.25 at 2147, 2153 and 2159 eV, respectively. Comparison between the Auger enhancement of phage killing and the number of Auger cascades indicated that one phosphorus Auger cascade in phage DNA caused about 0.41 (at 2153 eV) or 0.84 (at 2159 eV) lethal events.

INTRODUCTION

The Auger effect, characterized by a cascade of short-range electrons, leads to an intense local deposition of energy around the excited atom and correspondingly causes localized damage to the relevant molecule. Auger emitters, therefore, are an important tool in probing basic biological mechanisms. The effect of Auger cascades on several biological materials have been theoretically analyzed or experimentally tested with radioisotopes or quasi-monoenergetic fluorescent X rays (1). Excess damage was produced by X rays with energies above the K edge of the key atoms, leading to nearby DNA strand breaks. As a consequence, high relative biological effectiveness (RBE) was observed.

Several lines of study with nucleotides (2), DNA (3,4), phages (5), bacteria (6), and mammalian cells (7,8) have recently been performed to examine if an enhancement of the radiobiological effect of synchrotron X rays can be experimentally verified following their resonance absorption in brominated DNA. When bromouracil-labeled *E. coli* cells were irradiated with X rays, killing of cells was enhanced by 8% at energies above the absorption edge of bromine (13.49 keV) compared to below the edge (12.40 keV) (6). In the case of dried BrdU-labeled T1 phage, a larger (about 26%) enhancement was observed (5). This would partly be due to the associated suppression of radical mediated processes in *E. coli* cells.

Phosphorus, a constituent element of phosphodiester bonds of DNA, can be used to realize such an experimental situation. The results of the

experiment with K shell absorption of phosphorus atoms on yeast cells resulted in an enhancement factor of 1.4 (9). If one uses dry biological materials such as T1 phage for such an experiment, a larger enhancement could be expected because the indirect action of radiation can be completely suppressed. Therefore, an attempt was made in this study to investigate enhanced killing of bacteriophage T1 by Auger cascades following resonance absorption of X rays in the K shell of phosphorus atoms in the DNA. A large enhancement (about 67%) was obtained, as expected.

MATERIALS AND METHODS

Preparation of Bacteriophage T1

Host cells of *E. coli* strain B were cultured at 37°C with aeration in 400 ml of 3xD medium (4.5 g KH_2PO_4, 10.5 g Na_2HPO_4, 3.0 g NH_4Cl, 15 g casamino acid and 13 g glycerol per liter of distilled water with 1.2 mM $MgSO_4$, 0.15 mM $CaCl_2$ and 0.003% gelatin at the final concentrations). Phage T1 was added (multiplicity of infection, m.o.i. = 0.1) into the cell culture in logarithmic growth phase at 5×10^8 cells/ml, incubated at 37°C and phage lysate was obtained. After addition of 1 ml of chloroform and 1 μg/ml of both DNase and RNase, the lysate was incubated for 30 min at room temperature, and centrifuged (6,000 rpm, 30 min, 4°C) to remove cell debris. The supernatant was ultracentrifuged (100,000 X g, 2 hr, 4°C) and the phage pellet was saved. The pellet was resuspended in 1 ml of T1 absorption buffer (0.3 g KH_2PO_4 and 1.5 g Na_2HPO_4 per liter of distilled water with 40 μM $MgSO_4$, 50 μM $CaCl_2$ and 0.0025% gelatin at the final concentrations).

For calculation of a photoionization cross section of phage DNA, base composition of phage DNA was measured. Phage DNA was extracted, purified, hydrolyzed, and then analyzed with high performance liquid chromatography (HPLC) as reported previously (5). The GC content of T1 phage DNA was 0.49.

Source of Monoenergetic X rays

Irradiation was performed at the beam-line BL-1B and -11B of the 2.5 GeV storage ring at the Photon Factory, National Laboratory for High Energy Physics, Tsukuba, Japan. Details of the BL-11B system and the characteristics of the monoenergetic X rays were reported previously (10).

Briefly, white synchrotron radiation is focussed by a toroidal premirror, and monochromatized by an InSb double-crystal monochromator. The full width at half maximum of band width of the monoenergetic X rays around 2 keV was about 1 eV (10). The cross section of the X ray beam was about 2 X 5 mm. At BL-1B, the irradiation system consisted of a plane premirror and an InSb channel cut monochromator. The cross section of the beam was about 4 X 5 mm. X ray energy was chosen using the absorption spectrum of DNA film (see RESULTS).

Measurements of Exposure Rates and Calculation of Photon Fluences

Exposure rates in R were measured by a parallel-plate free air ionization chamber (11) in atmospheric air conditions. The monoenergetic X ray beam was transported from an irradiation chamber (~10^{-5} Pa) to the atmosphere through a Kapton (25 μm) window, a low pressure chamber (~10^{-1} Pa) and a Mylar (5 μm) window. Exposure rate at the sample position in the vacuum irradiation chamber was corrected by attenuation factors for the window materials and the air between Mylar window and the ionization chamber. The exposure rates at 100 mA of ring current at the sample position under a scanning condition (see below) were about 500 kR/min at BL-11B and 4 kR/min at BL-1B.

The photon fluences Φ (photons/m^2) per R were calculated by

$$\Phi = 2.58 \times 10^{-4} \times W \times 1/E \times 1/(\mu_{en}/\rho)_{air} \quad (1),$$

where $(\mu_{en}/\rho)_{air}$ is the mass energy absorption coefficient (m^2/kg) of air taken from Hubbell's data (12), W the energy required to produce an ion pair in normal room air (33.73 J/C), E the X ray photon energy in eV and 2.58 X 10^{-4} the conversion factor from R to C/kg.

Calculation of Absorbed Dose

Absorbed doses of X ray in Gy were calculated as the product of exposure in R and the Roentgen-Gray conversion factor (f-factor). The f-factor, given by equation 2, are listed in Table I.

$$f = 2.58 \times 10^{-4} \times W \times [(\mu_{en}/\rho)_s / (\mu_{en}/\rho)_{air}], \quad (2)$$

TABLE I

Mean Lethal Doses (D_0) for Bacteriophage T1			
	X ray energy		
	2147 eV	2153 eV	2159 eV
Mean lethal dose (kR)			
exp.1	525	389	461
exp.2	581	339	490
exp.3	556	268	351
Avg. ± se [a]	554 ± 20	332 ± 43	434 ± 52
f-factor [b]	0.00938	0.00938	0.00939
Mean lethal dose (kGy) [c]			
Avg. ± se [a]	5.21 ± 0.18	3.12 ± 0.40	4.07 ± 0.49
Relative sensitivity [d]	1.00	1.67	1.28

[a] Averaged value with standard error.

[b] Roentgen-Gray conversion factor; for calculation method, see text.

[c] D_0 (kGy) = D_0 (kR) x f-factor.

[d] Reciprocals of mean lethal doses (kGy) normalized at 2147 eV. Same values were obtained using the mean lethal doses in units of R.

where $(\mu_{en}/\rho)_s$ and $(\mu_{en}/\rho)_{air}$ are the mass energy absorption coefficients of the sample and the air, respectively. The mass energy absorption coefficient of the sample $(\mu_{en}/\rho)_s$ was calculated under the following assumptions. The sample (see below) consisted of 16 μg of dried nutrient broth containing about 100 pg of phage particles, which was assumed to be twice its DNA weight, 50 pg = $(1 \times 10^6) \times (3.1 \times 10^7) \times (1.66 \times 10^{-12})$, where 1×10^6 the number of phage particles in a sample, 3.1×10^7 the molecular weight of T1 DNA (13) and 1.66×10^{-12} the picogram per atomic mass unit. Thus the sample can be assumed as to be essentially pure NB, but its atomic composition is not known. Then the $(\mu_{en}/\rho)_s$ is assumed to be that of the solid components of striated muscle of ICRU, $(\mu_{en}/\rho)_m$ (12) and calculated by,

$$(\mu_{en}/\rho)_s = \{(\mu_{en}/\rho)_m - 0.6 \times (\mu_{en}/\rho)_w\} / 0.4, \quad (3)$$

where $(\mu_{en}/\rho)_w$ is the mass energy absorption coefficient of water and 0.6 the water content of the muscle which was assumed to be 60%. The mass energy absorption coefficients were taken from Hubbell's data (12).

Irradiation and Survival Assay of Phage

The stock suspension of bacteriophage T1 was diluted at 5×10^8 plaque forming units per 1 ml with 0.8% nutrient broth (Difco). Two µl of the phage suspension, which has 16 µg of dry weight, was placed on a small polypropylene sheet (2 X 10 mm with 0.2 mm of thickness) and dried in a silica-gel desiccator at room temperature (25°C). After being dried on the sheet, the shape of the phage sample was a disk with a diameter of about 1.5 mm and thickness of 9 µm ($= 16 \times 10^{-6}$ g / $(\pi \times 0.075^2)$ cm^2 / 1 g-cm^{-3}). Phage sample thickness was greater than the ranges of the photoelectrons, which have the maximum energy of about 2 keV corresponding to the range of 0.2 µm in unit density matter (14).

About 30 phage samples were attached to a sample holder in the vacuum irradiation chamber. These samples were sequentially and automatically irradiated under vacuum (about 10^{-5} Pa) with monoenergetic X rays at assigned doses with a computer aided program. Immediately after the last sample had been irradiated, each sample was resuspended in 0.5 ml of NB medium (0.8% NB and 0.5% NaCl, pH~7) and kept in the dark at 4°C. Phage survivals were determined by the usual agar layer method with indicator host cells of repair deficient *E. coli* B_{s-1}. After irradiated phage samples were adequately diluted with T1 absorption buffer, 100 µl of the diluted phage sample and 100 µl of the cell suspension cultured in NB medium overnight were added into 2.5 ml of pre-warmed (45°C) NB soft agar (0.75% agar). This mixture was quickly spread on an NB agar plate (1.5% agar). After the soft agar had hardened, the plate was incubated in the dark at 37°C for 16 h. Phage survivors were counted as numbers of phage plaques.

Measurement of DNA Absorption Spectrum

An absorption spectrum of a self-standing thin DNA film was measured. About one milliliter of calf thymus DNA (Sigma, Type I: Sodium Salt) solution dissolved at about 5 mg/ml in distilled water with sonication was dropped on a polypropylene sheet, and then dried in a silica-gel desiccator. After being dried, the DNA film was peeled off from the sheet.

This self-standing DNA film was inserted in front of the ionization chamber and the X ray intensity measured. The absorbance of the DNA film was calculated as $\log(I_0/I)$ from the output of the ionization chamber with (I) and without (I_0) the DNA film.

RESULTS

Confirmation of the Resonance Absorption of Phosphorus in DNA

The absorption spectrum of the DNA film (about 1 mg/cm^2) was measured around the K absorption edge of phosphorus (Fig. 1). The spectrum had a highly intense peak (resonance peak), the energy of which was defined as 2153 eV following the methods of Kobayashi *et al.* (9). This intense peak was assigned (15) to be the 1s to t_2* (3p-like) transition of phosphorus, and a next broad band in the higher energy region is the shape resonance (15,16).

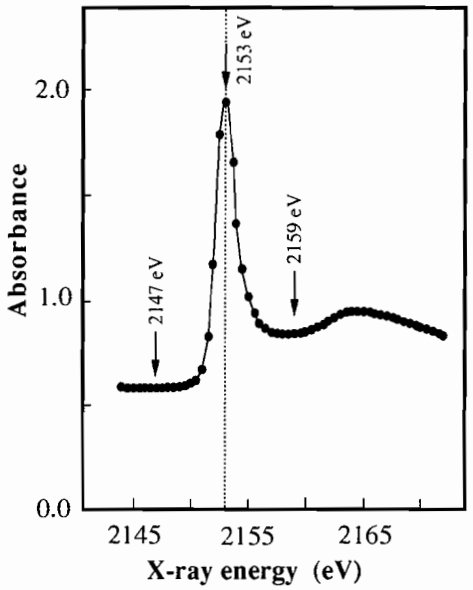

FIG. 1. Absorption spectrum of a thin film (about 1 mg/cm^2) of calf thymus DNA at energies around the K shell absorption edge of phosphorus. Arrows indicate the X ray energy used for irradiation experiments.

From this observation, three energies were chosen for phage irradiation: at the resonance peak (2153 eV), below (2147 eV) and above (2159 eV) the resonance peak.

Absorption Cross Section of Phage DNA at the Resonance Energy

Using the absorption spectrum, the absorption cross section of the phage DNA molecule was estimated as follows. Below the K absorption edge of phosphorus (2147 eV), the cross section was assumed to be the sum total of tabulated cross sections over atoms constituting the phage DNA molecule. For each constituent element, the number of atoms was calculated from the molecular weight (13) and the GC content (0.49) of the phage DNA molecule (see MATERIALS AND METHODS), and they were 1.13×10^6, 9.83×10^5, 3.77×10^5, 6.05×10^5, 1.01×10^5 and 1.01×10^5 for hydrogen, carbon, nitrogen, oxygen, sodium and phosphorus, respectively. The cross section at 2147 eV was obtained by extrapolation from the values at 1.5 and 2.0 keV of Scofield (17). The cross section of phage DNA at 2147 eV was calculated as 2.36×10^{10} barn, and thus those at 2153 eV (7.69×10^{10} barn) and 2159 eV (3.37×10^{10} barn) were the products of that at 2147 eV and the ratio of absorption spectrum, 3.26 at 2153 eV and 1.43 at 2159 eV. The cross section at 2159 eV calculated by extrapolation from the data of Scofield (17) at 3.0 and 4.0 keV agrees (3.47×10^{10} barn) with the result. The increments of the cross sections from 2147 eV must be the K shell cross section of phosphorus.

Enhanced Killing Effect at the Resonance Peak

Figure 2 shows survival curves of phages embedded in NB irradiated by monoenergetic X rays. The phage survivals were lowest at the peak energy (2153 eV, circles) and highest below the peak (2147 eV, inverted triangles). All of the survival data points collected at each X ray energy fell on a line on semi-logarithmic scale. Consequently, the experimental results can be conveniently expressed in terms of 37% exposure dose (D_0) calculated using an unweighted least squares method (Table I). The D_0 expressed in units of kR were converted into absorbed dose in units of kGy with the f-factors (Table I) for each X ray energy. The ratio of D_0 expressed in kR and kGy units were the same because the f-factors were not different for the different X ray energies. The sensitivity, as expressed by $1/D_0$, at the resonance peak (2153 eV) is clearly higher than both below and above the peak. The ratios of the averaged sensitivity normalized at 2147 eV were 1.68 at 2153 and 1.26 at 2159 eV (Table I).

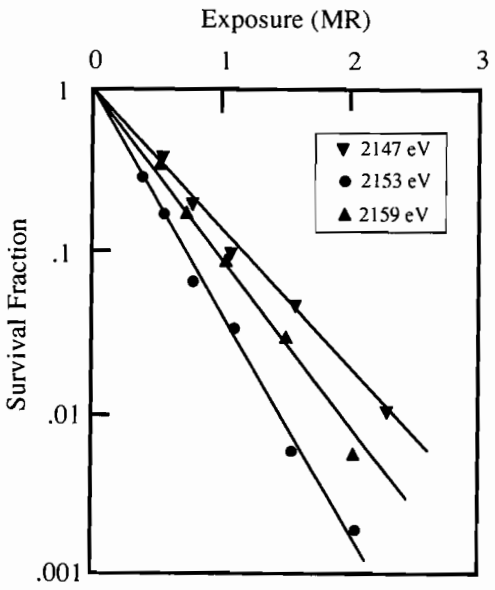

FIG. 2. Exposure-survival curves of bacteriophage T1 embedded in nutrient broth and irradiated with 2147, 2153 and 2159 eV monoenergetic X rays in vacuum condition.

DISCUSSION

Killing Efficiency of an Auger Cascade of Phosphorus

Absorbance of a DNA film (Fig. 1) sharply increased at 2153 eV by a factor of 3.26 (Table II) from 2147 eV. At the same energy, phage sensitivity increased by a factor of 1.67 (Table I). The enhanced sensitivity must be related to the K shell absorption of phosphorus, because the increment in absorbance was caused by K shell absorption of phosphorus in DNA. The number (N_P) of photoabsorption events by the K shell of phosphorus at the mean lethal dose (D_0) can be obtained as the products of three values: the number of phosphorus atoms in a phage DNA molecule, the photon fluence at D_0 from Eq. (1), and the photoionization cross section of the K shell of phosphorus. The number of phosphorus atoms and the cross sections were described in the RESULTS section. The calculated number of photoionizations of the K shell of phosphorus is shown in Table II. The number of Auger cascades per

D_0 in one phage (N_A in Table II) were calculated by $N_A = N_P \times 0.937$, where 0.937 is the Auger transition probability (18) for a K shell vacancy in phosphorus. Thus, 1.05 phosphorus K shell photoionizations, or 0.98 Auger cascades, occurred at 2153 eV in one phage DNA at one lethal hit (Table II). The 0.98 Auger cascades caused an increment in phage sensitivity of 1.20 MR^{-1} (= 3.01-1.81, Table II), or 0.40 (= 1.20/3.01) of fraction in total sensitivity. Thus, we suggest that the killing efficiency of Auger cascades induced at the resonance peak of the K shell of phosphorus is 0.41 (= 0.40/0.98).

At 2159 eV, the number of K shell photoabsorptions and the number of Auger cascades was calculated to be 0.27 and 0.25 per D_0 per phage DNA (Table II)

TABLE II

	X ray energy			Symbol
	2147 eV	2153 eV	2159 eV	
Normalized DNA absorbance	1.00 [f]	3.26	1.43	
Phage sensitivity (1/MR)				
total [a]	1.81	3.01	2.30	S_T
increased fraction [b]	-	1.20	0.49	S_I
Photoabsorption [c]				
1 / D_0 / phage	-	1.05	0.27	N_P
Auger enhancement				
1 / D_0 /phage [d]	0.00	0.98	0.25	N_A
efficiency [e]	-	0.41	0.84	E_A

[a] $S_T = 1 / D_0$. Data taken from Table I.

[b] $S_I = S_T$(at each energy) - S_T(at 2147 eV).

[c] For calculation method, see text.

[d] $N_A = N_P \times 0.937$, 0.937 is the transition probability (14) to Auger cascade from K-shell vacancy of phosphorus.

[e] $E_A = (S_I / S_T) / N_A$.

[f] 2.36×10^{10} barn (or 2.36×10^{-18} m^2) (for calculation method, see text).

using the same methods described above. The increment in the phage sensitivity was 0.49 MR^{-1} (Table II). A fraction of 0.21 (= 0.49/2.30) in total sensitivity was caused by the Auger cascades. Thus, the killing efficiency of an Auger cascade following K shell photoabsorption at 2159 eV was 0.84 (= 0.21/0.25). This result is twice that at the resonance peak. The reasons for this difference in the efficiency is not known at the present stage. It should be noted that very different mass-spectra (19) for fragmentation of simple molecules caused by a small difference in photon energy were shown around the K shell absorption edge. It may be possible that photolytic processes in DNA molecules are different at 2153 and 2159 eV, and the different photolytic processes may affect the killing efficiency of the Auger cascades.

Basal Sensitivity without the Auger Effect

For 2147 eV X rays, phosphorus atoms have no K shell photoionization cross section. Phages, however, were killed through photoabsorptions in outer-shells of phosphorus and in other elements in phage DNA, and through photoelectrons arising from phage and surrounding materials. Phage sensitivity at 2147 eV was 0.192 kGy^{-1} (= 1/5.21, Table I), which agrees with that (0.197 kGy^{-1}) obtained for monoenergetic synchrotron radiations at 12.4 keV under dry NB conditions (5) and that (0.235 kGy^{-1}) for ^{60}Co γ rays at high NB concentrations (13) at which the indirect effects is thought to be very small. The agreement of phage sensitivities over a wide range of energies of radiation indicates that phage sensitivities, except for the K shell photoabsorption of phosphorus, are determined by absorbed dose. We call this phage sensitivity the basal sensitivity.

At 2147 eV, the number of photoabsorptions by phage DNA, that is, photoabsorption at atoms except for phosphorus and outer-shells of phosphorus, could be superficially calculated by the same way described above: the number of photoabsorptions was 0.77 and the killing efficiency by the photoabsorption was 1.37 (= 1/0.77). The unexpected calculated result that the killing efficiency (1.37) of photoabsorptions other than in the K shell of phosphorus was higher than that (0.41 or 0.84) for Auger cascades of phosphorus is due to the fact that the basal sensitivity is due predominantly to the interactions in the phage DNA of secondary electrons originating from photoabsorption in the nutrient broth - as is the justification for the calculation of f-factors based on the nutrient broth (rather than DNA or phage) composition. These photoabsorptions, or their electrons, do not appear

at all in the above calculation and therefore the superficial, false result is obtained.

CONCLUSIONS

The absorption spectrum of a self-standing DNA film (calf thymus) at energies around resonance absorption energy of phosphorus showed a sharp peak at 2153 eV; the relative values were 1.00, 3.26 and 1.43 at 2147, 2153 and 2159 eV, respectively. Thus, bacteriophage T1 embedded in nutrient broth were irradiated under vacuum by monoenergetic X rays at energies of 2147, 2153 and 2159 eV and corresponding mean lethal doses of 553, 332 and 434 kR, respectively, were obtained. Fractional increments of the phage sensitivities due to Auger cascades following absorption in the K shell of phosphorus atoms in phage DNA were 0.67 at the resonance peak (2153 eV) and 0.28 above the resonance (2159 eV). The number of lethal events in phage per Auger cascade was 0.41 at 2153 eV and 0.84 at 2159 eV. The reason for lower efficiency at the resonance peak compared to above the resonance still remains to be studied. The basal sensitivity (Gy^{-1}), which was the sensitivity below the peak (2147 eV), was similar to the sensitivity at 12.4 keV previously reported, indicating that basal sensitivity was determined by absorbed dose.

ACKNOWLEDGMENTS

The authors are grateful to Dr. Takashi Ito and Dr. Shigefumi Okada for their valuable advice in the course of this work. We thank Dr. Akinori Yokoya and Noriko Usami of University of Tsukuba for their assistance in the operation of the irradiation system. This work was performed under the approval of the Photon Factory Program Advisory Committee (Proposal No. 86-098 and No. 86-101), and was partly supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan (59880014) and by a Tokai University School of Medicine Research Aid (1986,1987).

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DISCUSSION

Humm, J. The photon energies with which you are irradiating your specimens are extremely close to the K-absorption edge of phosphorus. You used the photoelectric cross section close to the edge. Did you consider the possibility of large errors in your estimated cross section and if so how?

Furusawa, Y. The cross section was experimentally measured as absorption spectrum. We found no special structure in the lower energy region than the highly intense resonance peak at 2153 eV. The cross section at 2147 eV (lower than the resonance peak) of 2.36×10^{10} barn/phage was obtained by extrapolation from Scofield's cross sections at 1.5 and 2.0 keV. Then we obtained the cross section at 2153 and 2159 eV with the value at 2147 eV and the measured absorption spectrum of DNA film normalized at 2147 eV. Also the cross section at 2159 eV was checked with extrapolated values from those at higher (3 & 4 keV) energies. The value calculated by the absorption spectrum 3.37×10^{10} barn/phage and the extrapolated value of 3.47×10^{10} barn/phage from the higher energy region showed good agreement.