AN ENDONUCLEASE ACTIVITY IN BACILLUS SUBTILIS SPECIFIC FOR UV-IRRADIATED DNA

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SUMMARY

An enzyme activity specific for UV-DNA* was found in the extract of Bacillus subtilis(Marburg 168). The enzyme preparation obtained from the extract by ammonium sulfate precipitation acts on UV-DNA endonucleolytically and induces single strand breaks. The number of single strand breaks introduced in DNA is proportional to UV dose.

INTRODUCTION

In the mechanism of excision repair in UV-damaged DNA, it seems that two DNases are involved. One is endonuclease which incises first in the 5'-end of pyrimidine dimer. Another, exonuclease which removes pyrimidine dimer in the form of oligonucleotide. And then, probably gaps made by these two enzymes, are repaired by DNA polymerase and ligase(1,2). In T4-phage, Yasuda and Sekiguchi reported that UV-endonuclease activity was absent in a UV-sensitive mutant of T4-phage and was identified to v gene product(3). This enzyme is thought to be responsible for the initial step of excision repair in T4-infected cells. They were also studying in the initial step of excision repair in Escherichia coli using T4-endonuclease(4).

Although in bacteria, UV-specific endonuclease was isolated and purified from Micrococcus luteus(5,6,7,8,9,10), it is not clear that UV-endonuclease acts as repair enzyme in vivo(11,12,13). UV-specific endonuclease activity was also

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* Abbreviations: UV, ultraviolet light; UV-DNA, UV-irradiated DNA.

detected in E. coli(14). More recently, Braun and Grossman isolated UV-specific endonuclease from E. coli and showed that UV-sensitive and excision defective mutants: uvrA,uvrB did not possess this endonuclease activity(15). In B. subtilis, Strauss et al reported that MMS*-treated DNA was degraded by its extract, but UV-DNA was not(16,17).

In this paper, we describe the existence of endonuclease in B. subtilis extract that preferentially acts on UV-DNA.

MATERIALS AND METHODS

Enzyme preparation. The strain used in this experiment was B. subtilis Marburg 168 thy^-ind^-**. The cells were grown overnight at 28C on tryptose blood agar base(TBAB:Difco). Cells were harvested from 3 plates, and then suspended to 1 liter of Spizizen's minimum medium containing 0.02% casamino acids (Difco) and 0.02% arginine(18). This culture was incubated at 37C on a rotary shaker. At an early stationary growth phase, cells were harvested by centrifugation at 8,000 rpm for 10 min at 4C. The harvested cells were resuspended in 40 ml of 20mM potassium phosphate buffer,pH 7.4 containing 0.5M sucrose, 10μg/ml thymine and 1mM EDTA, treated with 200μg/ml crystalline lysozyme(Sigma) at 37C for 40 min and followed by centrifugation at 50,000 rpm for 30 min using RP65-196 rotor in an Hitachi 55PA ultracentrifuge at 4C. The precipitate was suspended in above buffer containing 10mM 2-mercaptoethanol omitted sucrose and thymine, and then gently mixed with Triton X-100(Wako Pure Chemical Co.) to the final concentration of 0.01% in an ice bath for 30 min. The suspension was sonicated at 10 KC for two 30 sec at 0-4C(Ultrasonic Vibrator UR-200P:Tomy Seiko Co.) and was centrifuged at 50,000 rpm for 30 min at 4C. This supernatant was used as enzyme preparation under these experiments unless otherwise indicated.

Enzyme assay. Enzyme activity was measured as follows: reaction mixture(0.2 ml) containing 20mM pottasium phosphate buffer,pH 7.4, 0.2μg ^{14}C-labeled φ105C-DNA(about $6x10^3$ cpm), 10mM EDTA and enzyme preparation(400-500μg as protein) was

* ** Abbreviations: MMS, methyl methane sulfonate; thy^-ind^-, $thymine^-indole^-$.

incubated at 37C for 30 min and chilled in an ice bath. The reaction mixture(0.2 ml) was layered on 4.6 ml linear 5-20% (W/V) alkaline sucrose gradient prepared in 0.7M NaCl, 1mM EDTA and 0.3N NaOH. The centrifugation was carried out at 10C for 180 min at 38,000 rpm using RPS 40T-2 rotor in a Hitachi 55PA ultracentrifuge. After centrifugation, 3 drop-fractions were collected onto Toyo Glass Fiber filter papers(Type GB-100) from the bottom of the tube. The filter papers were dried and counted in toluene base scintillator using a Beckman DPM 100 type liquid scintillation spectrometer. In neutral sucrose, centrifugation was also carried out at 38,000 rpm at 10C for 120 min. Sucrose containing 0.9M NaCl, 10mM Tris-HCl,pH 7.5 and 1mM EDTA, was used for the preparation of the gradient. Other conditions were the same as for alkaline sucrose.

Preparation of DNA. Phage ϕ105C is a clear plaque mutant of B. subtilis temperate phage ϕ105. [^{14}C]Thymidine-labeled ϕ105C-DNA(3×10^4 cpm/μg) was prepared from purified ϕ105C phage particles grown in 100 ml of tryptone broth supplemented with 20 μCi of [2-^{14}C]Thymidine(62 mCi/mmol) according to the method reported by Rutberg and Rutberg(19). [2-^{14}C]Thymidine was purchased from the Radiochemical Centre(Amersham).

UV-irradiation of DNA. 0.3 ml of ^{14}C-labeled ϕ105C-DNA in 10mM pottasium phosphate buffer,pH 7.4(3μg/ml) on a small watch glass, was exposed to the 15W-Toshiba germicidal lamp at a distance of 19 cm.

RESULTS AND DISCUSSION

The extract of B. subtilis was found to introduce breaks in UV-DNA(4500 erg/mm^2)(Fig.1). In Fig.1a, there is little difference of sedimentation behaviors between unirradiated DNAs treated and untreated with extract. As shown in Fig.1b, however, the degradation of UV-DNA by extract occured clearly when the sedimentation of UV-DNA treated or untreated with extract was compared. The size of UV-DNA treated with extract was several times smaller than UV-DNA untreated. The relationship of endonuclease activity to UV dose is shown in Fig.2. DNA was irradiated by UV at indicated dose, treated with this extract and centrifuged in alkaline sucrose gradient. According to the elevation of UV dose, DNA gradually becomes slow sedimenting materials. The sedimentation behavior of un-

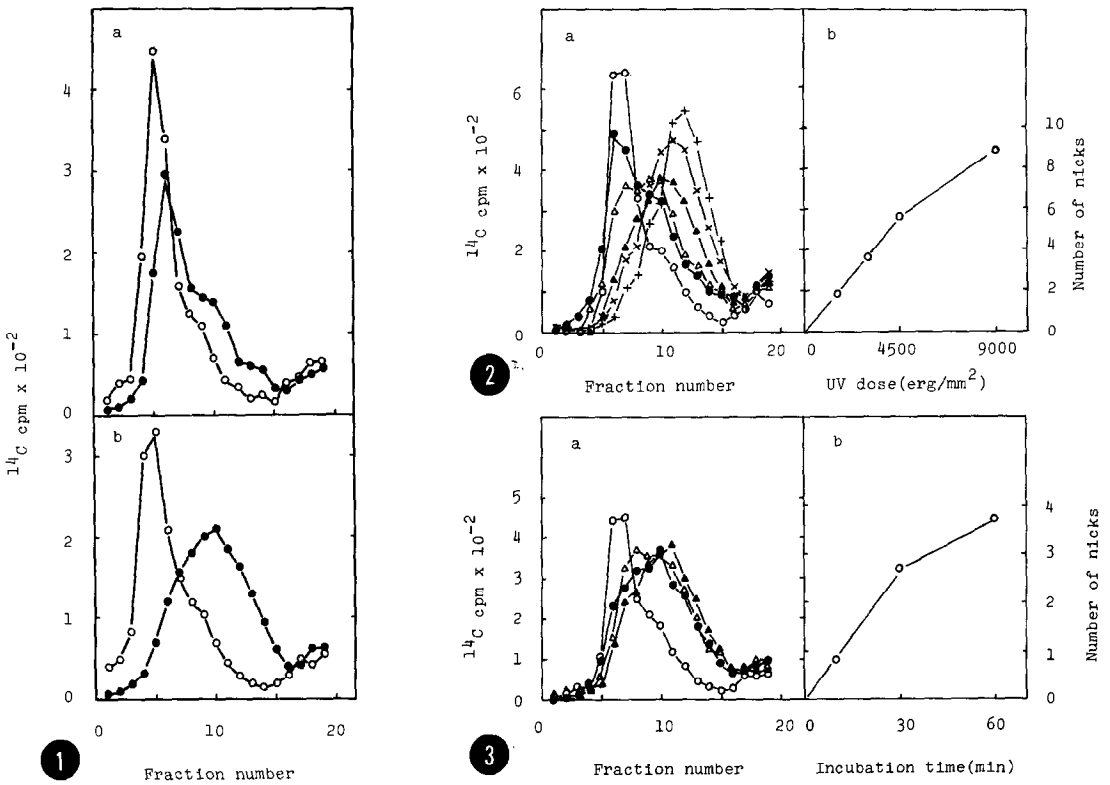

Fig.1. Alkaline sucrose gradients of ^{14}C-labeled unirradiated and UV-irradiated ϕ105C-DNA, untreated and treated with extract. Experimental details are presented in Materials and Methods. Sedimentation was from right to left in all gradients. a. Unirradiated DNA was used as substrate: treatment with extract(●—●), without extract(o—o). b. UV-DNA(4500 erg/mm^2) was used as substrate: treatment with extract(●—●), without extract(o—o).

Fig.2. UV dose dependency of enzyme activity. a. Profiles of alkaline sucrose gradients. ^{14}C-labeled UV-irradiated ϕ105C-DNAs at indicated dose were incubated with extract as described in Materials and Methods. Symbols: unirradiated DNA omitted extract(o—o), unirradiated DNA(●—●), 1500 erg(▲—▲), 3000 erg(▲—▲), 4500 erg(×—×), 9000 erg(+—+). b. The average number of nicks introduced in UV-DNA at indicated dose calculated on the equation of Abelson and Thomas.

Fig.3. Time course of enzyme activity. a. Profiles of alkaline sucrose gradients. ^{14}C-labeled UV-irradiated ϕ105C-DNA(4500 erg/mm^2) was incubated with extract at various intervals as described in Materials and Methods. Symbols: 0 min(extract omitted, o—o), 10 min(▲—▲), 30 min(●—●), 60 min(▲—▲). b. The average number of nicks introduced in UV-DNA at various incubation time calculated on the equation of Abelson and Thomas.

irradiated DNA which has been treated with extract has little difference from that of the control. The average number of nicks per intact double strand DNA is calculated on the

equation of Abelson and Thomas(20) and shown in Fig.2b. The relation of UV dose to nicks is linear under 4500 erg/mm^2. It indicates this endonuclease activity is proportional to UV dose, and it acts specifically on UV-DNA in the presence of EDTA(10mM). The relation of incubation time to nicks introduced in UV-DNA is linear up to 30 min(Fig.3a,b). In the presence or absence of Mg^{++}, there is no detectable acid soluble radioactivity, so, the action to UV-DNA appears to be endonucleolytical. Then, this endonuclease was partially purified by treatment with streptomycin sulfate and ammonium sulfate precipitation, and used in the experiment for precise mode of

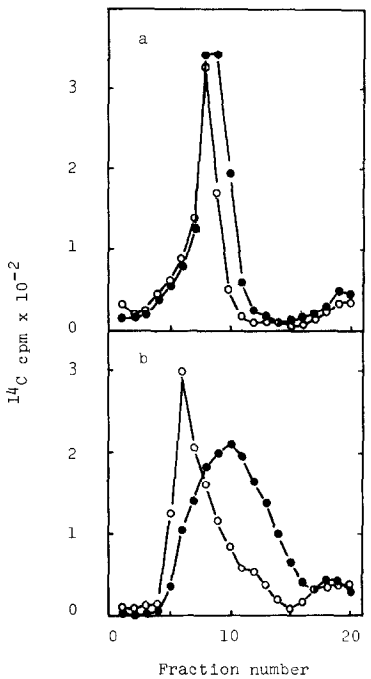

Fig.4. Sedimentation profiles in neutral and alkaline sucrose gradients. The enzyme preparation used in this experiment, was partially purified with streptomycin and ammonium sulfate treatments. 1.6% of streptomycin solution containing in 10mM potassium phosphate buffer,pH 7.4, 1mM EDTA and 10mM 2-mercaptoethanol, was added to the same volume of extract. Ammonium sulfate was added to the supernatant of the extract treated with streptomycin and 45-90% ammonium sulfate-saturated fraction was obtained as enzyme preparation. ^{14}C-labeled UV-irradiated ϕ105C-DNAs(4500 erg/mm^2) were incubated as described in Materials and Methods. a. Neutral sucrose gradients: treatment with enzyme(●—●), without enzyme(○—○). b. Alkaline sucrose gradients: treatment with enzyme(●—●), without enzyme (○—○).

action to UV-DNA. Fig.4 shows sedimentation profiles of UV-DNAs treated and untreated with the enzyme preparation in neutral and alkaline sucrose gradients. There is no significant difference of sedimentation behaviors in neutral sucrose. In alkaline sucrose, however, the enzyme treated-UV-DNA is sedimented at slower rate. Therefore, it may be suggested that this endonuclease induces single strand scissions in UV-DNA. This endonuclease appears to possess very similar characters to T4-phage, M. luteus and E. coli UV-endonucleases. But, it was reported by Strauss et al(16,17) that extract of B. subtilis acted on methylated DNA, but not on UV-DNA. It is not yet clear why they could not detect UV-endonuclease activity. One possibility is the enzyme preparation from cells had reduced levels of non specific nucleases, so, we could detect UV-DNA specific endonuclease activity without interferences from other nucleases. Another may be different method of enzyme preparation from them. In our enzyme preparation, it is not yet examined whether methylated DNA specific DNase is existed or not.

Now, we roughly estimated the ratio of nicks introduced in UV-DNA / thymine dimers induced in DNA by UV(4500 erg/mm^2, shown in Fig.2). The ratio is calculated to be 1/25. The average number of nicks introduced in UV-DNA seems to be relatively low in comparison with the number of thymine dimers induced in DNA by UV. In our opinion, it might be explained by low specific activity of the enzyme preparation used in these experiments. Preliminary results obtained by partially purified enzyme preparation showed that its activity was not inhibited by heat denatured ϕ105C-DNA in competition experiments. It seems that this endonuclease does not act on single-stranded region in UV-DNA where local denaturation has occured by UV-irradiation (21,22,23). We detected an endonuclease activity in extract of B. subtilis which introduced single strand scission in double strand UV-DNA in the presence of EDTA and is proportional to UV dose. We are now progressing in the purification of this endonuclease and studying in the role of this enzyme in vivo using various UV-sensitive mutants of B. subtilis .

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