Apurinic endonuclease from *Saccharomyces cerevisiae*

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An endonuclease cleaving depurinated and alkylated double-stranded DNA has been purified 500-fold from *Saccharomyces cerevisiae*, strain MB 1052. The enzyme has an M_r of $31\,000 \pm 2000$, a sedimentation value of 3.2S and a diffusion coefficient of $9.5 \times 10^{-7} \text{ cm}^2/\text{s}$. The enzyme was active only at apurinic/apyrimidinic sites, regardless of whether they were produced by heating the DNA at acidic pH or by alkylation with the ultimate carcinogen methyl methanesulphonate. Native DNA was not acted upon. U.v.-irradiated DNA and DNA treated with the ultimate carcinogen *N*-acetoxy-2-acetylaminofluorene were cleaved to an extent related to the extent of apurinic/apyrimidinic sites. Enzymic activity was not dependent upon Mg^{2+} , but was stimulated approx. 3-fold by 4 mM- Mg^{2+} . The enzyme did not bind to DEAE-cellulose or CM-cellulose at KCl concentrations greater than 160 mM. The endonuclease was obtained free of exonuclease and 3-methyladenine-DNA glycosylase activity in five chromatographic steps.

The genome of diploid *Saccharomyces cerevisiae* cells contains of the order of 7.3×10^7 bases (Mounolou, 1971). From the depurination rate of double-stranded DNA [$k = 10^{-11} \text{ s}^{-1}$ at pH 7.4 and 30°C (Lindahl & Nyberg, 1972)] and the generation time [100 min at 30°C (for references see: Stokes, 1971)], it can be calculated that approximately one purine base should be lost per yeast genome in each generation. The depurination rate is considerably enhanced at acidic pH (Lawley & Brookes, 1963; Lindahl & Nyberg, 1972) or after introduction of positive charges into purine ring systems, as occurs after alkylation of guanine and adenine in the *N*-7 and *N*-3 positions (Margison & O'Connor, 1973; Singer, 1975). AP sites caused by spontaneous loss of purines, or after DNA alkylation, can be repaired (Verly & Rassart, 1975; Lindahl, 1979). The first step consists of endonucleolytic cleavage on the 5' side of the AP site (Verly & Rassart, 1975). Subsequently, the deoxyribose moiety is removed by an 5'–3' exonuclease activity (Lindahl, 1979).

Abbreviations used: CHO cells, Chinese hamster ovary cells subclone K₁-BH₄; SDS, sodium dodecyl sulphate; MeSO₂OMe, methyl methanesulphonate; (Ac)₂ONFln, *N*-acetoxy-2-acetylaminofluorene; Me₂SO, dimethyl sulphoxide; PM2 DNA, superhelical DNA of the *Pseudomonas* phage PM2; AP endonuclease, apurinic/apyrimidinic endonuclease; AP sites, apurinic/apyrimidinic sites; Hepes, 4-(2-hydroxyethyl)-piperazine-ethanesulphonic acid.

Excision of the damage is accomplished by repolymerization and sealing of the DNA termini (Lindahl, 1979).

An endonuclease specific for AP sites has been discovered in 105 000 g supernatants of *S. cerevisiae* wild type and *rad* mutant cells (Chlebowicz & Jachymczyk, 1977). In a further study (Armell & Wallace, 1978) three endonuclease activities have been partially purified from the haploid strain LP98-1D on the basis of their activity against X-irradiated or depurinated double-stranded DNA. However, important physical properties of the AP endonuclease(s) remained undetermined so far. We have accordingly purified an AP endonuclease about 500-fold from crude extracts of the diploid yeast strain MB 1052, monitoring its activity on MeSO₂OMe-treated or heat/acid-treated DNA, and measured its sedimentation and diffusion constants. From these data we calculated the molecular weight, the Stokes' radius and the frictional coefficient ratio. Some catalytic properties of the enzymes were also investigated.

Materials and methods

Materials

Cells Strain MB 1052 *tmp1-3* of *S. cerevisiae* [diploid and rho[−] (Fäth & Brendel, 1975)] was generously supplied by Dr. M. Brendel, Arbeitsgruppe Mikrobengenetik im Fachbereich Bio-

logie der Johann-Wolfgang-Goethe-Universität, D-6900 Frankfurt am Main, Federal Republic of Germany.

Media and growth conditions. Cells were grown in YPD medium [1% Difco yeast extract, 2% Difco peptone, 3% D-(+)-glucose supplemented with 6 μ g of dTMP/ml at pH 7.4 (Fäth & Brendel, 1975)]. For preparing yeast on a preparative scale, cells were pregrown in YPD medium at 30°C in a model 76 gyrotory water bath shaker (NewBrunswick Scientific Co., New Brunswick, NJ, U.S.A.). With these cells, 75 litres of fresh medium were inoculated at a titre of approx. 2×10^6 /ml. When the cells had reached the mid-exponential phase they were harvested by centrifugation (3000 g) to yield 200 g (wet wt.) of cells, washed with buffer (50 mM-Tris/HCl/200 mM-KCl/2 mM-EGTA/20% glycerol, pH 7.4), resuspended in one vol. of the same buffer and slowly poured into liquid N₂.

Chemicals. Ethidium bromide and Seakem agarose were obtained from Serva; Cremophor EL, a polyethoxylated *Ricinus* oil, was obtained from BASF. dTMP was purchased from Pharma-Waldhof GmbH, Düsseldorf, Federal Republic of Germany.

Buffers. Buffer A consisted of 50 mM-Tris/maleate/1 mM-EGTA/3 mM-dithiothreitol/20% (v/v) glycerol, pH 7.45; buffer B contained in addition 10 mM-KCl; buffer C contained 160 mM-KCl instead. Buffer D was 50 mM-Tris/maleate/200 mM-KCl/3 mM-dithiothreitol/20% (v/v) glycerol, pH 7.45. Buffer E consisted of 50 mM-Tris/HCl-1 mM-dithiothreitol/0.1 mM-EGTA/10% (v/v) glycerol/0.02% NaN₃. Buffer F had the same composition as buffer E, except that the KCl concentration was 100 mM.

Chromatography materials. DEAE-cellulose (DE 52), CM-cellulose (CM 52) and phosphocellulose (P 11) were from Whatman Biochemicals. Hydroxyapatite (Bio-Gel HT) was from Bio-Rad. Sephadex G-100 (Superfine) was from Deutsche Pharmacia GmbH, Freiburg, Federal Republic of Germany.

Marker proteins. Cytochrome *c* prepared from horse heart, myoglobin prepared from equine skeletal muscle, carbonic anhydrase prepared from bovine erythrocytes, ovalbumin, bovine serum albumin, and aldolase prepared from rabbit muscle were all obtained from Serva.

DNA. PM2 DNA was prepared as described by Espejo *et al.* (1969).

Carcinogens. [³H]Me₂SO₄ (sp. radioactivity 3.3 Ci/mmol) was obtained from New England Nuclear. (Ac)₂ONFlu was synthesized according to published methods (Lotlikar *et al.*, 1965; 1966).

Polyacrylamide-gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out essentially as described in the LKB

Application note 306 (April, 1977) using the phosphate-buffer system. The equipment consisted of an LKB Multiphor Electrophoresis kit. The gel concentration was 10% (w/v). The current was adjusted to 20 mA for the first 10 min, then it was raised to 190 mA, and electrophoresis continued for 5 h. The cooling water temperature was approx. 10°C.

For denaturation, proteins were dissolved in 10 mM-NaH₂PO₄/5% (w/v) SDS/10% (v/v) 2-mercaptoethanol at pH 7.0, and heated for 5 min at 100°C.

Preparation of substrates for endonuclease test

Heat/acid treatment of PM2 DNA for introduction of AP sites. Heat-induced depurination/depyrimidination of PM2 DNA was performed essentially according to Lindahl & Nyberg (1972). Briefly, DNA was heated for various times in 100 mM-NaCl/10 mM-sodium phosphate/10 mM-sodium citrate, pH 4.8 at 70°C, chilled, and adjusted to pH 7.4 by the addition of 2 vol. of 200 mM-Tris/HCl/1 mM-EDTA at pH 8.1. Under these conditions, 0.17 alkali-labile (i.e. AP) sites/min per PM2 DNA molecule were introduced. This number was determined by using alkaline sucrose gradients (Thielmann, 1977). The DNA was used immediately.

U.v. irradiation of PM2 DNA. PM2 DNA solutions (60 μ M in 10 mM-Tris/HCl/1 mM-EDTA at pH 7.4) were irradiated with a germicidal lamp (model PCQ/X1, emitting predominantly 253.7-nm radiation; Ultra-Violet Products, Inc., San Gabriel, CA, U.S.A.). The intensity of u.v. radiation was 20 erg/mm² (2 J/m²), as measured by a Blak-Ray u.v.-dosimeter (model J-225, Ultra-Violet Products).

Reaction of PM2 DNA with carcinogens. PM2 DNA (6 nmol) was allowed to react in 100 μ l of buffer (40 mM-potassium phosphate/20 mM-NaCl/2 mM-sodium acetate at pH 7.4) with MeSO₂OMe or (Ac)₂ONFlu (200-fold or 55-fold molar excess respectively over DNA nucleotides). As determined by alkaline sucrose-gradient analysis, treatment of DNA with MeSO₂OMe introduced approx. one alkali-labile (i.e. AP) site per PM2 DNA molecule. The carcinogen solutions were always freshly prepared; (Ac)₂ONFlu was solubilized in Cremophor EL/Me₂SO (1/1, v/v). The final concentration of Me₂SO did not exceed 2.5% in the assays. After incubation at 37°C for 20 min, the modified DNAs were used immediately.

Determination of MeSO₂OMe-induced alkali-labile sites. Superhelical PM2 DNA was treated *in vitro* with MeSO₂OMe, and the number of alkali-inducible single-strand breaks was determined by using alkaline sucrose gradients (Thielmann, 1977) in order to correlate enzyme-catalysed cleavage of alkylated DNA with the capacity of the carcinogen

to introduce alkali-labile (i.e. AP) sites (see Fig. 4b). Aliquots of ^{32}P -labelled PM2 DNA (Espejo *et al.*, 1969) were treated with MeSO_2OMe (final concn. 1.5–8.9 mM) for 45 min at 37°C, kept an additional 45 min at 30°C, denatured at pH 13.2 for 15 min, loaded on top of alkaline sucrose gradients, and sedimented.

Determination of endonuclease activity

The standard reaction mixture (total vol. 25 μl : 40 mM-Tris/HCl/1 mM-EDTA/15% (v/v) glycerol at pH 7.4) contained 0.3 nmol of heat/acid-treated, u.v.-irradiated or carcinogen-modified PM2 DNA and 5–15 μl of column fractions. After 30 min at 30°C the reaction was stopped with 10 μl of 10% (w/v) SDS. Bromophenol Blue marker was added (5 μl of a 0.4% solution) together with glycerol (10 μl); covalently closed circular PM2 DNA was then separated from the nicked form by agarose-gel electrophoresis.

Gel electrophoresis. DNA samples were run in 0.9% agarose gels for 15 h at a constant voltage of 60 V at 20°C, stained with ethidium bromide, and photographed as described (Hecht & Thielmann, 1978).

Evaluation of the gels. Photographs were traced with a Zeiss KM3 spectrophotometer in order to obtain the relative quantities of the respective bands. The peak areas were determined using a MOP-AM 03 integrator (Kontron Messgeräte GmbH, Eching, Federal Republic of Germany). Endonuclease activity is expressed by the number of enzyme-catalysed breaks per PM2 molecule. The latter was calculated (Vinograd *et al.*, 1965) from the fraction of molecules which contained no breaks (α) by using the equation: $N = -\ln(\alpha)$. One unit of endonuclease activity was defined as the amount of enzyme that converted 0.14 nmol of MeSO_2OMe -treated or depurinated PM2 DNA into the nicked form under the standard conditions.

Velocity sedimentation in sucrose gradients

After dialysis against 50 mM-Tris/HCl (pH 7.6)/0.3 M-NaCl/1 mM-EDTA, 0.4 ml of purified endonuclease (fraction VI) was loaded on top of 12 ml neutral gradients [5–30% (w/v) sucrose in the above buffer] and centrifuged in a Beckman SW 40 rotor for 48 h at 40 000 rev./min at 4°C. Fractions (eight drops) were collected and assayed by using MeSO_2OMe -treated PM2 DNA as well as heat/acid-treated DNA. Marker proteins were determined by spectrophotometry.

Reaction of DNA with $[^3\text{H}]\text{Me}_2\text{SO}_4$

In 4 ml of buffered medium [80 mM-sodium cacodylate (pH 7.0)/0.13 M- NaClO_4 /1 mM-EDTA] 10.1 μmol (as nucleotides) of calf thymus DNA was

incubated with 5.0 mCi of $[^3\text{H}]\text{Me}_2\text{SO}_4$ (sp. radioactivity 3.3 Ci/mmol) for 5.5 h at 37°C [under these conditions $t_{1/2}$ of Me_2SO_4 is 44 min (Voss & Blanke, 1931)]. The solution was then transferred into a 50 ml stainless steel centrifuge tube, and DNA was precipitated with 16 ml of ethanol (10 min at -40°C) and sedimented (10 min at 8000 g). The pellet was redissolved in 2 ml of buffer (100 mM-Tris/HCl (pH 7.4)/1 mM-EDTA), and, after transferring the solution into a 14 ml glass tube, DNA was again precipitated with 6 ml of ethanol (20 min at -40°C) and pelleted (10 min at 8000 g). This procedure was repeated five times until the supernatant was free of ^3H radioactivity. Finally the pellet was dissolved in 1.2 ml of Hepes buffer (83 mM, pH 8.0). The total radioactivity thus obtained was 7.25×10^6 d.p.m. The sp. radioactivity of the methylated DNA was 1.06 Ci/mol.

Assay for DNA glycosylase activity

In 445 μl of buffer [64 mM-Hepes (pH 8.0)/7 mM-Tris/HCl/7 mM-KCl/0.3 mM-MgCl₂/0.01 mM-EGTA/1% glycerol] 260 nmol $[^3\text{H}]\text{Me}_2\text{SO}_4$ -methylated calf thymus DNA (6×10^5 d.p.m.) were incubated with 50 μl of the enzyme preparation to be tested for 90 min at 37°C (Laval, 1977; Brent, 1979). The reaction was terminated by addition of 50 μl of carrier DNA (2500 $\mu\text{g}/\text{ml}$) and 2 ml of ethanol. The sample was kept at -40°C for 15 min, centrifuged (10 min, 12 000 g), and 1 ml aliquots of the supernatant were counted in 4 ml of Rotiscint 23 (Carl Roth KG, Karlsruhe, Federal Republic of Germany) using a Berthold model BF8000 scintillation spectrometer. The counting efficiency of ^3H under these conditions was 33%.

Enzyme purification

All purification steps were carried out at 4°C unless otherwise indicated. Yeast cells (frozen beads; see under 'Media and growth conditions') were disrupted batchwise at -80°C in an X-press (X-25, LKB Instruments). The resulting homogenate was diluted with 3 litres of buffer A in which 125 g of DE-52 had been suspended. The suspension was carefully layered onto a column (10 cm diameter) which had been partially filled with 500 ml of settled DE-52 pre-equilibrated with buffer A. After washing with 4 litres of buffer B, the enzymic activity was eluted with 2 litres of buffer C. The first 500 ml of the effluent contained the enzymic activity and showed a pink colour. It was loaded onto a CM-cellulose column (5 cm $\times$ 15 cm) previously equilibrated with buffer C. Enzymic activity was eluted with 500 ml of buffer C. The effluent (pink colour) was applied onto a phosphocellulose column (5 cm $\times$ 15 cm) equilibrated with buffer D. The column was washed with 3 vol. of buffer D, followed by a linear gradient (1 litre) of 0.2–1.5 M-KCl in

buffer A. Fractions (56, each of 18 ml) were collected and aliquots were dialysed against 50 mM-Tris/HCl/50 mM-KCl/1 mM-dithiothreitol/20% (v/v) glycerol at pH 7.4, and assayed with both MeSO₂OMe-treated and depurinated PM2 DNA. Enzymic activity was found between 0.3–1.2 M-KCl (30 fractions, 540 ml). The active fractions were pooled, dialysed against 50 mM-Tris/maleate/70 mM-KCl/3 mM-dithiothreitol/20% (v/v) glycerol at pH 7.4, and applied to a hydroxyapatite column (2.6 cm × 13 cm) pre-equilibrated with 20 mM-potassium phosphate, and 20% (v/v) glycerol at pH 7.0. The column was washed with 3 vol. of the same buffer followed by a linear gradient (380 ml) of 20–400 mM-potassium phosphate/3 mM-dithiothreitol and 20% (v/v) glycerol at pH 7.0. Fractions (21 ml) were collected, dialysed (two changes) against 5 litres of buffer E, and tested with depurinated and MeSO₂OMe-treated PM2 DNA. Enzymic activity was eluted between 200–300 mM-potassium phosphate. Active fractions (118 ml) were combined, dialysed against buffer E, and concentrated by using an Amicon PM10 Diaflo membrane (which retains compounds with $M_r > 10000$) under N₂ (pressure 40 lb/in², 276 kPa) to 2 ml. A precipitate was removed by centrifugation. The supernatant was applied to a Sephadex G-100 (Superfine) column (1.6 cm × 60 cm) equilibrated with buffer F. Elution was performed with buffer F. Fractions (1 ml) were collected and tested. Fractions containing endonuclease activity with MeSO₂OMe-treated or depurinated PM2 DNA (fractions 69–73) were combined and stored at –8°C.

Results

Purification of an endonuclease acting on MeSO₂OMe-treated double-stranded DNA

The scheme of enzyme purification is shown in Table 1. Technical details are given in the Materials and methods section.

Briefly, yeast cells were disrupted and the resulting homogenate was diluted with buffer A. DNA and cell debris were removed by adsorption to DEAE-cellulose at low salt concentration. Active material was eluted with 160 mM-KCl in buffer. The effluent was then subjected to a second stepwise elution by using CM-cellulose. Active material was applied to a phosphocellulose column and eluted with a linear KCl gradient. The eluate was further chromatographed on hydroxyapatite. Final purification was achieved by elution from Sephadex G-100 (Superfine). A purification of approx. 500-fold was achieved with the procedure summarized in Table 1.

Although no particular care was taken to adjust the purification procedure so as to obtain an optimal yield of enzyme, yeast can be regarded as a rich and readily available source of the AP endonuclease. No technical difficulties were encountered when using an X-press for cell disruption. Membrane fragments present in the cell homogenate were retained by the DEAE-cellulose and CM-cellulose columns.

Physical characterization

The purified enzyme was assayed for homogeneity by electrophoresis in polyacrylamide gels (Fig. 1). The mobility of the protein band corresponded to an M_r of 30000 ± 3000 .

In determining the M_r , the Stokes' radius (a) and the frictional coefficient ratio (f/f_0) of the endonuclease, the procedure recommended by Siegel & Monty (1966) was adopted; this is based on velocity sedimentation in neutral sucrose gradients and diffusion measurements in porous gels (Laurent & Killander, 1964; Andrews, 1965). The sedimentation coefficient was measured to be 3.2 S (Fig. 2b). A calibrated gel filtration column was used (Sephadex G-100, Superfine) for determination of the diffusion coefficient of the endonuclease. $D_{20,w}^{20}$ was found to be 9.5×10^{-7} cm²/s (Fig. 2a).

Table 1. *Purification of endonucleolytic activity with MeSO₂OMe-treated DNA from MB 1052 S. cerevisiae cells*
Endonucleolytic activity was measured as described in the Materials and methods section. Protein was determined by the method of Lowry *et al.* (1951).

Step	Volume (ml)	Total protein (mg)	Activity with MeSO ₂ OMe-treated DNA (kU)	Yield (%)	Specific activity (kU/mg)	Purification (-fold)
1. Cell disruption	3300	22000	780	100	0.035	1
2. DE-52 step elution	500	904	226	29	0.25	7
3. CM-52 step elution	500	239	170	22	0.71	20
4. Phosphocellulose chromatography	540	28.1	110	14	3.9	100
5. Hydroxyapatite chromatography	118	5.5	25.3	3.2	4.6	130
Concentration by Diaflo membrane (fraction V)	2	—	—	—	—	—
6. Gel filtration (fraction VI)	5	0.26	4.4	0.56	16.6	470

Fig. 1. SDS/polyacrylamide-gel electrophoresis of fraction VI (b) and marker proteins (a)

Marker proteins were: cytochrome *c* (a), myoglobin (b), carbonic anhydrase (c), ovalbumin (d) and bovine serum albumin (e). Details are given in the Materials and methods section. The gel was stained with Coomassie Blue. The anode is on the left. The stained gel was photographed on 13 cm × 18 cm negative film and the negatives were traced with a Zeiss KM3 spectrophotometer.

Fig. 2. Determination of the diffusion constant $D_{20,w}$ and the sedimentation value of the endonuclease by gel filtration on Sephadex G-100 (Superfine) and velocity sedimentation

Marker proteins of known diffusion coefficient (in 10^{-7} cm²/s) were aldolase (4.6), bovine serum albumin (6.0), ovalbumin (7.8), carbonic anhydrase (10.5), myoglobin (11.3), cytochrome *c* (13.0), and for sedimentation, myoglobin (2.0 S), ovalbumin (3.6 S), bovine serum albumin (4.4 S), and aldolase (7.35 S). Marker proteins were assayed by using absorption techniques. $K_{av} = V_e - V_o / V_i - V_o$ (Laurent & Killander, 1964). V_o and V_i , determined with Dextran Blue and [³H]thymidine, were 39 and 117 ml, respectively.

With these values the Svedberg formula:

$$M_r = \frac{RTS}{D(1 - \bar{v}\rho)}$$

where M_r is the relative molecular mass, T the absolute temperature, $\bar{v}$ the partial specific volume [approx. 0.725 cm³/g (Martin & Ames, 1961)], and ρ the density of water at 20°C (0.998 g/cm³) can be resolved to give $M_r = 31\,000 \pm 2000$.

The Stokes' radius of the endonuclease protein was calculated by applying the equation:

$$a = \frac{kT}{6\pi\eta D}$$

where a is the Stokes' radius, k the Boltzmann constant, η the viscosity of water at 20°C (1.005×10^{-2} P) and D the diffusion coefficient. The Stokes' radius was found to be 2.1 nm. The frictional coefficient ratio (f/f_o) was derived from the formula:

$$f/f_o = \frac{a}{(3\bar{v}M_r/4\pi N)^{1/3}}$$

with M_r being the calculated relative molecular mass and N Avogadro's number. A value of 1.06 was obtained. Assuming 0.2 g water of hydration per g of protein, this corresponds to an ellipsoid of rotation and an axial ratio of approximately 1.3:1 (Edsall, 1943). This characterizes the endonuclease as a globular protein molecule. The physical properties of the AP endonuclease are summarized in Table 2.

Absence of DNA glycosylase and exonuclease activity

Fraction V (the concentrate of the active fractions from the hydroxyapatite chromatography) as well as fraction VI (the purified Sephadex G-100 fractions) were tested for DNA glycosylase activity by using 5×10^6 d.p.m. of $[^3\text{H}]\text{Me}_2\text{SO}_4$ -methylated calf thymus DNA as substrate.

As Table 3 shows, fraction V liberated 0.44% ethanol-soluble radioactivity above the background level (2.75%) of the protein-free control. Fraction VI, however, did not degrade DNA-bound radioactivity at all. Thus fraction VI appears free of 3-methyladenine-DNA glycosylase as well as exonuclease activity. Under the same assay conditions, crude extracts from both *Micrococcus luteus* and CHO cells rendered 12.1% and 4.3% of the DNA-bound radioactivity ethanol-soluble. Using cellulose chromatogram sheets and the solvent system 2-methylpropan-1-ol/ NH_4OH /water (7:1:2, by vol.) (Kirtikar & Goldthwait, 1974) for analysis, this material was found to be almost exclusively 3-methyladenine.

Substrates for endonuclease activity

Activity towards heat/acid-treated and MeSO_3OMe -treated DNA. PM2 DNA was heated at pH 4.65 for 9 min at 70°C in order to introduce a defined number of AP sites into the DNA substrate. This treatment gave rise to 1.5 AP sites per PM2 DNA molecule, as determined by sedimentation analysis in alkaline sucrose. Incubation of this DNA substrate with increasing amounts of fraction VI yielded a saturation curve (Fig. 3, lower curve). Endonucleolytic cleavage levelled off at 0.4 breaks per PM2 molecule (30% of the AP sites present). There is no simple explanation for the finding that not all of the AP sites present were converted into single-strand breaks under the experimental conditions. However, similar observations were made with AP endonucleases from mouse epidermal cells (Ludwig & Thielmann, 1979) and from *M. luteus* (Hecht & Thielmann, 1978).

In further experiments, PM2 DNA species containing increasing numbers of AP sites (0.33–2.0 AP sites per PM2 DNA molecule) were prepared. These DNA substrates were incubated with 8 μl of fraction VI. A linear relation between the number of enzyme-catalysed breaks and the number of AP sites was obtained (Fig. 4a). Using a double-reciprocal plot (Lineweaver & Burke, 1934) for the estimation of kinetic parameters (Fig. 5a), a K_m value of 11 nM-AP sites and a $V_{\max}$ value of 0.2 nM-breaks $\cdot \text{min}^{-1} \cdot \mu\text{g}$ of protein $^{-1}$ were calculated from the data shown in Fig. 4a.

For introduction of AP sites by treatment with

Table 2. *Physical properties of an AP endonuclease from S. cerevisiae*

Property	Unit	Value
M_r by SDS/polyacrylamide-gel electrophoresis	—	$30\,000 \pm 3000$
M_r by calculation using diffusion and sedimentation values	—	$31\,000 \pm 2000$
$S_{20,w}$	S	3.2
$D_{20,w}$	cm^2/s	9.5×10^{-7}
Stokes' radius	nm	2.1
Frictional coefficient ratio	—	1.06
Axial ratio	—	1.3:1

Table 3. *Test for DNA glycosylase and exonuclease activity of fraction V and fraction VI*

Enzyme preparation	Concentration of protein in the assay ($\mu\text{g}/\text{ml}$)	Radioactivity rendered ethanol-soluble (%)
Control	0	2.75
Fraction V	100	3.19
Fraction VI	0.23	2.70
Crude extracts from		
<i>M. luteus</i> *	4.9	14.8
CHO cells	2050	7.0

* For details of preparation, see Hecht & Thielmann, 1978.

Fig. 3. Cleavage of MeSO_2OMe -treated or heat/acid-treated PM2 DNA as a function of enzyme concentration

For methylation, 3.8 nmol of PM2 DNA were treated with 9 mM- MeSO_2OMe in 50 μl of buffer (100 mM-Tris/HCl (pH 7.6)/1 mM-EDTA) for 45 min at 37°C. As determined by sedimentation analysis in alkaline sucrose (Thielmann, 1977), approx. 2.2 AP sites per PM2 molecule were introduced by this procedure (results not shown). Enzyme assays with fraction VI were as follows: in 25 μl total vol. (55 mM-Tris/HCl (pH 7.6)/60 mM-KCl/5 mM- MgCl_2 /0.2 mM-EDTA/0.6 mM-dithiothreitol/0.06 mM-EGTA/6% (v/v) glycerol) 0.38 nmol of DNA were incubated with up to 10 μl of fraction VI for 45 min at 30°C. For introduction of AP sites, 3.8 nmol of PM2 DNA were heated for 9 min at 70°C in 50 μl of buffer (100 mM-NaCl/10 mM-sodium citrate/10 mM-sodium phosphate at pH 4.65). Then 50 μl of 200 mM-Tris/HCl (pH 8.1)/1 mM-EDTA were added and the sample was chilled in ice. By this procedure, 1.5 AP sites per PM2 DNA molecule were created (results not shown). Aliquots (10 μl) of this solution (controls: corresponding amounts of untreated PM2 DNA) were incubated with the indicated quantities of fraction VI in 25 μl total vol. [110 mM-Tris/HCl (pH 7.6)/4 mM-sodium phosphate/60 mM-KCl/20 mM-NaCl/8 mM- MgCl_2 /2 mM-sodium citrate/0.6 mM-dithiothreitol/0.06 mM-EGTA/6% (v/v) glycerol] for 45 min at 30°C. $\square$, MeSO_2OMe -treated DNA; $\bullet$, depurinated DNA.

Fig. 4. Endonucleolytic cleavage of PM2 DNA by fraction VI as a function of either the MeSO_2OMe concentration used for DNA modification (b) or the number of AP sites (a)

MeSO_2OMe , PM2 DNA was incubated with 9 mM- MeSO_2OMe for 45 min at 37°C. Alkaline sucrose gradient analysis revealed that 2.2 alkali-labile (i.e. AP) sites per PM2 molecule were introduced by this treatment. The substrate was incubated with increasing amounts of fraction VI (Fig. 3, upper curve). As can be seen, enzymic activity reached a saturation level at which approx. 0.7 breaks per PM2 molecule were measured (30% of the AP sites present).

In further experiments, aliquots of PM2 DNA were treated with increasing concentrations of

PM2 DNA, 1.52 nmol, was treated with MeSO_2OMe at various concentrations of up to 8.9 mM in 20 μl of buffer (50 mM-Tris/HCl (pH 7.6)/1 mM-EDTA) for 45 min at 37°C. Aliquots of 0.76 nmol of these DNA preparations were further incubated with fractions VI (8 μl) in 30 μl of buffer (42 mM-Tris/HCl (pH 7.6)/50 mM-KCl/4.2 mM- MgCl_2 /0.3 mM-EDTA/0.5 mM-dithiothreitol/0.05 mM-EGTA/5% (v/v) glycerol) for 45 min at 30°C. For introduction of AP sites, PM2 DNA was heated as described in the legend to Fig. 3 for various times. Incubations were of 0.76 nmol DNA with 8 μl of fraction VI in 40 μl of buffer [66 mM-Tris/HCl (pH 7.6)/60 mM-KCl/25 mM-NaCl/8 mM- MgCl_2 /2.5 mM-sodium citrate/2.5 mM-sodium phosphate/0.3 mM-dithiothreitol] for 45 min at 30°C.

Fig. 5. AP endonuclease activity in fraction VI as a function of AP sites using either heat/acid-treated (a) or MeSO₂OMe-treated (b) PM2 DNA as substrate. The values of the double-reciprocal plot are derived from those shown in Fig. 4.

MeSO₂OMe (1.5–8.9 mM) for 45 min at 37°C. The resulting DNA species were then subjected to two types of measurement: (1) determination of the respective numbers of alkali-labile sites using sedimentation analysis in alkaline sucrose (see the second abscissa in Fig. 4b), and (2) determination of the number of single-strand breaks catalysed by 8 μl of fraction VI (Fig. 4b). As Fig. 4(b) shows, the number of enzyme-catalysed breaks was linearly related to both the MeSO₂OMe concentration used for DNA pretreatment and—correspondingly—to the concentration of AP sites resulting from MeSO₂OMe treatment of DNA. From a $1/v$ versus $1/S$ plot (Lineweaver & Burke, 1934) of the data shown in Fig. 4(b), a K_m value of 15 nM-AP sites and a V_{max} value of 0.12 nM breaks · min⁻¹ · μg of protein⁻¹ were calculated (Fig. 5b). Within the limits of accuracy of the analysis applied, these values are in satisfactory agreement with those determined with depurinated (heat/acid-treated) DNA, indicating that fraction VI contains but one enzyme which cleaves both depurinated and methylated-apurinic DNA.

Activity towards u.v.-irradiated DNA. PM2 DNA was irradiated with u.v.-light so as to introduce

approx. 30 pyrimidine dimers per DNA molecule (Woodworth-Gutai *et al.*, 1977). It was then incubated with increasing amounts of AP endonuclease (fraction VI). Fig. 6 shows (lower curve) that enzymic activity increased until 4 μl were added. A further increase beyond this amount did not result in a higher yield of breaks. This contrasts with the activity of the u.v. endonuclease from *M. luteus*. The same DNA substrate was not saturated by increasing amounts of *M. luteus* enzyme which had been purified by phosphocellulose chromatography (Hecht & Thielmann, 1978). This means that the AP endonuclease (fraction VI) did not act on pyrimidine dimers. The small number of enzyme-catalysed breaks in u.v.-irradiated DNA corresponds to the number of alkali-labile sites present (0.13/PM2 DNA molecule; H. W. Thielmann, unpublished work).

When PM2 DNA was pretreated with the 'u.v.-like' carcinogen (Ac)₂ONFlu, so as to fix covalently approx. 100 *N*-2-fluorenylacetamido

Fig. 6. Enzymic activities of fraction VI and u.v. endonuclease from *M. luteus* on u.v.-irradiated PM2 DNA as a function of enzyme concentration.

U.v.-endonuclease from *M. luteus* (protein concentration 10 μg/ml) was prepared as described [fraction III (Hecht & Thielmann, 1978)]. Irradiation of DNA [1500 erg/mm² (150 J/m²)] and assays for endonucleolytic activity were performed as described in the Materials and methods section. (●) Activity of u.v.-endonuclease from *M. luteus*; (○) activity of fraction VI.

residues per PM2 DNA molecule, and then incubated with fraction VI, only marginal endonucleolytic cleavage was observed (data not shown). The frequency of breaks was out of all proportion to the number of *N*-2-fluorenylacetamido residues and was therefore attributed to the presence of AP sites. These occur to very low extents as a result of DNA modification (Thielmann, 1977).

Divalent cations. Testing of fraction VI with MeSO₂OMe-treated PM2 DNA in the presence of

increasing concentrations of MgCl₂ revealed a maximum of activity between 2.5–6 mM-MgCl₂ (Fig. 7a). Although the enzyme does not require Mg²⁺ for activity, it was stimulated approx. 3-fold by the optimal Mg²⁺ concentration (4 mM).

Monovalent cations. The enzymic activity was strongly influenced by KCl. As Fig. 7(b) shows, inhibition of cleavage of MeSO₂OMe-treated PM2 DNA was observed at KCl concentrations greater than 50 mM. At 100 mM-KCl, inhibition was 50%, at 200 mM-KCl the activity was practically abolished.

Discussion

For enzyme purification 200 g (wet weight) yeast cells were disrupted, and the resulting homogenate was freed from DNA and cell debris by stepwise elution from DE-52 cellulose with buffered 160 mM-KCl. The fractions active with MeSO₂OMe-treated and depurinated double-stranded DNA were subjected to stepwise elution from CM-52 (160 mM-KCl). The active fractions were further purified by chromatography on phosphocellulose, hydroxyapatite and Sephadex G-100 (Superfine). The chromatographic steps led to an approx. 500-fold purification of an activity (fraction VI) that cleaved MeSO₂OMe-treated and depurinated DNA. Fraction VI was inactive with native double-stranded DNA both in the presence and the absence of Mg²⁺. Neither pyrimidine dimers nor covalently attached *N*-2-fluorenylacetamido residues were recognized by the enzyme, as u.v.-irradiated and (Ac)₂ONFl-treated DNAs were nicked only to extents which corresponded to the amounts of alkali-labile sites present in the respective DNA species.

Enzymes with similar substrate specificities have been isolated from a number of prokaryotes (Pater-son, 1978) as well as from eukaryotic cells, namely rat liver (Verly *et al.*, 1973), calf liver (Kuebler & Goldthwait, 1977), calf thymus (Ljungquist & Lindahl, 1974), placenta (Linsley *et al.*, 1977) and seed embryos of the plant *Phaseolus multiflorus* (Thibodeau & Verly, 1977). Endonucleases active on depurinated DNA have also been shown to exist in barley (Svachulová *et al.*, 1978), xeroderma pigmentosum fibroblasts (Kuhnlein *et al.*, 1978), in malignant mouse epidermal cells grown in culture (Ludwig & Thielmann, 1979), and in extracts of HeLa cells (Teebor & Duker, 1975) and of a mouse cell line (Nes & Nissen-Meyer, 1978). Chlebowski & Jachymczyk (1977) reported on endonuclease activity specific for AP sites in 105 000g supernatants from wild type RAD yeast cells, and from the MeSO₂OMe-sensitive mutants *rad6*, *rad18-2*, and *rad21*. By eluting a DNA-free extract from DNA-agarose, Armel & Wallace (1978) partially purified three endonuclease activities called fractions IVA, IVB, and IVC from *S. cerevisiae* LP98-D which

Fig. 7. Endonucleolytic cleavage of MeSO₂OMe-treated PM2 DNA as a function of the MgCl₂ (a) or KCl (b) concentration

PM2 DNA was modified under standard conditions with 23.7 mM-MeSO₂OMe for 45 min at 37°C before aliquots (0.3 nmol) were incubated with 8 µl of fraction VI in 40 µl final vol. The buffer composition was 40 mM-Tris/HCl (pH 7.6)/30 mM-KCl/20 µM-EGTA/0.2 mM-dithiothreitol and variable concentrations of MgCl₂ (a) or 40 mM-Tris/HCl (pH 7.6)/0.2 mM-dithiothreitol/20 µM-EGTA and variable concentrations of KCl (b). For further details see the Materials and methods section.

cleaved depurinated, but not native, PM2 DNA. When tested with heavily u.v.-irradiated PM2 DNA, the same fractions introduced approximately 0.4% nicks per pyrimidine dimer. From this low yield of enzyme-catalyzed breaks the authors concluded that their endonucleases did not recognize pyrimidine dimers.

Our enzyme was active in the presence of EDTA, although 2.5–6 mM-MgCl₂ caused a 3-fold stimulation. Similarly, the fractions IVA-C described by Armel & Wallace (1978) were found to be active in the presence of 2 mM-EDTA, and were stimulated 2–5-fold by 8 mM-Mg²⁺ and 2 mM-ATP. The AP endonuclease from calf thymus (Ljungquist & Lindahl, 1974), calf liver (Kuebler & Goldthwait, 1977) and *Phaseolus multiflorus* (Thibodeau & Verly, 1977) have also been reported to be stimulated by Mg²⁺.

In testing for DNA glycosylase and exonuclease activity, [³H]Me₂SO₄-treated calf thymus DNA was incubated with fraction VI. No DNA-bound radioactivity was rendered ethanol-soluble under assay conditions in which crude extracts from both *M. luteus* cells and CHO cells liberated 12.1% and 4.3% ethanol-soluble 3-methyladenine from the DNA substrate. Therefore fraction VI can be considered free of 3-methyladenine-DNA glycosylase and exonuclease activity, as holds true for the eukaryotic endonucleases known so far (Kuebler & Goldthwait, 1977; Ljungquist & Lindahl, 1974; Ljungquist *et al.*, 1975; Linsley *et al.*, 1977; Thibodeau & Verly, 1977; Verly & Paquette, 1973).

Applying the Svedberg formula, the *M_r* of the enzyme was calculated to be 31 000 ± 2000 from the diffusion coefficient (9.5 × 10⁻⁷ cm²/s) and the sedimentation value (3.2 S). A comparison of the present endonuclease with the corresponding activities from yeast so far described is not possible, as the physical properties of the latter are not known. However, similar *M_r* values have been reported for the AP endonucleases from *E. coli* (Verly & Rassart, 1975; Ljungquist & Lindahl, 1977), *M. luteus* (Hecht & Thielmann, 1978; Tomilin & Barenfel'd, 1977), *Bacillus stearothermophilus* (Bibor & Verly, 1978), and mouse epidermal cells (Ludwig & Thielmann, 1979). The molecular weights of the AP endonucleases from calf thymus (Ljungquist & Lindahl, 1974), calf liver (Kuebler & Goldthwait, 1977), and placenta (Linsley *et al.*, 1977) also lie around 30 000. However, two such enzymes with a higher *M_r* (Thibodeau & Verly, 1977; Bibor & Verly, 1978) and one enzyme with a lower *M_r* (Svachulová *et al.*, 1978) have been found.

The present endonuclease resembles the analogous enzymes from calf liver, placenta and mouse epidermal cells in some of its chromatographic properties. None of the four enzymes binds to DEAE-cellulose (and/or CM-cellulose). This

could be an indication that the isoelectric point of these enzymes is at alkaline pH.

The present enzyme is certainly not identical with the endonucleases specific for u.v.-induced pyrimidine dimers, which, apart from their different substrate specificity, are either considerably smaller (Van Lancker & Tomura, 1974; Braun & Grossman, 1974; Kaplan *et al.*, 1969; Tomura & Van Lancker, 1980) or larger in size (Seeborg, 1978). With regard to its biological function, it appears that the enzyme plays a role in the excision repair of depurinated DNA regardless of whether this depurination might be spontaneous (Lindahl & Nyberg, 1972) or alkylation-induced (Lawley & Brookes, 1963). It was reported that the (u.v.-sensitive) mutants *rad6* and *rad21* showed less survival following treatment with MeSO₂OMe than the RAD wild type (Jachymczyk *et al.*, 1977). In keeping with this observation, strand rejoining after MeSO₂OMe treatment was slower in the *rad6* and *rad21* mutants than in the control strain (Jachymczyk *et al.*, 1977). However, when 105 000 g supernatants of the same strains were tested with alkylated-depurinated *E. coli* DNA, the endonucleolytic activities of the wild type and mutant cells were found to be approximately the same (Chlebowicz & Jachymczyk, 1977). Yet, this finding does not exclude a role for the present AP endonuclease in the repair of alkylated DNA, as steps preceding DNA incision might be defective in the mutants. It is well known that treatment of double-stranded DNA with MeSO₂OMe results in 7-methylguanine (88%) and 3-methyladenine (9%) residues, respectively (Singer, 1975). On the one hand these modifications lead to spontaneous depurination [e.g. at 37°C and neutral pH with *t*_{1/2} 144 h and 24 h (Margison & O'Connor, 1973)] while, on the other hand, 3-methyladenine residues can be enzymically liberated by a specific DNA glycosylase (Lindahl, 1979). In each case AP sites arise, the excision of which can be initiated by AP endonucleases (Laval, 1977; Lindahl, 1979).

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References

- Andrews, P. (1965) *Biochem. J.* **96**, 595–606
- Armel, P. R. & Wallace, S. S. (1978) *Nucleic Acids Res.* **5**, 3347–3356
- Bibor, V. & Verly, W. G. (1978) *J. Biol. Chem.* **253**, 850–855
- Braun, A. & Grossman, L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1838–1842

- Brent, T. P. (1979) *Biochemistry* **18**, 911–916
- Chlebowicz, E. & Jachymczyk, W. J. (1977) *Mol. Gen. Genet.* **154**, 221–223
- Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides* (Cohn, E. J. & Edsall, J. T., eds.), pp. 419–443. Reinhold, New York
- Espejo, R. T., Canelo, E. S. & Sinsheimer, R. L. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 1164–1168
- Fäth, W. W. & Brendel, M. (1975) *Z. Naturforsch.* **30c**, 804–810
- Hecht, R. & Thielmann, H. W. (1978) *Eur. J. Biochem.* **89**, 607–618
- Jachymczyk, C., Chlebowicz, E., Swietlinska, Z. & Zuk, J. (1977) *Mutat. Res.* **43**, 1–10
- Kaplan, C. J., Kushner, S. R. & Grossman, L. (1969) *Proc. Natl. Acad. Sci. U.S.A.* **63**, 144–151
- Kirtikar, D. M. & Goldthwait, D. A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2022–2026
- Kuebler, J. P. & Goldthwait, D. A. (1977) *Biochemistry* **16**, 1370–1377
- Kuhnlein, U., Lee, B., Penhoet, E. E. & Linn, S. (1978) *Nucleic Acids Res.* **5**, 951–960
- Laurent, T. C. & Killander, J. (1964) *J. Chromatogr.* **14**, 317–330
- Laval, J. (1977) *Nature (London)* **269**, 829–832
- Lawley, P. D. & Brookes, P. (1963) *Biochem. J.* **89**, 127–138
- Lindahl, T. (1979) *Prog. Nucleic Acid Res. Mol. Biol.* **22**, 135–192
- Lindahl, T. & Nyberg, B. (1972) *Biochemistry* **11**, 3610–3618
- Lineweaver, H. & Burke, D. (1934) *J. Chem. Soc.* **56**, 658–666
- Linsley, W. S., Penhoet, E. E. & Linn, S. (1977) *J. Biol. Chem.* **252**, 1235–1242
- Ljungquist, S. & Lindahl, T. (1974) *J. Biol. Chem.* **249**, 1530–1535
- Ljungquist, S. & Lindahl, T. (1977) *Nucleic Acids Res.* **4**, 2871–2879
- Ljungquist, S., Nyberg, B. & Lindahl, T. (1975) *FEBS Lett.* **57**, 169–171
- Lotlikar, P. D., Miller, E. C., Miller, J. A. & Margreth, A. (1965) *Cancer Res.* **25**, 1743–1752
- Lotlikar, P. D., Scribner, J. D., Miller, J. A. & Miller, E. C. (1966) *Life Sci.* **5**, 1263–1269
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Ludwig, G. & Thielmann, H. W. (1979) *Nucleic Acids Res.* **6**, 2901–2917
- Margison, G. P. & O'Connor, P. J. (1973) *Biochim. Biophys. Acta* **331**, 349–356
- Martin, R. G. & Ames, B. N. (1961) *J. Biol. Chem.* **236**, 1372–1379
- Mounolou, J. C. (1971) in *The Yeasts* (Rose, A. H. & Harrison, J. S., eds.), vol. 2, pp. 309–333, Academic Press, London and New York
- Nes, I. F. & Nissen-Meyer, J. (1978) *Biochim. Biophys. Acta* **520**, 111–121
- Paterson, M. C. (1978) *Adv. Radiat. Biol.* **7**, 1–53
- Seeberg, E. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2569–2573
- Siegel, L. M. & Monty, K. J. (1966) *Biochim. Biophys. Acta* **112**, 346–362
- Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 219–284
- Stokes, J. L. (1971) in *The Yeasts* (Rose, A. H. & Harrison, J. S., eds.), vol. 2, pp. 119–134, Academic Press, London and New York
- Svachulová, J., Savata, J. & Veleminský, J. (1978) *Eur. J. Biochem.* **87**, 215–220
- Teebor, G. W. & Duker, N. J. (1975) *Nature (London)* **258**, 544–547
- Thibodeau, L. & Verly, W. G. (1977) *J. Biol. Chem.* **252**, 3304–3309
- Thielmann, H. W. (1977) *Z. Krebsforsch.* **90**, 37–69
- Tomilin, N. V. & Barenfel'd, L. S. (1977) *Biokhimiya* **42**(6) 764–771
- Tomura, T. & Van Lancker, J. L. (1980) *Arch. Biochem. Biophys.* **201**, 636–639
- Van Lancker, J. L. & Tomura, T. (1974) *Biochim. Biophys. Acta* **353**, 99–114
- Verly, W. G. & Paquette, Y. (1973) *Can. J. Biochem.* **51**, 1003–1009
- Verly, W. G. & Rassart, E. (1975) *J. Biol. Chem.* **250**, 8214–8219
- Verly, W. G., Paquette, Y. & Thibodeau, L. (1973) *Nature New Biol.* **244**, 67–69
- Vinograd, J., Lebowitz, J., Radloff, R., Watson, R. & Laipis, P. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **53**, 1104–1111
- Voss, W. & Blanke, E. (1931) *Liebigs Ann. Chem.* **485**, 258–283
- Woodworth-Gutai, M., Lebowitz, J., Kato, A.-C. & Denhardt, D. T. (1977) *Nucleic Acids Res.* **4**, 1243–1256