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Purification and characterization of an AP endonuclease/DNA 3' repair diesterase from mouse ascites sarcoma cells

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Abstract

Purification and characterization of a DNA repair enzyme having 5' apurinic/apyrimidinic (AP) endonuclease activity are reported. The enzyme extracted from mouse ascites sarcoma (SR-C3H/He) cells with 0.2 M potassium phosphate buffer (pH 7.5) was purified by successive chromatographies on phosphocellulose, DEAE-cellulose, phosphocellulose (a second time) and single-stranded DNA cellulose, and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified enzyme has an apparent molecular mass of 30 kDa as determined by SDS-PAGE. It was shown to have nicking activity on acid-depurinated DNA but not on intact DNA, and to have priming activities for DNA polymerase on acid-depurinated DNA and bleomycin-treated DNA. The results indicate that it is a multifunctional DNA repair enzyme having 5' AP endonuclease and DNA 3' repair diesterase activities. The enzyme activity is dependent upon the presence of a divalent cation such as Mg^{2+}. Its amino-terminal amino acid and internal amino acid sequences are determined.

1. Introduction

Apurinic/apyrimidinic (AP) sites resulting from loss of bases are one of the most common lesions generated in DNA [1-5]. The AP sites are produced by spontaneous hydrolysis, by various chemicals and radiations and by DNA glycosylases which remove particular abnormal bases from DNA [1-5]. Single-strand breaks with 3'-blocked termini are also a major class of DNA lesion produced by free radical pathways caused by ionizing radiation or other sources of oxygen radicals [1,4,6-8]. These lesions (AP sites and 3'-blocked single-strand breaks) are generally repaired by cellular DNA repair systems, but sometimes these mechanisms fail, resulting in genetic changes or cell death [8-10]. Repair of these lesions is thought to be mostly initiated by AP endonuclease/DNA 3' repair diesterase [1-5]. Although multiple forms of AP endonucleases have been reported in mammalian cells (1-5, and therein), so far only one form (the major 5' AP endonuclease designated as HAP1, APEX, APE or Ref-1 protein) of AP endonuclease/DNA 3' repair diesterase is clearly identified [11-15].

Recently, in addition to the major AP endonuclease, a 5' AP endonuclease/DNA 3' repair diesterase of 48-60 kDa was reported by Chen et al. [16]. Winters et al. [17] resolved human HeLa DNA 3' repair diesterases into 3 types by Mono P chromatofocusing. Previously, Nes [18] reported partial purification and characterization of a major AP endonuclease having 28 kDa molecular mass from a mouse plasmacytoma cell line. We also detected a type of AP endonucleases having an apparent molecular mass of 30 kDa, although the enzyme was not a major but a minor AP endonuclease in mouse sarcoma cells.

We describe here purification, enzymatical characterization and partial amino acid sequence determination of the 30 kDa AP endonuclease/DNA 3' repair diesterase (30 kDa enzyme) isolated from mouse ascites sarcoma cells.

2. Materials and methods

2.1. Materials

The reagents used in these experiments were obtained from the following sources: [1H]dTTP from Amersham Japan, Tokyo, Japan; ribonucleoside triphosphates (NTPs)
and deoxyribonucleoside triphosphates (dNTPs) from Seikagaku Kogyou, Tokyo, Japan; the large fragment (Klenow polymerase) of DNA polymerase I from Takara Shuzo, Kyoto, Japan; calf thymus DNA from Pharmacia, Uppsala, Sweden. The other reagents used were obtained as described previously [19]. Mouse ascites sarcoma cells were obtained and maintained as described previously [20]. pUC18 plasmid DNA and bleomycin-Fe(II)-treated DNA were prepared as described [21,22].

2.2. Preparation of acid-depurinated DNA

pUC18 DNA and calf thymus DNA were depurinated by incubating them in three volumes of 50 mM sodium citrate (pH 3.5) at 60°C for 15 min essentially as described by Niwa and Moses [12,23]. After the incubation, the mixture was chilled to 0°C and dialyzed against 50 mM Tris-HCl (pH 7.5) for 3 h and then against distilled water overnight. The dialyzed DNA solution was used to measure AP endonuclease activity. The acid treatment produced about six alkali-sensitive sites per pUC18 DNA molecule, as determined previously [24].

2.3. Assay of AP endonuclease activity

The SDS-PAGE-purified enzyme was extracted from the gel pieces, denatured and renatured according to the methods of Hager and Burgess [25-27]. The assay mixture (10 μl in the final volume adjusted with deionized water) for AP endonuclease activity contained 0.25 μg (0.14 pmol) acid-depurinated pUC18 DNA, 2 μl of 5-fold-concentrated Triton-buffer B (Triton-buffer B: 0.0175% Triton X-100, 0.25 M sucrose, 10 mM Tris-HCl, 4 mM MgCl2, 1 mM EDTA and 6 mM 2-mercaptoethanol, pH 8.0 adjusted at 25°C) and the renatured enzyme at an appropriate amount. After incubating the assay mixture at 37°C for 30 min, the reaction was stopped by chilling the reaction tube to 0°C and then adding 2 μl of 6-fold-concentrated gel loading buffer (0.25% bromophenol blue, 0.25% Xylene cyanol and 30% glycerol in H2O) to the mixture. The sample was loaded into a slot of a submerged 0.8% agarose gel. Electrophoretic analysis of conformation of DNA was conducted as described previously [22,28].

2.4. Assay of priming activity of the 30 kDa enzyme for DNA polymerase on bleomycin-damaged or acid-depurinated DNA

Priming activity of the 30 kDa enzyme for DNA polymerase on bleomycin-damaged DNA or acid-depurinated DNA was measured by a two-step- (priming and repair DNA synthesis step-) method [19,28]. Incubation for the priming step on bleomycin-damaged DNA was conducted at 37°C for 30 min in a reaction mixture consisting of 40 μl Triton-buffer B supplemented with 6 μg calf thymus DNA, 20 μM Fe(II) (ferrous ammonium sulfate), 0.2 μg bleomycin and an appropriate amount of the 30 kDa enzyme. Incubation for the priming step on acid-depurinated DNA was conducted in the same way as on the bleomycin-damaged DNA except that 6 μg acid-depurinated DNA was added to the incubation mixture in place of 6 μg calf thymus DNA, 20 μM Fe(II) and 0.2 μg bleomycin. After the incubation, the reaction mixture was incubated at 60°C for 10 min to inactivate the priming enzyme and then chilled to 0°C. Twenty μl of a substrate mixture for DNA synthesis (100 mM Tris-HCl, 7.5 mM MgCl2, 240 mM NaCl, 150 μM dATP, 150 μM dCTP, 150 μM dGTP and 7.5 μM [3H]dTTP at 5 Ci/mmol, pH 8.0 at 25°C) supplemented with 0.04 U Klenow polymerase (or DNA polymerase β with an equivalent activity) was added to the preincubated reaction mixture [19,28]. DNA synthesis was carried out at 37°C for 30 min. The radioactivity incorporated into acid-insoluble materials was measured by the disc method. The sample was pipetted onto a glass fibre disc (Whatman GF/C) numbered with India ink, and the discs were batch-washed in 5% trichloroacetic acid, 95% ethanol and ether. The radioactivity was measured as described [19,28].

2.5. Formic acid treatment of the purified enzyme

To get peptide fragments, the SDS-PAGE-purified 30 kDa protein was treated at 37°C for 48 h with 70% formic acid, which preferentially hydrolyzes peptide bonds between aspartate and proline. It was cleaved into two major fragments. They were purified by SDS-PAGE and their partial amino acid sequences were determined by an Applied Biosystem Model 477A automated protein sequencer.

2.6. Other methods

Preparation of DNA polymerase β [19], agarose gel electrophoresis [24] and polyacrylamide gel electrophoresis [29] were conducted as described previously. Protein concentrations were determined by the BCA protein Assay (Pierce, Illinois, USA), using bovine serum albumin as the standard.

3. Results

3.1. Purification of the 30 kDa enzyme

The enzyme was extracted from permeable mouse ascites sarcoma cells with 0.2 M potassium phosphate (KPi) buffer containing 6 mM 2-mercaptoethanol (pH 7.5) [19]. After adjustment of 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 0.3 M KPi. The eluant (fraction N2) adjusted the KPi concentration to 0.15 M was passed through a DEAE-cellulose column equilibrated with 0.15 M KPi mainly to remove nucleic acid. After adjustment of the KPi concentration to 0.075 M, the flow
through fraction (fraction $N_3$) 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 enzyme was eluted around 0.26 M KPi. The fractions containing the 30 kDa enzyme were collected and dialyzed against TEMG (0.02 M Tris-Cl, pH 8.0, 1 mM EDTA, 6 mM 2-mercaptoethanol and 10% glycerol) supplemented with 0.01 M NaCl. The dialyzed sample (fraction $N_4$) was loaded onto single-stranded DNA cellulose column equilibrated with the 0.01 M NaCl-TEMG [12]. Proteins were eluted with a linear gradient of NaCl from 0.01 to 1.2 M. The enzyme was eluted around 0.26 M NaCl. The fraction containing the enzyme was concentrated by ultrafiltration using Centricut U-10 (molecular weight limit of filtration, 10,000; Kurabo, Osaka, Japan) (Fraction $N_5$). The fraction $N_5$ was electrophoresed on a large sodium dodecyl sulfate (SDS)-polyacrylamide gel (17 x 15 x 0.1 cm) according to the method of Laemmli [26,27,29]. The 30 kDa protein band was cut out from the gel in which the band was well resolved from its surroundings. The 30 kDa enzyme was eluted from the gel band, denatured and renatured as described previously [25-27]. Fig. 1 shows the result of SDS-PAGE of fractions obtained in purification steps of the enzyme.

3.2. AP endonuclease activity of the 30 kDa enzyme

When acid-depurinated pUC18 DNA was treated at 37°C for 30 min with varying concentrations of the 30 kDa protein in Triton-buffer B, nicked open circular DNA increased in a protein dose-dependent fashion (Fig. 2). The nicking activity of the SDS-PAGE purified, renatured 30 kDa enzyme was estimated to be about 5.0 nmol of acid-depurinated DNA nicked/mg protein per 30 min. Non-specific endonuclease activity was not detected in the purified enzyme preparation (data not shown).

3.3. Factors affecting AP endonuclease activity of the enzyme

Divalent cations Mg$^{2+}$ and Co$^{2+}$ stimulated the enzyme activity at 4 to 8 mM and 4 mM, respectively, being the optimal concentrations, while Ca$^{2+}$ ions inhibited the reaction (Fig. 3). Mg$^{2+}$ had higher stimulatory effect than Co$^{2+}$ (Fig. 3). Addition of chelating agents such as EDTA (5 mM) inhibited the AP endonuclease activity. The AP endonuclease activity was dependent upon ionic strength. The activity was optimized with potassium chloride (KCl) within the concentrations of 20 and 100 mM and decreased to about a half of this optimum activity without KCl or with 200 mM KCl (Fig. 4).

3.4. New uses for AP endonuclease activity of the enzyme

Acid-depurinated DNA was treated with the purified AP endonuclease preparation (Fig. 5). The activity increased in a progressive manner with increasing concentration of the enzyme (Fig. 5). The activity was not affected by the presence of Mg$^{2+}$ or Co$^{2+}$ ions (Fig. 5). Addition of EDTA (5 mM) inhibited the AP endonuclease activity. The AP endonuclease activity was dependent upon ionic strength. The activity was optimized with potassium chloride (KCl) within the concentrations of 20 and 100 mM and decreased to about a half of this optimum activity without KCl or with 200 mM KCl (Fig. 4).
3.4. Priming activity of the 30 kDa enzyme for DNA synthesis on bleomycin-damaged DNA

DNA damage by bleomycin requires Fe(II) [30]. Bleomycin-damaged DNA itself is a poor template-primer for DNA synthesis by Klenow polymerase [19,23], because 3' ends of the damaged DNA are not free but phosphoglycolated [30,31]. The results shown in Table 1 suggested that removal of the 3' phosphoglycolate termini and release of 3'-hydroxyl termini were brought about by the 30 kDa enzyme. The enzyme was heat sensitive and inactivated almost completely by incubating at 60°C for 10 min (Table I).

3.5. Indication of the presence of DNA 3' repair diesterase and 5' AP endonuclease activities both in the 30 kDa enzyme

The heat labile property of the enzyme made possible to separate the bleomycin-induced DNA synthesis into two steps, priming step and DNA synthesis step, as described in 'Materials and Methods' and shown in Fig. 5.

Table 1

<table>
<thead>
<tr>
<th>Reaction conditions</th>
<th>pmol of ([^3]H)dTMP incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>complete</td>
<td>1.75</td>
</tr>
<tr>
<td>minus 30 kDa enzyme</td>
<td>0.04</td>
</tr>
<tr>
<td>minus bleomycin</td>
<td>0.28</td>
</tr>
<tr>
<td>minus Fe(II)</td>
<td>0.25</td>
</tr>
<tr>
<td>minus Klenow polymerase</td>
<td>0.00</td>
</tr>
<tr>
<td>minus calf thymus DNA</td>
<td>0.00</td>
</tr>
<tr>
<td>minus 30 kDa enzyme plus heat-treated</td>
<td>0.02</td>
</tr>
<tr>
<td>30 kDa enzyme b</td>
<td></td>
</tr>
</tbody>
</table>

\[^a^1\] The complete reaction mixture contained calf thymus DNA, Fe(II), 2-mercaptoethanol, bleomycin, 2.65 \(\mu\)g of the 30 kDa enzyme, Klenow polymerase and four deoxynucleoside triphosphates \(([^3]H)dTTP\), as described in 'Materials and Methods'. Reaction was conducted at 37°C for 30 min.

\[^b^2\] The 30 kDa enzyme pretreated at 60°C for 10 min was used in place of the non-treated one.

Using the two step method, effects of varying the concentrations of the 30 kDa enzyme on DNA synthesis on the bleomycin-damaged DNA or acid-depurinated DNA were studied. Fig. 6 (A and B) clearly shows that these DNA syntheses depended on the doses of the 30 kDa enzyme, indicating that the enzyme has priming activity for DNA polymerase on bleomycin-damaged and acid-depurinated DNAs. Considering that the major DNA lesions induced by the bleomycin treatment are single-strand DNA breaks with 3'-phosphoglycolate termini and that the 30 kDa enzyme has priming activity for DNA polymerase on bleomycin-damaged DNA, the enzyme is thought to have DNA 3' repair diesterase activity. And also considering that the 30 kDa enzyme has AP endonuclease activity and
priming activity for DNA polymerase on acid-depurinated DNA, the enzyme is said to have 5' AP endonuclease (or class II AP endonuclease) activity.

3.6. Amino terminal amino acid sequence and formic acid-degradation pattern of the 30 kDa enzyme

Automated Edman degradation and gas phase amino acid sequencing of the 30 kDa enzyme were performed and the following amino-terminal amino acid sequence was obtained: N-Ala-Val-Ala-Val-Gly-Lys-Pro-Arg-Gly-Gly-Gly-Asp-Ala-Asp-Thr-Arg. Treatment of the 30 kDa enzyme with formic acid that is known to selectively hydrolyze peptide bonds between aspartate and proline cleaved the enzyme into 2 major fragments of about 18- and 12-kDa (Fig. 7). These fragments were purified from SDS-polyacrylamide gels, and the peptides were extracted, denatured and renatured according to the method described by Hager and Burgess [25]. These peptides did not show AP endonuclease activity. The 18 kDa peptide was thought to be an amino terminal fragment of the 30 kDa enzyme, because its amino terminal amino acid sequence was identical to that of the enzyme. The 12 kDa peptide having the sequence of (Asp)-Pro-Lys-Val-Ser-His was thought to be a carboxy terminal fragment formed by cleavage of the aspartyl-prolyl bond in the enzyme.

4. Discussion

AP endonucleases are classified into two major classes, classes I and II [1–5]. Class I AP endonucleases, otherwise known as AP lyases or 3' AP endonucleases, cleave the 3' side of AP sites to produce 3'-termini bearing the 2,3-unsaturated abasic residue 4-hydroxy-2-pentenal and 5'-phosphomonoester nucleotide termini [1–5,31]. 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 [1–5,31]. Class I AP endonucleases have no priming activity, but class II AP endonucleases have priming activity for DNA polymerase on acid-depurinated DNA [2,4]. The present 30 kDa enzyme is thought to belong to class II AP endonucleases because of its priming activity on acid-depurinated DNA. The presence of multiple forms of class II AP endonucleases in mammalian cells has been reported [1,4,5]. However, only the major AP endonuclease (designated as APEX nuclease, or as HAP1, APE or Ref-1 protein) has been identified by determining its primary structure [11,13–15]. Our previous studies on APEX nuclease showed that a small part of APEX nuclease degraded into the 31, 33 and 35 kDa C-terminal fragments during the purification process and that those fragments have 5' AP endonuclease activity [32,33]. We suggested that this is a possible cause of heterogeneity of mammalian apurinic/apyrimidinic endonuclease [33]. To discriminate a newly purified AP endonuclease from the active fragments of APEX nuclease, comparison of primary structures between them is thought to be important.

Single-strand breaks with 3'-blocked termini are frequently produced by free radical pathways caused by bleomycin, ionizing radiation and other sources of oxygen radicals [1,6–8,30]. Repair of single-strand breaks with 3'-blocked termini is known to be initiated by enzymes having DNA 3' repair diesterase activity which can remove the blocked termini and release 3'-hydroxyl termini. Such enzymes have been reported to be multiple and generally associated with 5' AP endonuclease activity [4,17]. APEX nuclease is also one of such enzymes.

The 30 kDa enzyme also have 5' AP endonuclease and DNA 3' repair diesterase activities, although both activities are approx. 1/40 and 1/100 of those of APEX nuclease, respectively. The relatively low activities of the 30 kDa enzyme may be partly due to its incomplete renaturation in the denaturation-renaturation step adopted for getting active preparation from the SDS-PAGE-purified enzyme.

The determined partial amino acid sequences of the 30 kDa enzyme have no homology to any regions of the primary structure of APEX nuclease. Homology search was also performed with proteins in SWISS-PROT (released Feb. 1995) and NBRF (released Dec. 1994) Protein Sequence Data Bases. Proteins having amino acid sequences more than 50% identity with the N-terminal 16 amino acid sequence of the 30 kDa protein were not detected in these Data Bases. The mouse 28 kDa AP endonuclease reported by Nes [18] is apparently identical to the 30 kDa enzyme reported here. Both enzymes have almost the same molecular weight and the similar requirement for ionic strength and divalent cations on the enzyme.
activities, although further definite comparision between them is difficult, because the Nes’s paper has no informations on partial amino acid sequences, DNA 3’ repair diesterase activity and peptide fragments of the 28 kDa AP endonuclease from a mouse plasmacytoma cell line [18].

We are now trying to clone cDNA for the 30 kDa enzyme using oligonucleotide probes synthesized according to its partial amino acid sequences.

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