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Purification and Characterization of a Novel DNA Repair Enzyme from the Extremely Radioresistant Bacterium *Rubrobacter radiotolerans*

EZAT ASGARANI[1], HIROAKI TERATO[1], KENJIRO ASAGOSHI[1], HAMID REZA SHAHMOHAMMADI[1], YOSHIHIKO OHYAMA[1], TAKESHI SAITO[2], OSAMU YAMAMOTO[3] and HIROSHI IDE[1]*

[1]Department of Mathematical and Life Sciences, Graduate School of Science, Hiroshima University, Higashi-Hiroshima 739–8526, Japan
[2]Radioisotope Center, Hiroshima University, Higashi-Hiroshima 739–8526, Japan
[3]Department of Clinical Radiology, Faculty of Health Science, Hiroshima International University, Kurose, Hiroshima 724–0695, Japan
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Rubrobacter radiotolerans is an extremely radioresistant bacterium. It exhibits higher resistance than the well-known radioresistant bacterium *Deinococcus radiodurans*, but the molecular mechanisms responsible for the radioresistance of *R. radiotolerans* remain unknown. In the present study, we have demonstrated the presence of a novel DNA repair enzyme in *R. radiotolerans* cells that recognizes radiation-induced DNA damages such as thymine glycol, urea residues, and abasic sites. The enzyme was purified from the crude cell extract by a series of chromatography to an apparent physical homogeneity. The purified enzyme showed a single band with a molecular mass of approximately 40 kDa in SDS-polyacrylamide gel electrophoresis, and was designated as R-endonuclease. R-Endonuclease exhibited repair activity for thymine glycol, urea residues, and abasic sites present in plasmid DNA, but did not act on intact DNA, UV-irradiated DNA and DNA containing reduced abasic sites. The substrate specificity together with the salt and pH optima suggests that R-endonuclease is a functional homolog of endonuclease III of *Escherichia coli*.

INTRODUCTION

Rubrobacter (formerly *Arthrobacter*) *radiotolerans,* which was isolated from muddy water at the radon-containing hot spring in Japan, is extremely resistant to the lethal effects of

*Corresponding author: Phone & Fax; +81–824–24–7457, E-mail; ideh@ipc.hiroshima-u.ac.jp

ionizing radiation. The resistance of this bacterium is even greater than the well-known radioresistant bacterium *Deinococcus radiodurans*[1,2]. *R. radiotolerans* and *D. radiodurans* show characteristic reddish color due to the presence of carotenoids, whose major components have been identified as bacterioruberin and deinoxanthin, respectively[3,4]. Since these carotenoids act as effective antioxidants *in vitro*, the resistance of these bacteria to ionizing radiation appears to be partly attributed to the carotenoids[5]. Recently we have also shown that *Halobacterium salinarium,* another red-pigmented bacterium, containing bacterioruberin exhibits considerably high resistance against DNA-damaging agents such as ionizing radiation and hydrogen peroxide[6,7]. Consistent with the *in vitro* results, the mutants of *D. radiodurans* and *H. salinarium* deficient in the carotenoids show higher sensitivities than the wild type strains against ionizing radiation and hydrogen peroxide[7,8]. These *in vitr*o and *in vivo* data suggest that bacterioruberin present in *R. radiotolerans* plays an important role in the radioresistance of this bacterium.

In view of the distinctive feature of high resistance of *R. radiotolerans* and *D. radiodurans* against ionizing radiation, the carotenoids can not be the sole explanation for the high resistance of these bacteria. Indeed, *D. radiodurans* has been suggested to have very proficient DNA repair mechanisms that may contribute the radioresistance[9]. There are also reports on the nucleotide excision[10,11] and base excision[12–14] repair enzymes in *D. radiodurans*. In contrast, there is no report on the DNA repair activity in *R. radiotolerans* cells until now.

Ionizing radiation and many other oxidative agents produce different types of DNA lesions including single- and double-strand breaks, base damage, and DNA-DNA and DNA-protein cross-links. Among them, base damage constitutes a major part of DNA lesions. Thymine glycol, urea residues, and abasic sites are identified as common radiolysis products of thymine in DNA[15]. Thymine glycol and ring fragmentation products such as urea residues have been shown to block DNA replication[16,17], and constitute lethal lesions in transfecting phage DNA[18]. Urea residues, but not thymine glycol, are also premutagenic[19]. Abasic sites and single-strand breaks are produced at equal frequencies by ionizing radiation. Since abasic sites constitute replicative blocks and direct incorporation of purine nucleotides during DNA replication, they could be lethal and mutagenic[20]. Thus, these DNA lesions need to be removed by cellular repair enzymes to prevent their cytotoxic and mutagenic effects on cells.

Considering the high resistance of *R. radiotolerans* against ionizing radiation, it is reasonable to assume the presence of DNA repair systems in this organism that allow cells to survive after exposure to high doses of irradiation. In the present study, a DNA repair enzyme that recognizes thymine glycol, urea residues, and abasic sites has been purified from *R. radiotolerans*. To our knowledge, this is the first report on the DNA repair enzyme of *R. radiotolerans* that acts directly on radiation-induced DNA lesions.

MATERIALS AND METHODS

Bacteria and enzymes

Rubrobacter radiotolerans JCM 2153T (IAM 12072, strain p-1) was obtained from Japan Collection of Micro-Organisms. *E. coli* endonuclease III (endo III) was overexpressed in *E. coli* harboring plasmid containing the *nth* gene (gift from S. S. Wallace and Z. Hatahet, University of Vermont) and purified as described[21]. *E. coli* exonuclease III (exo III) was purchased from Amersham Pharmacia Biotech. T4 endonuclease V (endo V) was a gift from E. Ohtsuka[22].

Preparation of substrates

Supercoiled pDEL19 DNA (4.8 kbp) was purified from the lysate of *E. coli* JM109/pDEL19 with Plasmid Maxi Kit (QIAGEN). Thymine glycol was introduced in pDEL19 DNA by the treatment with 0.08% osmium tetroxide at 65°C for 12 min[23]. After the treatment, DNA was extensively dialyzed against TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) at 4°C. Urea residues were selectively introduced into DNA by the treatment of DNA containing thymine glycol with alkali[24]. DNA containing thymine glycol was dialyzed against 40 mM Na_2HPO_4-NaOH (pH 12.0) and 2 mM EDTA at 25°C for 24 h, followed by dialysis against TE buffer overnight at 4°C. Abasic sites were introduced into DNA by incubation in 30 mM KCl and 10 mM sodium citrate (pH 5.0) at 70°C for 10 min[25]. To prepare DNA containing reduced abasic sites, abasic DNA (3 μg/50 μl) in TE buffer was incubated with sodium cyanoborohydride (0.5 M) at room temperature for 3 h[26]. Sodium cyanoborohydride was then removed by extensive dialysis at 4°C. UV-irradiated DNA was prepared by irradiation of pDEL19 DNA in 10 mM sodium phosphate buffer (pH 7.0) at room temperature with a Toshiba GL15 germicidal lamp at a fluence rate of 0.98 $J \cdot m^{-2} \cdot s^{-1}$ to a final dose of 30 $J \cdot m^{-2}$. DNA solution was stirred during irradiation.

Activity assay

The repair activity of enzyme at various purification stages was monitored by DNA nicking assay. Conversion of pDEL19 DNA from supercoiled (Type I) to open circular form (Type II) was assayed by agarose gel electrophoresis. Standard reaction mixture contained 100 ng of damaged pDEL19 DNA, enzyme fraction (5 ng – 13 μg as protein), 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl in a total volume of 10 μl. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 2 μl of the gel loading solution containing 0.25% bromophenol blue, 0.25% xylene cyanol FF, and 30% glycerol. The sample was analyzed by agarose gel electrophoresis at 100 V for 50 min. The amounts of supercoiled (Type I) and nicked (Type II) DNA in the ethidium bromide-stained gel were determined by an Imaging Analyzer FAS II (Toyobo). The number (n) of nicks per pDEL19 molecule was determined by the relation n = -ln (Type I fraction)[26]. One unit of R-endonuclease activity was defined as 1 fmol of nicks per min with OsO_4-treated pDEL19 DNA that contained an average of two thymine glycol sites per molecule. In

control experiments, the activities of endo III (10 ng) and exo III (0.4 ng) for damaged pDEL19 DNA were determined in manners similar to that of the *R. radiotolerans* enzyme, except that the buffer composition was 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 100 mM NaCl for endo III and 60 mM Tris-HCl (pH 8.0), 6.6 mM $MgCl_2$, and 1 mM ß-mercaptoethanol for exo III. The incubation was performed at 37°C for 30 min (endo III) or 10 min (exo III).

To assess the activity of R-endonuclease for pyrimidine photodimers, UV-irradiated DNA (50 ng) in appropriate buffers (10 μl) was treated with 5 ng of R-endonuclease as described above or 6 ng of T4 endo V for control. The endo V buffer contained 3.2 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM NaCl, and 0.02% bovine serum albumin[22]. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by addition of 2 μl of the gel loading solution. The nick formed in the plasmid was measured by agarose gel electrophoresis as described above.

The pH optimum of R-endonuclease was determined using the assay described above, except that Tris-HCl was used for the pH range between 7.8 and 9.0, and Hepes-KOH for the pH between 6.0 and 7.8.

Cell culture

R. radiotolerans was grown in R-broth medium in a jar fermentor with aeration at 48°C. R-broth contained 10 g of trypton, 5 g of yeast extract, 5 g of casamino acids, 2 g of meat extract, 5 g of malt extract, 1 g of $MgSO_4 \cdot 7H_2O$, 1.59 ml of glycerol per liter[27]. The cells were harvested at logarithmic phase by centrifugation. The cell paste was stored at −80°C until use.

Enzyme purification

All procedures were performed at 4°C unless otherwise indicated. Cells (100 g) were suspended in 350 ml of washing buffer A containing 50 mM Tris-HCl (pH 7.6), 10 mM EDTA, 0.3 M NaCl. The cells were centrifuged at 5000 *g* for 20 min, and the pellet was resuspended in the same volume of buffer A containing 0.15 mM PMSF. The cell suspension was subjected to freeze-thaw cycles three times. The DNA was sheared by sonication in an ice-water bath at 145 watt output (20 s pulse on, 40 s pulse off) for a total time of 10 min with a Tomy UR-200P ultrasonic disrupter. The sonicated solution was stirred for 1 h on ice and cell debris was removed by centrifugation at 35000 *g* for 30 min. DNA was precipitated by addition of 56 ml of 10% (w/v) streptomycin sulfate over 30 min with stirring, followed by centrifugation at 35000 *g* for 30 min. To the supernatant was added 225 g of ammonium sulfate (75% saturated solution) over 30 min with stirring. The solution was stirred for further 30 min and centrifuged at 35000 *g* for 30 min. The precipitate was resuspended in a minimum volume (ca. 20 ml) of storage buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol, 1 mM EDTA, and 20% glycerol and kept frozen at −20°C until further use (Fraction I).

(i) Q-Sepharose chromatography. The crude extract (Fraction I, 20 ml) was dialyzed against buffer B containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, and

50 mM NaCl and loaded on a Q-Sepharose FF column (Amersham Pharmacia, 2.6 cm × 20 cm) pre-equilibrated with buffer B. The column was eluted with a linear gradient of NaCl from 0.05 to 1.0 M in buffer B (total 264 ml). Fractions containing the activity (0.28–0.64 M NaCl) were pooled (Fraction II).

(ii) SP-Sepharose chromatography. The pooled fraction from the Q-Sepharose column (Fraction II) was dialyzed against buffer C containing 20 mM Hepes-KOH (pH 7.0), 50 mM NaCl, 1 mM EDTA, and 1 mM dithiothreitol and loaded on an SP-Sepharose column (Amersham Pharmacia, 1.6 cm × 10 cm) equilibrated with buffer C, and the bound protein was eluted with a linear gradient of NaCl from 0.05 to 1.0 M in buffer C (total 200 ml). The most active fractions (0.53–0.61 M NaCl) were pooled (Fraction III).

(iii) Phosphocellulose chromatography. The pooled fraction from the SP-Sepharose column (Fraction III) was dialyzed against buffer D containing 50 mM Hepes-KOH (pH 7.0), 50 mM NaCl, 0.1 mM EDTA, and 1 mM dithiothreitol. The dialyzed solution was loaded on a phosphocellulose column (Whatman P-11, 1.6 cm × 20 cm). The column was washed with buffer D (50 ml) and eluted with a linear gradient of NaCl from 0.05 to 1.0 M in buffer D (total 100 ml). Active fractions (0.47–0.75 M NaCl) were pooled and stored at –20°C (Fraction IV).

(iv) Mono S chromatography. A fast protein liquid chromatography system (FPLC, Amersham Pharmacia) was employed. Fraction IV was dialyzed against buffer C. The dialyzed sample was loaded on an HR 5/5 Mono S cation-exchange column (Amersham Pharmacia). Protein was eluted with a linear gradient of NaCl from 0.05 to 1.0 M in buffer C (total 20 ml). The active fraction around 0.31 M NaCl was taken and stored at –20°C (Fraction V).

Protein analysis

The protein concentration of Fractions I and II was determined with BCA protein assay reagent kit (Pierce) and bovine serum albumin (BSA) as the standard. The protein concentration of Fractions III–V containing diluted proteins were measured by SDS-PAGE analysis. The protein bands were visualized by silver staining and band intensities were analyzed and integrated by NIH Image (ver 1.55) using varying amounts of BSA as the standard. The following standard proteins were used in SDS-PAGE for molecular mass markers, myosin (M_r = 200 kDa), ß-galactosidase (116 kDa), BSA (66 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa) and myoglobin (17.2 kDa).

RESULTS

Purification of R-Endonuclease

The damage-specific endonuclease activity (designated as R-endonuclease after *Rubrobacter radiotorelans*) for plasmid DNA containing thymine glycol was observed in the supernatant after streptomycin-sulfate precipitation, although this fraction also contained non-specific activity acting on intact DNA. The activity in the ammonium sulfate-precipitated

Table 1. Purification of R-endonuclease

Fraction	Step	Protein conc. (mg/ml)	Total protein (mg)	Specific activity[a] (unit/mg)	Purification (X-fold)	Total activity (unit)	Yield[b] (%)
I	crude extract[c]	11.8	235	30.7	1	7210	—
II	Q-Sepharose	1.04	83.2	289	9.4	24000	100
III	SP-Sepharose	0.010	0.15	46000	1500	6900	29
IV	Phosphocellulose	0.005	0.04	64000	2100	2600	11
V	Mono S	0.003	0.003	190000	6200	570	1.6

[a]Specific activity was determined using nicking assay of thymine glycol-containing pDEL19 DNA.
[b]Percent yield was calculated starting with fraction II after removal of nucleic acids by Q-Sepharose chromatography.
[c]Ammonium sulfate-precipitated fraction.

sample (Fraction I) appeared to be inhibited for unknown reasons based on the comparison with the activity in the subsequent step of purification (Table 1).

R-Endonuclease was purified based on the endonuclease activity to thymine glycol-containing pDEL19 DNA. The purification steps employing a series of ion-exchange chromatography are summarized in Table 1. The activity was eluted from the Q-Sepharose column with 0.28–0.64 M NaCl (Fraction II). Active fractions were pooled and further purified by cation-exchange chromatographies on SP-Sepharose and phosphocellulose columns. The activity was eluted 0.53–0.61 (Fraction III) and 0.47–0.75 M NaCl (Fraction IV) with SP-Sepharose and phosphocellulose columns, respectively. Finally, chromatography on a Mono S column resulted in enzyme purification approximately 6200-fold and 1.6% yield from the Q-Sepharose fraction. The result of protein analysis of Fractions I–V by SDS-PAGE is shown in Fig. 1. The most purified preparation (Fraction V) appeared to be homogeneous in SDS-PAGE, showing a single protein band after silver staining. Fraction V was used for the subsequent characterization of the repair activity of R-endonuclease. The apparent molecular mass of purified protein was estimated as 40 kDa based on the mobility in SDS-PAGE.

Substrate Specificity

Ionizing radiation produces distinct types of base damage in DNA[15]. Thymine glycol, urea residues, and abasic sites are all radiolysis products of DNA. These lesions were selectively introduced into pDEL19 DNA to characterize the substrate specificity of R-endonuclease. The activity of R-endonuclease (Fraction V) was examined by nicking assay based on the conversion of Type I plasmid to Type II. As shown in Fig. 2A, R-endonuclease incised pDEL19 DNA containing thymine glycol (lane 6), urea residues (lane 11) and abasic sites (lane 16), but not intact DNA (lane 2). In parallel experiments, the nature of DNA lesions present in the substrates was further confirmed by the known activities of endo III and exo III on these substrates. Endo III incised DNA substrates containing thymine glycol (lane 4), urea residues (lane 9), and abasic sites (lane 14). In contrast, exo III incised those containing urea residues (lane 10) and abasic sites (lane 15), but not thymine glycol (lane 5). The activities of

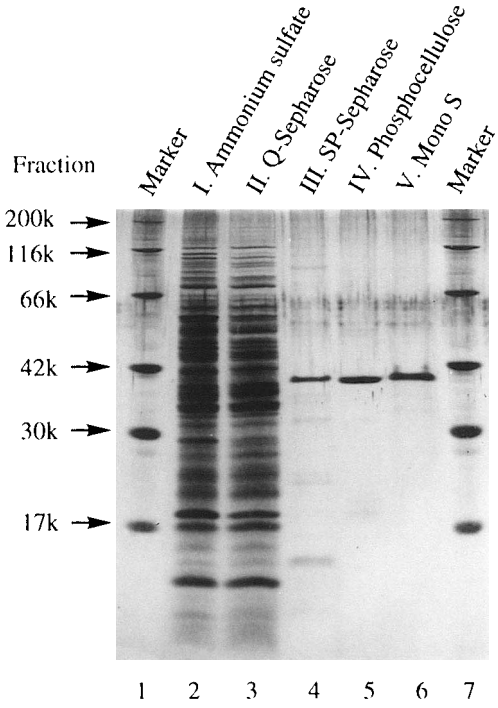

Fig. 1. SDS-Polyacrylamide gel electrophoresis of fractions obtained in each purification step of R-endonuclease. Lanes 1 and 7, molecular mass standards: myosin (200 kDa), ß-galactosidase (116.2 kDa), BSA (66.3 kDa), aldolase (42.4 kDa), carbonic anhydrase (30 kDa), myoglobin (17.2 kDa). The fraction and purification step are shown on the top of the gel.

R-endonuclease for thymine glycol, urea residues and abasic sites were all inactivated by the heat treatment at 90°C for 5 min (lanes 7, 12 and 17).

Substrate specificity of R-endonuclease for UV-irradiated DNA containing pyrimidine photodimers was also examined (Fig. 2B). R-Endonuclease did not nick UV-irradiated DNA (lane 6), although T4 endo V effectively incised the same substrate (lane 5).

Activity to reduced abasic sites

Hydrolysis of the *N*-glycosidic bond of DNA produces base-free deoxyribose moieties in DNA. There are two structures for the baseless sugar, a "closed" furanose form and an "opened" form containing C-1' aldehyde group. The C-1' aldehyde is required for the AP lyase activity of base excision repair enzymes but not the hydrolytic AP endonuclease activity[20]. In this study, it was examined whether a C-1' aldehyde was required for enzymatic cleavage of abasic sites by R-endonuclease. pDEL19 DNA containing abasic sites was treated with sodium cyanoborohydride to convert the aldehyde to a C-1' hydroxyl group. The effect of C-1' structure on cleavage by R-endonuclease, endo III, or exo III was analyzed

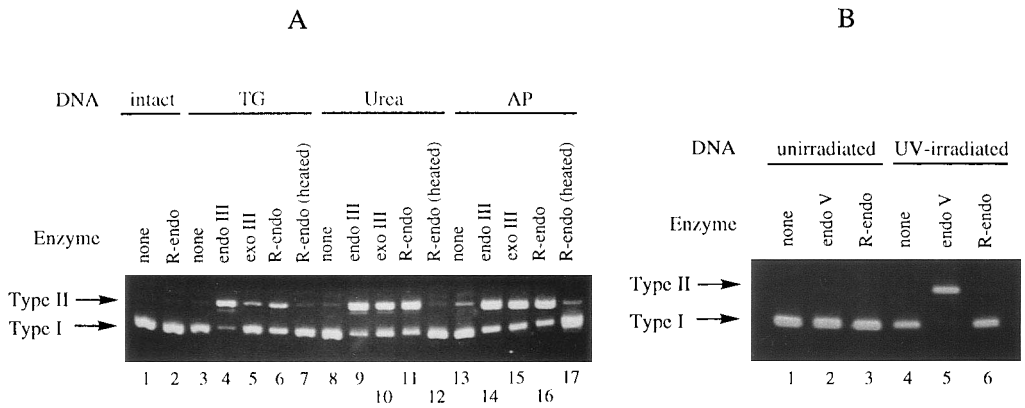

Fig. 2. Substrate specificity of R-endonuclease. (A) Supercoiled Type I pDEL19 DNA (100 ng) containing thymine glycol (TG), urea residues (Urea) or abasic sites (AP) was treated with the indicated repair enzymes for 30 min at 37°C, and run on an agarose gel. Lanes 1 and 2, intact DNA; lanes 3–7 thymine glycol-containing DNA; lanes 8–12 urea- containing DNA; lanes 13–17, AP-DNA. DNA substrates were treated without enzyme (lanes 1, 3, 8, and 13) or with endo III (10 ng) (lanes 4, 9, and 14), exo III (0.4 ng) (lanes 5, 10, and 15), R-endonuclease (5 ng) (lanes 2, 6, 11, and 16), and heat-inactivated R-endonuclease (5 ng, 90°C for 5 min). (B) UV-irradiated pDEL19 DNA containing pyrimidine photodimer (50 ng) was treated with T4 endo V (6 ng) or R-endonuclease (5 ng) at 37°C for 30 min and run on an agarose gel. Lanes 1–3, unirradiated DNA; lanes 4–6, UV-irradiated DNA. Lanes 1 and 4, without enzyme; lanes 2 and 5, samples treated with endo V, lanes 3 and 6 samples treated with R-endonuclease.

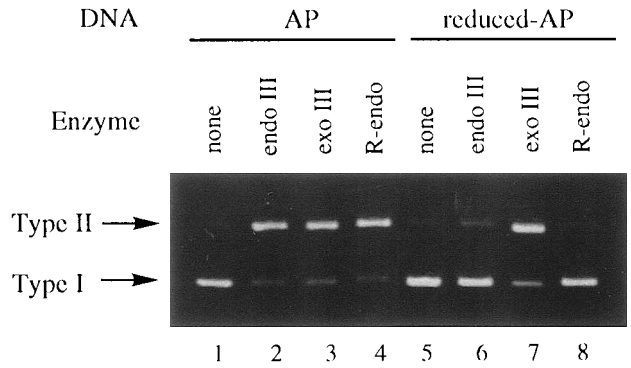

Fig. 3. Activity of R-endonuclease on natural and reduced absic sites. Supercoiled Type I pDEL19 DNA (100 ng) containing natural and reduced abasic (AP) sites was incubated with the indicated repair enzymes at 37°C for 30 min and run on an agarose gel. Lanes 1–4, AP-DNA; lanes 5–8, reduced-AP DNA. The substrates were treated without enzyme (lanes 1 and 5) or with endo III (10 ng) (lanes 2 and 6), exo III (0.4 ng) (lanes 3 and 7), and R-endonuclease (5 ng) (lanes 4 and 8).

(Fig. 3). Sodium cyanoborohydride reduction of abasic sites markedly decreased the incision activity of R-endonuclease (lanes 4 and 8) and endo III (lanes 2 and 6), whereas the activity of exo III was virtually independent of the C-1' structure (lanes 3 and 7). These data suggest that the C-1' aldehyde group is required for cleavage of abasic sites by R-endonuclease.

Comparison of the repair activities of R-endonuclease and endo III

The Type II form resulting from the incision of plasmid DNA containing thymine glycol increased with the increasing amount of R-endonuclease (Fig. 4), showing that the repair activity was dependent on the amount of protein. Using these assay conditions, relative repair activities of R-endonuclease for different lesions were quantitatively compared. Figure 5A shows the time courses of nicking reactions by R-endonuclease for plasmids containing thymine glycol, urea residues, and abasic sites. These results were compared to those for endo III using the same substrates (Fig. 5B). Both enzymes preferred DNA containing abasic sites and urea residues over DNA containing thymine glycol as substrates. The activities for these three lesions were compared based on the initial rate of the nicking reaction (up to 10 min). The activities of R-endonuclease for abasic sites and urea residues were 5.7 and 4.7 times higher than that of thymine glycol, respectively. The corresponding values for endo III were 2.0 and 1.7 for abasic sites and urea residues, respectively. A similar comparison also indicated that the specific activities of R-endonuclease for urea residues, abasic sites, and thymine glycol were 2 to 5 times higher than those of endo III.

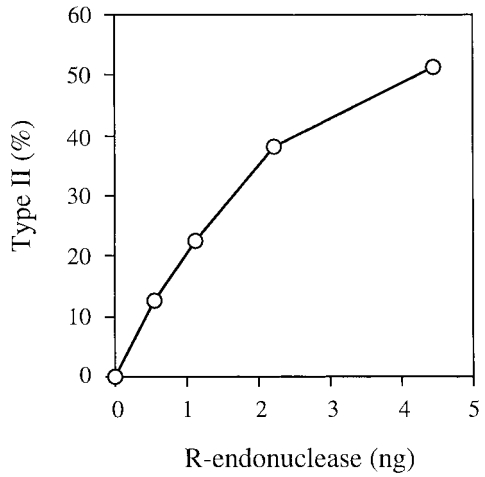

Fig. 4. Correlation between the amount of R-endonuclease and repair activity. Increasing amounts of R-endonuclease were incubated with thymine glycol-containing pDEL19 DNA (100 ng) at 37°C for 30 min. The percent of Type II DNA was measured by agarose gel electrophoresis and plotted against the amount of R-endonuclease used.

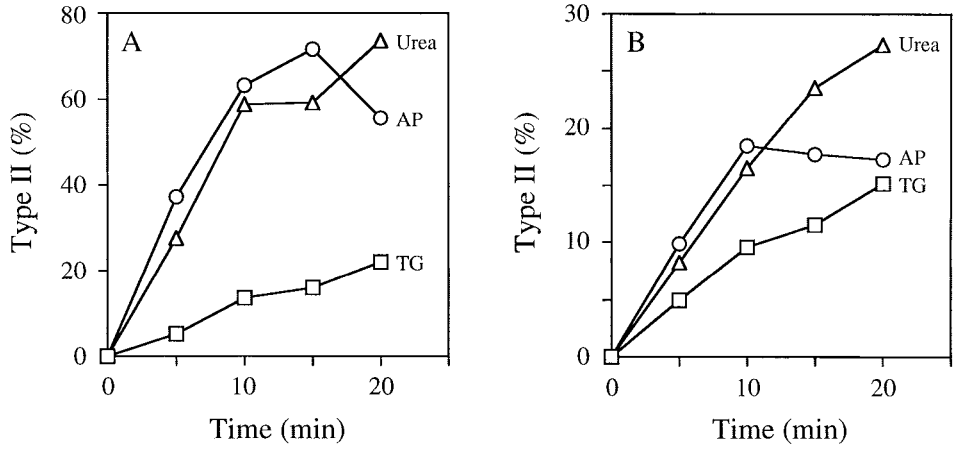

Fig. 5. Comparison of repair activities of R-endonuclease and endo III for different substrates. Supercoiled pDEL19 DNA containing thymine glycol (TG, □), urea residues (Urea, △) or abasic sites (AP, ○) was treated with (A) R-endonuclease (3 ng) and (B) endo III (4 ng) at 37°C for the indicated periods. Enzyme activities were measured as the percent of Type II DNA after incubation by agarose gel electrophoresis.

Requirements for activity and reaction optima

The purified R-endonuclease did not require the magnesium ion for the activity and the activity was not affected by the addition of EDTA. R-endonuclease activities for DNA containing thymine glycol, urea residues, and abasic sites were essentially retained in the presence of 5 mM $MgCl_2$ (data not shown). This result indicated that the magnesium ion was not inhibitory to the endonucleolytic reaction of R-endonuclease. This is in contrast to the

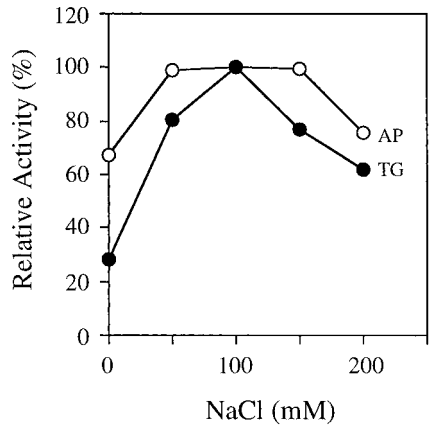

Fig. 6. Effect of NaCl on the activity of R-endonuclease. Supercoiled Type I pDEL19 DNA containing abasic sites (AP, ○) or thymine glycol (TG, ●) was incubated with R-endonuclease at the indicated concentrations of NaCl. The relative activity was determined based on the analysis of Type II DNA and plotted against the concentration of NaCl.

property of endo III that the magnesium ion was a very effective inhibitor of the reaction when abasic DNA was used as a substrate[28].

Salt optimum studies showed some differences in the response of R-endonuclease activity towards DNA substrates containing thymine glycol and abasic sites. The optimal salt concentrations of the activity for thymine glycol and abasic sites were both around 100 mM NaCl. However, the activity for thymine glycol showed a narrow optimum of the salt concentration, while that for abasic sites was considerably broad with the optimal range extended between 50–150 mM (Fig. 6). Endo III has a salt optimum at 100 mM when thymine glycol-containing DNA is used as a substrate, and the salt optimum for abasic sites is between 50-100 mM[23]. Thus, salt optima of R-endonuclease were similar to those of *E. coli* endo III. The pH optima of R-endonuclease were 7.8 and 7.0 when DNA containing thymine glycol and abasic sites were used as substrates respectively (Fig. 7), while those for endo III are 7 and 7.5, respectively[23].

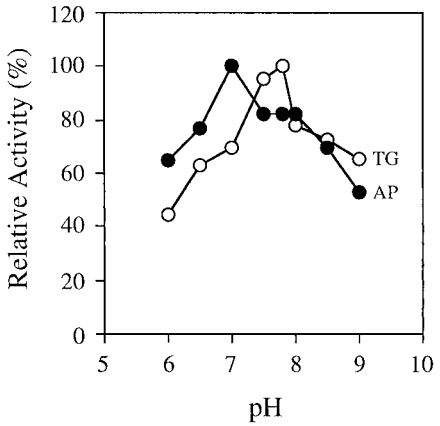

Fig. 7. Effect of pH on the activity of R-endonuclease. Supercoiled pDEL19 DNA containing abasic sites (AP, ●) or thymine glycol (TG, ○) was incubated with R-endonuclease at the indicated pH. The relative activity was determined based on the analysis of Type II DNA and plotted against pH.

DISCUSSION

In the present study, we have demonstrated the presence of DNA repair activity in *R. radiotolerans* that may function in the repair of DNA damage produced by ionizing radiation. The repair enzyme was purified by a series of chromatography (Table 1). The most purified fraction showed a single protein band in SDS-PAGE with an apparent molecular mass of 40 kDa (Fig. 1). We designated this enzyme as R-endonuclease after *Rubrobacter radiotolerans*.

The total activity of the crude cell extract of *R. radiotolerans* (Fraction I) appears to be

underestimated probably due to the presence of nucleic acids in this preparation. For this reason, the yield of the activity was given following the removal of nucleic acids, starting with Fraction II (Table 1). R-Endonuclease was purified to an apparent homogeneity after five purification steps from the ammonium sulfate-precipitated fraction. The third purification step employing SP-Sepharose chromatography removed most of the contaminating non-specific DNA endonuclease activity. The SP-Sepharose chromatography was also a very effective purification step since R-endonuclease activity was purified by 160-fold by a single column. The R-endonuclease activity adsorbed to phosphocellulose, and this property was employed to remove the trace amount of remaining non-specific endonuclease activity. In SDS-PAGE, the Mono S-purified enzyme fraction (Fraction V) showed a single protein band (Fig. 1, lane 6).

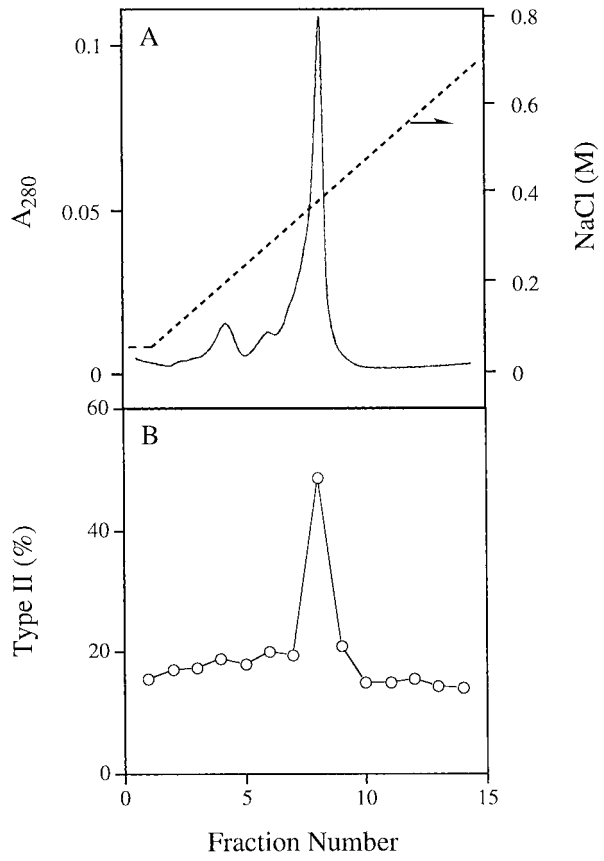

Fig. 8. Elution profiles of protein and activity in Mono S chromatography. (A) The active fraction from the phosphocellulose chromatography was subjected to the Mono S column. Elution profile of protein (solid line) was monitored by UV absorption at 280 nm. The gradient of NaCl is indicated by the broken line. (B) Elution profile of the activity for pDEL19 DNA containing thymine glycol. Endonuclease activity was determined by nicking assay using 1 μl aliquots of the column fraction.

Furthermore, the activity to thymine glycol comigrated with the major protein peak in the Mono S chromatography (Fig. 8) used in the last step of the purification. In light of these two results, the protein band observed in the SDS-PAGE analysis of Fraction V was attributed to R-endonuclease.

R-Endonuclease specifically cleaved DNA containing thymine glycol, urea residues, and abasic sites, but not intact DNA or UV-irradiated DNA (Figs. 2A and 2B). Moreover, R-endonuclease and endo III preferred abasic sites and urea residues over thymine glycol as substrates (Fig. 5), although R-endonuclease exhibited higher specific activities than endo III for these substrates. Thus, substrate specificity of R-endonuclease is similar to that of *E. coli* endo III when pDEL19 DNA containing these lesions were utilized as substrates for comparison.

The distinctive activities of R-endonuclease for natural and reduced abasic sites (Fig. 3) provide some evidence concerning the reaction mechanism by which the R-endonuclease catalyzes the incision of abasic sites. Two distinct reaction mechanisms are known for AP endonucleases. Endo III and its homologues that contain both *N*-glycosylase and AP endonuclease activities act as class I AP endonucleases, producing 5'-phosphate and 3' baseless sugar termini *via* ß-elimination[20]. Class II AP endonucleases such as exo III and endo IV hydrolyze the phosphodiester bond 5' to the abasic site. The present study demonstrated that reduction of the C-1' aldehyde of abasic sites essentially abolished the cleavage of DNA by R-endonuclease and endo III, but not exo III (Fig. 3). These results indicate a similarity in the action mechanism of R-endonuclease and endo III, and further suggest that R-endonuclease catalyzes the ß-elimination reaction as a lyase when acting on abasic sites.

The R-endonuclease was also similar to endo III in the following features. Like endo III, R-endonuclease exhibited no requirement of divalent cations for the activity. Therefore, the activity was not affected by addition of EDTA. The salt requirements of R-endonuclease were also close to those of endo III[23]. R-endonuclease had a salt optimum of 100 mM when thymine glycol was a substrate with 20–25% inhibition at 50 and 150 mM NaCl, whereas the salt optimum for abasic sites was extended between 50–150 mM with 20% inhibition at 20 and 190 mM NaCl (Fig. 6). Endo III shows optimum activity to thymine glycol at 100 mM KCl with 50% inhibition at 50 and 150 mM KCl, while the salt optimum for abasic sites is between 50 and 100 mM KCl with 50% inhibition at 20 and 120 mM KCl[23]. Figure 7 shows the pH optima of R-endonuclease for thymine glycol and abasic DNA as substrates. R-Endonuclease exhibited optimum at pH 7.0 for abasic sites with 20–25% inhibition at 0.5 pH unit on either sides. The activity for thymine glycol had optimum activity at 7.8 with 30% inhibition at pH 7.0 and pH 8.0. Similarly, endo III shows the activity for abasic DNA with a sharp pH optimum at pH 7.5 with 50% inhibition at 0.3 pH unit on either sides of this pH. The activity for thymine glycol DNA has a broader optimum at pH 7.0 with 50% inhibition at pH 6.2 and pH 8.1[23].

Despite many similarities between R-endonuclease and *E. coli* endo III, R-endonuclease differs from endo III with respect to the apparent molecular mass and some other properties. R-Endonuclease has a molecular mass of 40 kDa, whereas that of endo III encoded by the *nth* gene is 23.4 kDa[29]. In addition, the magnesium ion was not inhibitory to the activity of R-endonuclease on DNA containing thymine glycol, urea residues, and abasic sites. For endo

III, the magnesium ion has no effect on the endonucleolytic reaction when *N*-glycosylase and AP lyase activities are coupled as in the case for thymine glycol[28]. In contrast, when abasic DNA is used as a substrate for endo III, magnesium is a very effective inhibitor of the reaction, which was not the case for R-endonuclease.

R-Endonuclease may be a functional homolog of not only *E. coli* endo III but also *M. luteus* γ-endonuclease, yeast Nth-spo and NTG1/NTG2, and mammalian NTH1/Nthl1, all of which contain activity for thymine glycol and abasic sites[26,30–32]. Thus, the finding of R-endonuclease indicates that enzymes similar to endo III are conserved among prokaryotes including radioresistant bacteria, and eukaryotes. It is also noted that *E. coli* contains a second enzyme (endo VIII) that recognizes thymine glycol, urea residues, and abasic sites, though endo VIII in the cell extract represents only minor activity to these lesions[33,34]. Therefore, the relationship between R-endonuclease and endo VIII, if any, also need to be assessed in forthcoming studies.

The identification of a *R. radiotolerans* enzyme that specifically recognizes DNA containing thymine glycol, urea residues and abasic sites suggests that this activity may initiate repair of DNA damage formed by ionizing radiation and oxidative agents. Elucidation of the exact role played by R-endonuclease in the unusual resistance of this bacterium to ionizing radiation awaits cloning of the gene and isolation of the mutant lacking the enzyme.

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