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Abstract

We purified an apurinic/apyrimidinic (AP) endonuclease from mouse ascites sarcoma (SR-C3H/He) cells. The enzyme showed nicking activity on acid-depurinated DNA but not on untreated, intact DNA. It also showed priming activity for DNA polymerase on both acid-depurinated and bleomycin-damaged DNA. The priming activity on bleomycin-damaged DNA was two times higher than that on an acid-depurinated DNA. The enzymatic properties indicate that the enzyme is a class II AP endonuclease having DNA 3' repair diesterase activity. The purified enzyme has a molecular weight of 39,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH for AP endonuclease activity was 8.0 in 50 mM Tris-HCl buffer. The AP endonuclease activity depended on divalent cation such as Mg²⁺ and Co²⁺ ions, and was inhibited by 2 mM EDTA with no addition of the divalent cation. An appropriate concentration of sodium or potassium salt stimulated the activity. Partial digestion of the AP endonuclease with *Staphylococcus aureus* V8 protease produced 4 major peptide fragments which may be used for protein sequencing.

KEYWORDS: AP endonuclease, DNA 3' repair diesterase, DNA repair enzyme, mouse ascites sarcoma cells

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Purification and Characterization of a 39kDa Apurinic/aprimidinic Endonuclease from Mouse Ascites Sarcoma Cells

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We purified an apurinic/aprimidinic (AP) endonuclease from mouse ascites sarcoma (SR-C3H/He) cells. The enzyme showed nicking activity on acid-depurinated DNA but not on untreated, intact DNA. It also showed priming activity for DNA polymerase on both acid-depurinated and bleomycin-damaged DNA. The priming activity on bleomycin-damaged DNA was two times higher than that on an acid-depurinated DNA. The enzymatic properties indicate that the enzyme is a class II AP endonuclease having DNA 3' repair diesterase activity. The purified enzyme has a molecular weight of 39,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The optimal pH for AP endonuclease activity was 8.0 in 50mM Tris-HCl buffer. The AP endonuclease activity depended on divalent cation such as Mg²⁺ and Co²⁺ ions, and was inhibited by 2mM EDTA with no addition of the divalent cation. An appropriate concentration of sodium or potassium salt stimulated the activity. Partial digestion of the AP endonuclease with *Staphylococcus aureus* V8 protease produced 4 major peptide fragments which may be used for protein sequencing.

Key words: AP endonuclease, DNA 3' repair diesterase, DNA repair enzyme, mouse ascites sarcoma cells

Apurinic/aprimidinic (AP) sites in DNA are formed frequently (more than 10,000 per mammalian cell/day) by a variety of mechanisms that include spontaneous or chemically or physically induced hydrolysis of N-glycosylic bonds and enzymatic removal of modified bases by DNA glycosylases (1-6). Single-strand breaks with 3' termini blocked by nucleotide fragments are

also produced frequently by oxidative DNA damage (2-6). These lesions (AP sites and single-strand breaks with 3'-blocked termini) are potentially lethal and mutagenic (6-9). Repair of these lesion is generally initiated by 5' AP endonuclease and DNA 3' repair diesterase activities residing in DNA repair enzymes such as exonuclease III and endonuclease IV in *Escherichia coli* (2-6, 9). Although multiple forms of AP endonucleases have been reported in mammalian cells (3-6, 9), only the cDNA for a mammalian homolog of exonuclease III has been cloned independently by 4 groups and was designated as APEX (Apex for the mouse homolog) (10-13), HAP1 (14, 15), APE (16, 17) and Ref1 (18).

Recently, in addition to the major AP endonuclease (APEX nuclease), a 5' AP endonuclease/DNA 3' repair diesterases of 48-60kDa was reported by Chen *et al.* (19). Winters *et al.* (20) resolved HeLa cell DNA 3' repair diesterases into 3 types by Mono P chromatofocusing. Sarker *et al.* (21) reported a 30-kDa enzyme having 5' AP endonuclease and DNA 3' repair diesterase activities. Kelley *et al.* (22) reported that the *Drosophila* AP3 gene product, homologous to human ribosomal-associated protein PO, shows 5' AP endonuclease and DNA 3' repair diesterase activities.

We describe here the purification and characterization of a 39-kDa AP endonuclease/DNA 3' repair diesterase (39-kDa AP endonuclease) isolated from mouse ascites sarcoma cells, which is thought to be different from the identified major AP endonuclease.

Materials and Methods

Materials. The reagents used in these experiments were purchased from the following sources: [³H]

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dTTP from Amersham Tokyo, Japan; ribonucleotides (NTPs) and deoxyribonucleotides (dNTPs) from Seikagaku Kogyo, Tokyo, Japan; the large fragment (Klenow polymerase) of DNA polymerase I from Takara Shuzo, Kyoto, Japan; calf thymus DNA from Pharmacia, Uppsala; Ponceau S, Nacalai Tesque Inc., Kyoto, Japan; prestained molecular weight marker (middle range) from Daiichi Kagaku, Tokyo, Japan; polyvinylidene difluoride (PVDF) membrane (Trans-Blot cell) from Bio-Rad Japan, Tokyo, *Staphylococcus aureus* V8 protease from Takara Shuzo, Kyoto. The other reagents used were obtained as described previously (23). Mouse ascites sarcoma cells were obtained and maintained as described (24). pUC18 plasmid DNA and bleomycin-Fe(II)-treated DNA were prepared as described (25).

Preparation of acid-depurinated DNA.

pUC18 DNA and calf thymus DNA were depurinated as described previously (10, 26-28). The acid treatment produced approx. six alkali-sensitive sites per pUC18 DNA molecule, as determined previously (23).

AP endonuclease assay. A 39-kDa AP endonuclease/DNA 3' repair diesterase (tentatively designated as 39-kDa AP endonuclease) was purified from permeabilized SR-C3H/He mouse ascites sarcoma cells as described in the following Results section. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified enzyme was extracted from the gel pieces, denatured and renatured according to the method of Hager and Burgess (29, 30). AP endonuclease activity was assayed in the standard assay mixture (10 ml in the final volume adjusted with deionized water) containing 250 ng (0.14 pmol) of acid-depurinated pUC18 DNA, 2 ml of a buffer containing 0.0875 % Triton X-100, 1.25 M sucrose, 250 mM Tris-HCl (pH 8.0), 25 mM MgCl₂ and the renatured 39-kDa AP endonuclease at an appropriate amount, unless otherwise indicated. After incubation at 37 °C for 30 min, the reaction mixture was chilled at 0 °C to stop the reaction. Then, 2 ml of 6-fold-concentrated gel loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol and 30 % glycerol in H₂O) was added to the mixture. The sample was loaded into a slot of a submerged agarose gel which was prepared at 0.8 % agarose in TBE buffer (89 mM Tris-borate, 89 mM boric acid and 2 mM EDTA, pH 8.0) supplemented with ethidium bromide (EtdBr) at 0.5 mg/ml (25, 27). The electrophoresis was conducted at 50 volts for 45 min using a Mini Gel Electrophoresis System (Mupid 2) of Cosmo Bio Co., Ltd., Tokyo, Japan and TBE buffer supplemented with EtdBr at 0.5

mg/ml as the electrophoresis buffer. A linear correlation between the DNA amount in an electrophoresed, EtdBr-stained band of pUC18 DNA and the absorbance measured with the densitometer (Scanning Imager 300SX, Molecular Dynamics, CA, USA) was observed. The absorbance increased 1.4-fold by converting the superhelical form of pUC18 DNA into the nicked, open circular form. Based on this, AP endonuclease activity of the enzyme was determined from the conversion of superhelical to nicked, open circular molecules after densitometric scanning (25, 27, 31). % Activity was tentatively expressed as the percentage of the acid-depurinated DNA (250 ng per assay) converted into the nicked, open circular form. Specific activity for AP endonuclease is expressed as nmoles of acid-depurinated pUC18 DNA nicked/mg protein per 30 min by measuring the amount of enzyme required to convert one-half of the superhelical acid-depurinated pUC18 DNA to nicked, open circular DNA.

Assay of the enzyme that enhances the template-primer activity of acid-depurinated or bleomycin-damaged DNA for DNA polymerase. In the present paper the term 'template-primer' activity of DNA is used to indicate the ability to serve as a template-primer for DNA polymerase. The term 'priming activity (or enzyme)' on bleomycin-damaged or acid-depurinated DNA is used to denote the activity (or enzyme) that enhances the template-primer activity of bleomycin-damaged or acid-depurinated DNA by removing the 3' tags or by introducing single-strand breaks. The priming activity was measured by the two-step (priming and repair DNA synthesis step) method as described previously (23, 27). Incubation for bleomycin-induced DNA damage and the priming step was conducted at 37 °C for 30 min in a reaction mixture (40 ml in final volume) consisting of 6 mg of calf thymus DNA, 20 mM Fe (II) (ferrous ammonium sulfate), 0.2 mg of bleomycin, 10 mM Mg²⁺, 100 mM KCl, 0.0175 % Triton X-100, 250 mM sucrose and 6 mM 2-mercaptoethanol and an appropriate amount of the 39-kDa AP endonuclease. Incubation for the priming step on acid-depurinated DNA was conducted in the same way as for the priming step on the bleomycin-damaged DNA except that 6 mg of acid-depurinated DNA was added to the incubation mixture in place of 6 mg of calf thymus DNA, 20 mM Fe (II) and 0.2 mg of bleomycin. After finishing the priming step, the reaction mixture was incubated at 60 °C for 10 min to inactivate the enzyme. After chilling at 0 °C for 5 min,

20 ml of the substrate mixture for DNA synthesis (100 mM Tris-HCl, 7.5 mM MgCl₂, 240 mM NaCl, 150 mM dATP, 150 mM dGTP and 7.5 mM [³H] dTTP at 5 Ci/mmol, pH 8.0 at 25 °C) supplemented with 0.04 u of Klenow polymerase were added to the mixture. DNA synthesis was conducted at 37 °C for 30 min. The radioactivity incorporated into acid-insoluble materials was measured by the disc method (23, 27). The priming activity is expressed in terms of pmoles of [³H] dTMP incorporated into the DNA.

Partial digestion of the enzyme with *S. aureus* V8 protease and peptide mapping.

The purified 39-kDa AP endonuclease was dissolved in a loading buffer consisting of 125 mM Tris-HCl (pH 6.8), 0.1 % (W/V) SDS and 10 % glycerol, and digested with *S. aureus* V8 protease essentially by the method of Cleveland *et al.* (32). The sample was loaded on the stacking gel for SDS-PAGE. Then, V8 protease dissolved in 125 mM Tris-HCl (pH 6.8), 0.1 % SDS and 5 % glycerol was overlaid gently by a micro pipet over the sample layer. The electrophoresis buffer consisting of 125 mM Tris-HCl (pH 6.8) and 0.1 % SDS, was overlaid on the V8 protease layer. The electrophoresis was started and continued at 2 watts until the sample migrated to the margin of the stacking and separating gels. At this position, the electric current was turned off for 30 min, and then the electrophoresis was regained and conducted at 4 watts. Peptide fragments on a SDS-polyacrylamide gel were stained with Coomassie brilliant blue R-250 (CBB).

Protein sequencing. The peptide fragments on an SDS-polyacrylamide gel were electroblotted onto a PVDF membrane essentially according to the method of Matsudaira (33) or directly adsorbed (34). The electroblotting buffer was modified by reducing the concentration of methanol to 5 %. The electroblotting was conducted at 10 watts for 30 min under 4 °C. The electroblotted membrane was washed three times, 15 min each in distilled water, and then stained with ponceau S.

The direct adsorption of proteins onto PVDF membranes was carried out under 4 °C. The desired band was cut out from the polyacrylamide gel stained with CBB. Then, it was soaked and agitated in 5 volumes of Hagar-Burgess elution buffer (29) with the omission of BSA for 24 h. To remove glycine contaminated at the elution step, the eluant showing blue color of CBB was diluted with 5 volumes of water and ultrafiltered at 5,000 rpm with Centricut U-10 (molecular weight limit of

filtration, 10,000; Kurabo, Osaka, Japan). A small piece of PVDF membrane (5 mm × 7 mm) was prewetted with 100 % methanol in a few seconds, and soaked in distilled water until used. Then the prewetted PVDF membrane was soaked, and agitated in 200 μl of filtrated eluant for 24 h to adsorb protein on the membrane. The electroblotted or directly adsorbed membrane was air-dried, and applied for protein sequencing on the model 476A protein sequencer (ABI, Tokyo, Japan) in the Central Research Laboratory of Okayama University Medical School.

Results

Enzyme purification. The enzyme was extracted from permeable mouse ascites cells with 0.2 M potassium phosphate (KPi) buffer containing 6 mM 2-mercaptoethanol (pH 7.5). After adjusting the KPi concentration to 0.075 M, the extract (fraction N1) was mixed with packed phosphocellulose equilibrated with 0.075 M KPi. Proteins bound to phosphocellulose were eluted with 0.30 M KPi. The eluant (fraction N2) with the KPi concentration adjusted to 0.15 M was passed through a DEAE-cellulose column equilibrated with 0.15 M KPi mainly to remove nucleic acids. After adjustment of the KPi concentration to 0.075 M, the flow-through fraction (fraction N3) was loaded onto a phosphocellulose column equilibrated with 0.075 M KPi buffer. Proteins were eluted with a linear gradient of KPi from 0.075 to 0.35 M. The fractions containing the 39 kDa AP endonuclease, which were eluted at around 0.16 M KPi, were dialyzed against TEMG (0.02 M Tris-HCl, pH 8.0, 1 mM EDTA, 6 mM 2-mercaptoethanol and 10 % glycerol) supplemented with 0.01 M NaCl. The dialyzed sample (fraction N4) was loaded onto a single-stranded DNA cellulose column equilibrated with the 0.01 M NaCl-TEMG (26). Proteins on the column were eluted with a linear gradient of NaCl from 0.01 to 1.2 M. The 39-kDa AP endonuclease was eluted in fractions with 0.20–0.25 M NaCl. The fraction (fraction N5) eluted with NaCl between 0.20 and 0.25 M were dialyzed against PMG (0.1 M KPi, 6 mM 2-mercaptoethanol and 10 % glycerol). The dialyzed sample was loaded onto a hydroxyapatite column equilibrated with PMG (26). Proteins were eluted with a linear gradient of KPi from 0.05 to 0.5 M. The fraction containing the 39-kDa AP endonuclease (fraction N6) was eluted from 0.15 M to 0.20 M KPi. The fraction N6 was electrophoresed on a large SDS-polyacrylamide gel (17 × 15 ×

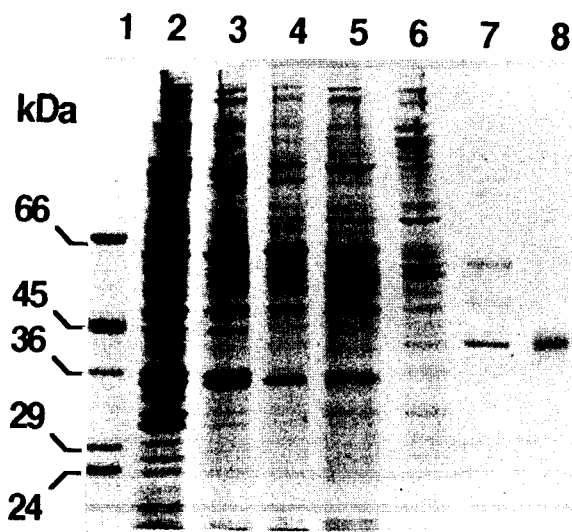


Fig. 1 SDS-PAGE of fractions obtained in purification steps of the 39-kDa AP endonuclease. Samples were electrophoresed on a 0.1% SDS-12% polyacrylamide gel. The sample and amount of protein loaded per lane were as follows: Lane 1, molecular weight marker; Lane 2, 48 μ g of fraction N₁; Lane 3, 56 μ g of fraction N₂; Lane 4, 78 μ g of fraction N₃; Lane 5, 45.6 μ g of fraction N₄; Lane 6, 12 μ g of fraction N₅; Lane 7, 3 μ g of fraction N₆; Lane 8, 3 μ g of SDS-PAGE-purified 39-kDa AP endonuclease.

0.1 cm) according to the method of Laemmli (30, 35). The 39-kDa protein band was cut out from the gel in which the band was well resolved from its surroundings. The 39-kDa AP endonuclease was eluted from the gel band as described previously (29, 30). Fig. 1 shows SDS-PAGE of the fractions obtained in the purification steps of the 39-kDa AP endonuclease.

Optimal pH and factors affecting AP endonuclease activity of the enzyme. Maximal AP endonuclease activity of the enzyme occurred at pH 8.0 in 50mM Tris-HCl buffer adjusted at 25°C. The activity could not be detected in the presence of 2mM EDTA with no addition of any divalent cations. The divalent cations Mg²⁺ and Co²⁺ stimulated enzyme activity (Fig. 2). The optimal concentration of Mg²⁺ was 6 to 14mM. Co²⁺ also enhanced the AP endonuclease activity. The optimum concentration, however, could not be determined, because Co²⁺ concentrations higher than 5mM caused aggregation of the sample in the agarose gel slot for the electrophoretic analysis. The enzyme activity measured in the presence of Mg²⁺ was stimulated by the

addition of the salt such as NaCl and KCl. The optimal concentration of the salt was about 100mM (50 to 150mM) (Fig. 3).

Specific activity to acid-depurinated DNA and stability of the enzyme. The nicking (AP endonuclease) activity of the 39-kDa AP endonuclease was assayed under optimal conditions consisting of 0.0175% Triton-X, 250mM sucrose, 50mM Tris-HCl (pH 8.0 at 25°C), 10mM MgCl₂, 100mM KCl, 250ng of acid-depurinated pUC18 DNA and the renatured 39-kDa AP endonuclease at an appropriate amount. The nicking activity of the enzyme was estimated to be about 0.85nmol of acid-depurinated DNA nicked/mg of the enzyme per 30min. The enzyme was heat-sensitive and inactivated completely by incubation at 60°C for 10min.

Priming activity of the enzyme for DNA synthesis on acid-depurinated and bleomycin-damaged DNA. Both acid-depurinated and bleomycin-treated DNAs are poor template-primers for DNA synthesis by Klenow polymerase or DNA polymerase β . The two-step method for assaying the priming activity revealed that the 39-kDa AP endonuclease has priming activity for DNA polymerase on both acid-depurinated and bleomycin-damaged DNAs (Fig. 4). Comparing the priming activity of the enzyme between

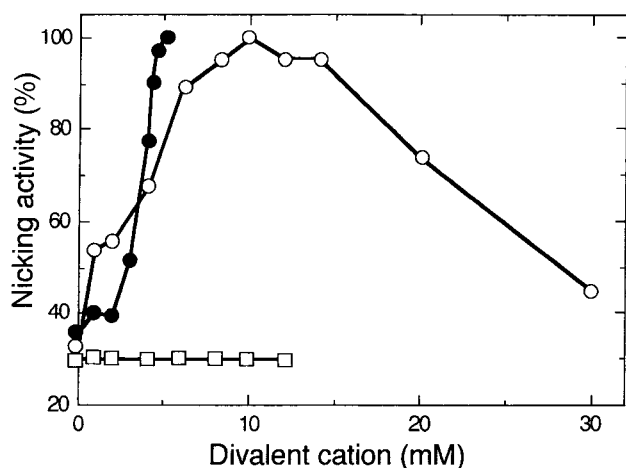


Fig. 2 Effect of varying concentration of a divalent cation on AP endonuclease activity. Nicking activity was assayed under standard assay conditions except for the variables, and 100ng of the renatured enzyme was used. Divalent cation (Mg²⁺, Co²⁺ or Ca²⁺) was added to the reaction mixture at the indicated final concentration. Activity (%) was defined as described in Materials and Methods. Open circles, Mg²⁺; solid circles, Co²⁺; open squares, Ca²⁺.

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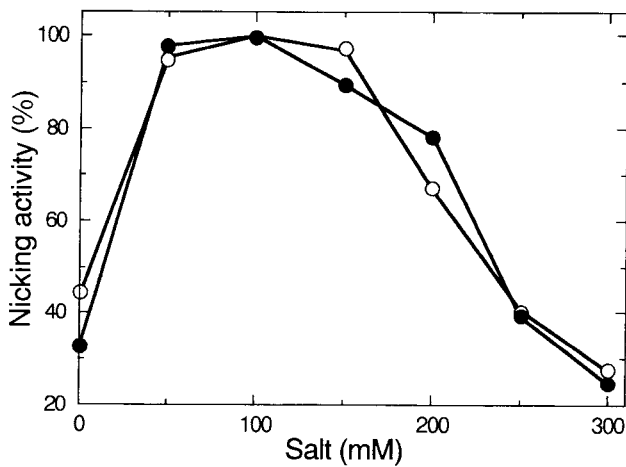


Fig. 3 Effect of varying salt concentration on AP endonuclease activity. Nicking activity was measured under standard assay conditions except for the variables, and 100 ng of the renatured enzyme was used. Open circles, NaCl; solid circles, KCl. Activity (%) was defined as described in Materials and Methods.

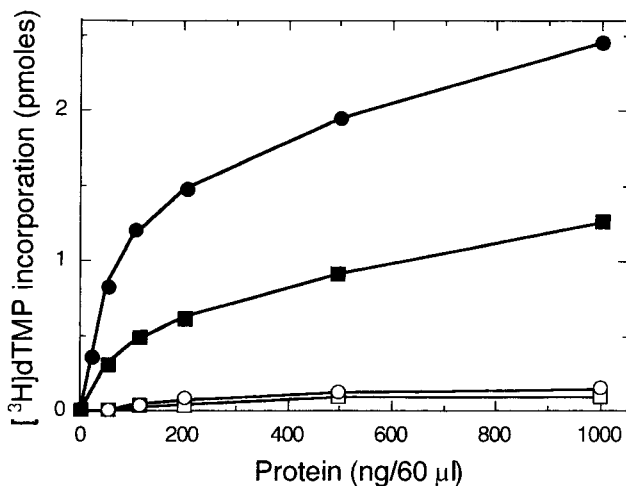


Fig. 4 Effects of varying concentration of the 39-kDa AP endonuclease on the template-primer activity of acid-depurinated DNA or bleomycin-damaged DNA. The template-primer activity for Klenow polymerase of the damaged DNA was measured by the two-step method as described in Materials and Methods. The amount of the AP endonuclease in the reactidon mixture was varied as indicated. The template-primer activity is expressed as pmoles of [^3H] dTMP incorporated into 6 μg of DNA. Closed circles, bleomycin-treated DNA incubated with the 39-kDa AP endonuclease; closed squares, acid-depurinated DNA with the enzyme; open circles, bleomycin-damaged DNA without enzyme; open squares, acid-depurinated DNA without the enzyme.

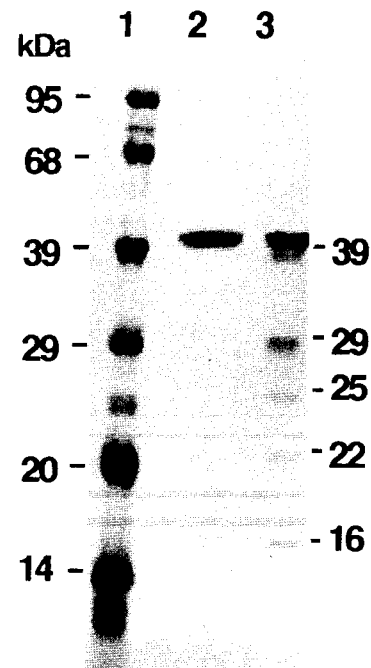


Fig. 5 The peptide map of the 39-kDa AP endonuclease partially digested with *S. aureus* V8 protease. The procedure is described in the Materials and Methods section. Lane 1, prestained molecular weight marker; lane 2, 5 μg of the purified 39-kDa enzyme; lane 3; the purified enzyme (5 μg) digested with V8 protease.

bleomycin-damaged DNA and acid-depurinated DNA, the activity on bleomycin-damaged DNA was two or more times higher than that on acid-depurinated DNA.

Peptide map of 39-kDa AP endonuclease. The purified enzyme, a 39-kDa protein on SDS-polyacrylamide gels (Fig. 1), was applied on a protein sequencer to determine a partial amino acid sequence from the amino-terminal end. However, we could not get any amino acid sequence possibly because of the blockade of the N-terminal. Treatment of the enzyme with 75% formic acid for 48h did not produced any significant peptide fragments. These properties of the 39kDa AP endonuclease were quite different from those of the mammalian major AP endonuclease, APEX nuclease (10, 11, 26). To determine partial amino acid sequences of the 39-kDa AP endonuclease, it was digested with *S. aureus* V8 protease. Fig. 5 shows a peptide map of the partially digested 39kDa enzyme. Four significant fragments were observed on the SDS-polyacrylamide gel. The molecular

mass of these fragments was estimated to be about 16, 22, 25 and 29 kDa, respectively. Partial amino acid sequence of the 16 kDa and 22 kDa peptides were determined. The 29-kDa fragments did not give a reproducible sequence, possibly because of impurity of the peptide fragments. Sequences highly homologous to the determined N-terminal amino acid sequences (data not shown) of 16 and 22 kDa peptides have not been detected in the SWISS-PROT (released 1995, Sept.) and NBRF Protein (released 1995, Nov.) Sequence data bases.

Discussion

AP endonucleases are classified into two major classes, classes I and II (2-6, 36, 37). Class I AP endonucleases, otherwise known as AP lyases or 3' AP endonucleases, cleave on the 3' side of AP sites to produce 5' termini with 5'-phosphate nucleotide and 3' termini bearing the 2, 3-unsaturated abasic residue 4-hydroxy-2-pental (4-6, 9, 37, 38). This class AP endonucleases are also characterized by the virtual absence of no affinity to phosphocellulose (3). Class II AP endonucleases (5' AP endonucleases) catalyze incision of the 5' side of AP sites to produce 3' hydroxynucleotide and 5'-deoxyribose-phosphate termini (4-6, 9, 37). Class II AP endonucleases have priming activity for DNA polymerase on acid-depurinated DNA. They have affinity to phosphocellulose (3).

The present 39-kDa AP endonuclease has priming activity for DNA polymerase on acid-depurinated DNA, and has affinity to phosphocellulose. The results indicate that the 39-kDa AP endonuclease is classified into class II. Besides the 5' AP endonuclease activity, this enzyme is thought to have relatively high DNA 3' repair diesterase activity as indicated by the presence of priming activity for DNA polymerase on bleomycin-induced single-strand breaks which have 3'-blocked termini. Mg^{2+} ion-dependency (or EDTA-sensitivity) of the 39-kDa AP endonuclease is also a characteristic property.

So far, multiple forms of AP endonucleases have been reported, although it is not clear yet how many types of AP endonucleases are actually present in mammalian cells (2-6, 36, 37). Some of the enzyme heterogeneity based on molecular weight difference of AP endonucleases are suggested to be caused by proteolytic (and chemical) cleavage of a species of AP endonucleases during extraction and purification (3, 5, 39). There is insufficient information to compare the present enzyme

directly with previously reported mammalian AP endonucleases. The difficulty of identification of AP endonucleases has been demonstrated clearly by the previous studies on a mammalian major AP endonuclease. We suggested previously that the mammalian major AP endonuclease is an *Escherichia coli* exonuclease III-type enzyme of class II AP endonucleases (23, 26). Although the majority of previous studies had indicated that the exonuclease III-type enzyme had not been detected in mammalian cells (3, 5), the cDNA cloning in 1991 and the determination of the primary structure of the mammalian major AP endonuclease clearly showed that the enzyme is a mammalian homolog of exonuclease III (10, 11, 14, 16).

Now, we are trying to determine partial amino acid sequences of the 39-kDa AP endonuclease for cDNA cloning of the enzyme. The enzyme was digested partially by V8 protease, and the peptide fragments were separated on SDS-polyacrylamide gels. Four major peptides (16, 22, 25 and 29 kDa) were detected on the gel. Preliminary study showed that the N-terminal amino acid sequence of the 22-kDa fragment was identical to that of the 25-kDa fragment. So far, no sequences highly homologous to the determined sequences have been detected in the SWISS-PROT and NBRF Protein Sequence data bases.

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