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The Apurinic/Apyrimidinic Endodeoxyribonuclease of Rat-Liver Chromatin

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Supplementary Material

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The extract from rat liver chromatin contains two apurinic/apyrimidinic (AP) endodeoxyribonucleases named 0.2 M and 0.3 M isozymes according to the phosphate concentration necessary to elute them from an hydroxyapatite column. The 0.3 M isozyme is the main and perhaps the only chromatin AP endodeoxyribonuclease in the living cell. This 0.3 M isozyme was purified by successive chromatographies on hydroxyapatite, phosphocellulose, heparin-Sepharose and alkylated-depurinated DNA-cellulose. It has a molecular weight of approximately 39000; its optimum pH is around 8.0; it needs Mg^{2+} or Mn^{2+} to be active and the optimum concentration for Mg^{2+} is between 5 mM and 10 mM. The 0.3 M isozyme has no action on intact DNA strands or on alkylated sites; it cuts the phosphodiester bridge which is the immediate neighbour of the AP site on its 5′ side leaving 3′-hydroxyl and 5′-phosphate ends. It has no associated exonuclease activity. To hydrolyze the phosphoester bond near the AP site, the enzyme makes a close contact with three base residues in the large groove of the DNA molecule.

More than 90 % of the AP (apurinic/apyrimidinic) endodeoxyribonuclease activity in rat liver cells are in chromatin. Native chromatin has little action on an added foreign DNA containing AP (apurinic or apyrimidinic) sites, but the activity is found in the non-histone proteins after dissociation of the chromatin [1]. Chromatography of the chromatin proteins on DEAE-cellulose separates two AP endodeoxyribonucleases. In the initial work done in this laboratory, the species emerging at the lower ionic strength was the most abundant; it was completely purified and its properties were studied [2,3].

In more recent experiments, it was found convenient to start the purification of the chromatin proteins with an hydroxyapatite chromatography which also separates the two AP endodeoxyribonuclease isozymes: the species which had been completely purified is eluted with 0.2 M potassium phosphate whereas the other species emerges with 0.3 M potassium phosphate. These two species were called 0.2 M and 0.3 M isozymes respectively. The ratio of the two isozymes varies according to details in the procedure followed before they are separated. The 0.2 M isozyme is brought below 10 % when 0.5 mM phenylmethylsulfonyl fluoride is used in all solutions and the time for completion of the hydroxyapatite chromatography is kept to a minimum (see miniprint supplement). It seems finally that most, if not all, of the 0.2 M isozyme is an artifact and that the important AP endodeoxyribonuclease in the living cell chromatin is the 0.3 M isozyme. This paper deals with the purification and properties of the 0.3 M AP endodeoxyribonuclease isozyme of rat liver chromatin.

Abbreviations. AP, apurinic or apyrimidinic; PMSF, phenylmethylsulfonyl fluoride.

Enzymes. Apurinic/apyrimidinic (AP) endodeoxyribonucleases (EC 3.1.25.2); pancreatic deoxyribonuclease (EC 3.1.21.1); alkaline phosphatase (EC 3.1.3.1); polynucleotide 5′-hydroxyl-kinase (EC 2.7.1.78); snake venom phosphodiesterase (EC 3.1.4.1).

MATERIALS AND METHODS

NaCl/Cit Buffers

NaCl/Cit = 150 mM NaCl, 15 mM sodium citrate; the pH was 7.0 or 8.0 as specified. It was sometimes diluted 10-fold (0.1 NaCl/Cit).

Substrates

Untreated, Alkylated and Alkylated-depurinated [^{3}H]DNAs. DNA, prepared from *Escherichia coli* B41 grown in the presence of [^{3}H]thymidine (20 Ci/mmol) had a specific radioactivity of about 70000 dis. $min^{-1} \cdot \mu g^{-1}$. Alkylation with methyl methanesulfonate and partial depurination were carried out as previously described [4]. The alkylated [^{3}H]DNA contained about 140 methyl groups and the alkylated-depurinated [^{3}H]DNA about 50 AP sites/10^3 nucleotides. The labelled double-stranded DNA (untreated, alkylated or alkylated-depurinated) was dissolved in 0.1 NaCl/Cit, 20 mM $MgCl_2$, pH 8.0, to a concentration of 20 $\mu g \cdot ml^{-1}$.

Acid-depurinated [^{3}H]DNA. To 1 vol. of untreated [^{3}H]DNA solution (200 $\mu g \cdot ml^{-1}$) was added 1 vol. of 0.5 M sodium acetate buffer, pH 3.7. After an 120-h incubation at 37 °C, it was dialyzed against 0.1 NaCl/Cit, 20 mM $MgCl_2$, pH 8.0. The depurinated [^{3}H]DNA contained 13 AP sites/10^3 nucleotides.

Nicked [^{3}H]DNA. [^{3}H]DNA (16 µg) in 800 µl of 0.1 NaCl/Cit, 20 mM $MgCl_2$, pH 8.0, was mixed with 80 µl 0.15 M KCl, 0.05 % bovine serum albumin, 0.01 % 2-mercaptoethanol containing 0.04 unit of pancreatic deoxyribonuclease (Boehringer) and incubated 15 min at 37 °C. The nuclease was then inactivated by heating 10 min at 77 °C to obtain double-stranded nicked [^{3}H]DNA. This substrate contained about 11 breaks/10^3 nucleotides.

Nicked [^{3}H]DNA with 5′-[^{32}P]phosphate Ends. Nicked [^{3}H]DNA prepared as in the previous section was successively

treated with alkaline phosphatase and polynucleotide 5′-hydroxyl-kinase together with [γ-^{32}P]ATP following the method of Weiss et al. [5]. After phenol deproteinization and precipitation with ethanol, the double-labelled DNA (70000 dis. $^{3}H\ min^{-1} \cdot \mu g^{-1}$; 200000 dis. $^{32}P\ min^{-1} \cdot \mu g^{-1}$) was dissolved in 10 mM Tris/HCl, 0.1 mM EDTA, pH 8.0.

Doubly-labelled Uracil-containing Poly(dA-dT). The details of the preparation were given previously [3,6]. The polymer was labelled with ^{3}H in the adenine residues and with ^{32}P 5′ to the uracil residues.

Enzyme Assay

To 20 µl of alkylated-depurinated [^{3}H]DNA (0.4 µg) solution were added 20 µl of enzyme preparation. After a 15-min incubation at 37 °C, the tubes were cooled in ice, then 100 µl NaCl/Cit, pH 7.0, containing 200 µg of unlabelled DNA and 860 µl 5.8 % perchloric acid were added successively. The tubes were shaken vigorously, then centrifuged at $12000 \times g$ for 10 min; 500 µl from the supernatants were used to measure the acid-soluble radioactivity. Break frequencies were calculated from the acid-soluble fractions (unpublished). Results were corrected for controls without enzyme which always contained very few breaks.

One unit (U) of AP endodeoxyribonuclease is the enzyme activity which hydrolyzes 1 pmol phosphoester bonds near AP sites/min in the conditions described above [7].

Assay for Protein

Protein concentration was measured by the micromethod of Peterson [8] using bovine serum albumin as standard.

Chromatography on Bio-Gel P-100

A column (1.6×95 cm) of Bio-Gel P-100 was equilibrated with 50 mM Tris/HCl, 0.1 M ammonium sulfate, pH 7.5. The void volume (V_0) was measured with blue dextran, the total volume (V_t) with riboflavin, and the column was calibrated with four proteins of known molecular weights: cytochrome *c* (12500), chymotrypsinogen (25000), ovalbumin (45000) and bovine serum albumin (67000). Each of these proteins or the enzyme was dissolved in 2 ml of buffer and applied on the column. The elution was carried out at a rate of $8\ ml \cdot h^{-1}$; 3.1-ml fractions were collected and the absorbance at 280 nm or the activity on alkylated-depurinated [^{3}H]DNA was measured to determine the elution volume (V_e).

Acid-Solubility

This always refers to solubility in 5 % perchloric acid.

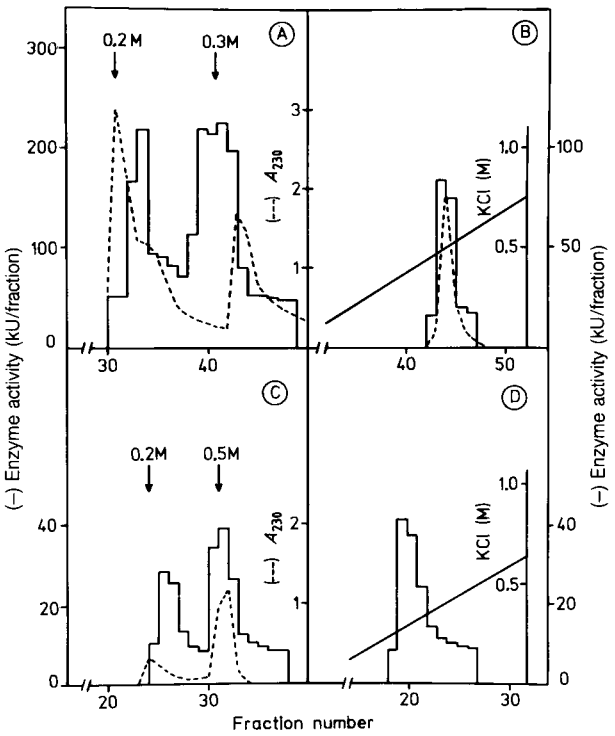

Fig. 1. *Purification of the 0.3 M isozyme of AP endodeoxyribonuclease.* (A) Hydroxyapatite chromatography. The 0.5 M KCl extract from rat liver chromatin was poured on a column (1.6×15 cm) of hydroxyapatite. Elution was carried out with 100 ml of 0.2 M and 100 ml of 0.3 M potassium phosphate, pH 6.80; 9-ml fractions were collected. The arrows indicate when the eluent emerging from the column had reached 0.2 M and 0.3 M respectively (refractory index determination). (B) Phosphocellulose chromatography. Fractions 39–43 from the hydroxyapatite chromatography were pooled and dialyzed against buffer B. The solution was poured on a column (1×8 cm) of phosphocellulose. After washing with 40 ml of buffer B, the column was eluted with 100 ml of a 0–1 M KCl linear gradient in buffer B; 3.1-ml fractions were collected. The straight line represents the gradient as it emerged from the column. (C) Heparin-Sepharose chromatography. Fractions 44 and 45 from the phosphocellulose chromatography were pooled and dialyzed against 10 mM phosphate, pH 7.30. The solution was poured on a column (1×6 cm) of heparin-Sepharose. After a 65-ml washing, the column was eluted by steps with 20 ml each of 0.2 M, 0.5 M, and 1.0 M KCl in the same buffer; 3-ml fractions were collected. The arrows indicate when the eluent emerging from the column had reached the indicated KCl concentrations (refractory index determination). (D) Alkylated-depurinated DNA-cellulose chromatography. Fractions 31–33 from the heparin-Sepharose chromatography were pooled and dialyzed against buffer D. The protein solution was poured on a column (1×10 cm) of alkylated-depurinated DNA-cellulose. After washing with 25 ml of buffer D, the column was eluted with 100 ml of a 0–1 M KCl linear gradient in buffer D; 3-ml fractions were collected. The straight line represents the gradient as it emerged from the column. An aliquot of each fraction, after dilution with buffer E, was used to measure the activity on alkylated-depurinated [^{3}H]DNA. The values are the minimum since the determinations were not always done under maximal conditions

EXPERIMENTS AND RESULTS

Preparation of Chromatin and Purification of the 0.3 M Isozyme

All the solutions used to prepare the chromatin extract contained 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The nuclei were isolated from rat liver and chromatin prepared from the purified nuclei as described by Thibodeau and Verly [1]. The chromatin was dissociated with heparin-Ultrogel (LKB) and the heparin-Ultrogel-DNA-protein complex was extracted twice with 0.5 M KCl, 10 mM potassium phosphate, 10 mM Tris/HCl, pH 8.0, as described in the miniprint supplement (see also [9]). The pooled extracts (Prep I; 237 ml) contained 100 mg protein and 3610 kU of AP endodeoxyribonuclease activity.

Prep I was directly poured onto a column (1.6×15 cm) of hydroxyapatite equilibrated with 5 mM potassium phosphate, pH 6.8. The column was eluted by steps with 100 ml of 0.2 M and 100 ml of 0.3 M potassium phosphate, pH 6.8, at a rate of $15\ ml \cdot h^{-1}$. Absorbance at 230 nm and activity on alkylated-depurinated [^{3}H]DNA were measured on the collected 9-ml

Table 1. *Purification of the 0.3 M isozyme of AP endodeoxyribonuclease from rat liver chromatin*
Prep I refers to the proteins extracted from the heparin-Ultrogel-DNA-protein complex with 0.5 M KCl; Prep II is the second peak of the hydroxyapatite chromatography (0.3 M isozyme) after dialysis; Prep III is the active fractions from the phosphocellulose chromatography after dialysis; Prep IV is the second peak from the heparin-Sepharose chromatography after dialysis; Prep V is the single peak from alkylated-depurinated DNA-cellulose chromatography. The 0.3 M isozyme activity in Prep I has been calculated taking into account the ratio of 0.2 M to 0.3 M isozyme found in the hydroxyapatite chromatographies and assuming the same yields for the two isozymes

Prep	Enzyme activity		Yield	Protein	Specific activity		Purification
	total	0.3 M isozyme			total	0.3 M isozyme	
	kU		%	mg	kU/mg protein		-fold
I	3610	2635	100	99.8	36.2	26	1
II		1374	52	7.9		174	7
III		620	23	1.8		344	13
IV		546	21	0.94		560	22
V		496	19	0.32		1550	59

fractions. Fig. 1A shows that two peaks of activity were separated: the 0.2 M isozyme represented 27 % and the 0.3 M isozyme 73 % of the recovered activity; the overall yield was 52 %. Only the 0.3 M isozyme was processed further.

Fraction 39 – 43 from the hydroxyapatite chromatography were pooled and dialyzed over night against 4 l of buffer B (15 mM potassium phosphate, 0.1 mM EDTA, 0.1 mM 2-mercaptoethanol, pH 7.2). A small precipitate which formed in the dialysis bag was discarded by centrifugation. The 47-ml supernatant (Prep II) containing 7.9 mg protein and 1370 kU enzyme was poured at a rate of 20 ml · h^{-1} onto a column (1 × 8 cm) of phosphocellulose equilibrated in buffer B. After washing with 40 ml of the same buffer, the column was eluted, at a rate of 8 ml · h^{-1}, with 100 ml of a 0 – 1 M KCl linear gradient in buffer B. Absorbance at 230 nm and activity on alkylated-depurinated [^{3}H]DNA were measured on each 3.1-ml fraction. Fig. 1B shows a protein peak eluted at 0.5 M KCl corresponding to the single peak of enzyme activity.

Fractions 44 and 45 from the previous chromatography were pooled and dialyzed three times against 1 l of buffer C (10 mM potassium phosphate, pH 7.3). The small precipitate which appeared in the dialysis bag was discarded by centrifugation. The 6-ml supernatant (Prep III), containing 1.8 mg protein and 620 kU enzyme, was poured onto a column (1 × 6 cm) of heparin-Sepharose (Pharmacia) equilibrated with buffer C. The column was washed with 65 ml of the same buffer, then eluted by steps with 20 ml of 0.2 M, 20 ml of 0.5 M and 20 ml of 1.0 M KCl in buffer C at a rate of 8 ml · h^{-1}. Absorbance at 230 nm and activity on alkylated-depurinated [^{3}H]DNA were measured on each 3-ml fraction. Fig. 1C shows two peaks: a minor one eluted with 0.2 M potassium phosphate which is an unexplained accident since it was never observed in any other heparin-Sepharose chromatography, and the major one eluted with 0.5 M potassium phosphate.

Fractions 31 – 33 from the heparin-Sepharose chromatography were pooled and dialyzed three times against 1 l of buffer D (20 mM Tris/HCl, 1 mM EDTA, 0.2 mM dithiothreitol, pH 8.1, containing 10 % glycerol). The dialyzed solution (Prep IV; 6.3 ml), containing 0.94 mg protein and 546 kU enzyme, was poured onto a column (1 × 10 cm) of alkylated-depurinated DNA cellulose equilibrated with buffer D. After washing with 25 ml of the same buffer, the column was eluted with 100 ml of a 0 – 1 M KCl linear gradient in buffer D at a rate of 8 ml · h^{-1}. The proteins were undetectable at 230 nm. The activity on alkylated-depurinated [^{3}H]DNA was measured on the 3-ml fractions. Fig. 1D shows a single peak of enzyme activity, eluted with 0.3 M KCl, containing 496 kU of AP endodeoxyribonuclease activity (Prep V). After addition of 0.023 % bovine serum albumin (Boehringer), Prep V was shared between several tubes which were stored at 4 °C.

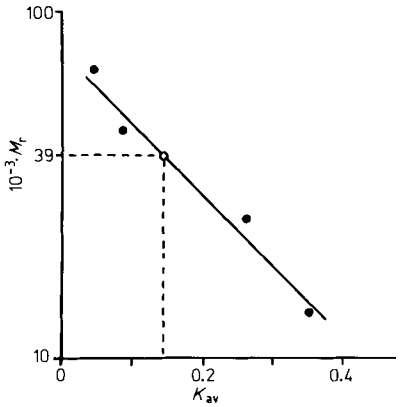

Fig. 2. *Molecular weight of the 0.3 M isozyme of AP endodeoxyribonuclease.* A BioGel P-100 column (1.6 × 95 cm) was calibrated with four proteins of known molecular weights (cytochrome c = 12500; chymotrypsinogen = 25000; ovalbumin = 45000; bovine serum albumin = 67000). The K_{av} values were calculated from the elution volumes in each case and plotted against the logarithm of the molecular weights (black dots). The K_{av} for the AP endodeoxyribonuclease was also calculated and its position (open dot) on the best straight line passing through the black dots indicated a molecular weight of about 39000

The results of the four purification steps are summarized in Table 1.

Molecular Weight of the Enzyme

Prep V was 10-fold diluted with the buffer used to equilibrate the BioGel P-100 column. A 2-ml aliquot of the diluted enzyme solution was placed on the column and the elution was carried out as described in Materials and Methods; the AP endodeoxyribonuclease activity was measured on each fraction to determine V_e. In Fig. 2, $K_{av} = (V_e - V_0)/(V_t - V_0)$ is plotted against the logarithm of the molecular weight; the value for the enzyme as measured on the straight line obtained from the standards gives a molecular weight of 39000.

Specificity of the Endonuclease Activity

Prep V was diluted 30-fold with buffer E (50 mM Tris/HCl, 50 mM NaCl, pH 8.0). Aliquots (20 µl) of the diluted enzyme solution (37 units) or the same volumes of buffer E were mixed with 20 µl of solutions containing 0.4 µg of untreated, alkylated or alkylated-depurinated [^{3}H]DNA and incubated at 37 °C for 15 min before measuring the acid-soluble fraction. In half of the tubes, the incubation with or without enzyme was followed by addition of 40 µl of 0.4 M NaOH and a further 15-min incubation at 37 °C (a treatment known to produce a break near each AP site [4]), before measuring the acid-soluble fraction. Break frequencies were calculated from the acid-soluble fractions. Table 2 shows that the enzyme had no action on untreated DNA; its action on alkylated or alkylated-depurinated DNA was restricted to alkali-labile sites, i.e. AP sites, since a preliminary incubation with a large amount of enzyme did not significantly increase the break frequency given by an exposure to NaOH.

Table 2. *Action of the 0.3* M *isozyme of AP endodeoxyribonuclease on untreated, alkylated or alkylated-depurinated DNA*
[^{3}H]DNA (0.4 µg) (untreated, alkylated or alkylated-depurinated) was incubated for 15 min at 37 °C with or without 37 units of Prep V in 40 µl of solution. The acid-soluble fraction was measured either directly or after a 15-min treatment at 37 °C with NaOH (0.2 M final concentration). Break frequencies were calculated from the acid-soluble fractions

DNA	Enzyme	NaOH	Breaks/10^3 nucleotides
Untreated	–	–	3.8
	+	–	3.8
	–	+	4.5
	+	+	5.3
Alkylated	–	–	5.3
	+	–	18.5
	–	+	18.0
	+	+	19.0
Alkylated-depurinated	–	–	7.0
	+	–	47.0
	–	+	54.0
	+	+	55.0

Absence of Exonuclease Activity

Prep V was 40-fold diluted with buffer E. Two different experiments were carried out.

In the first experiment, 50-µl aliquots of the diluted enzyme solution were mixed with 50 µl of nicked [^{3}H]DNA solution (1.0 µg) and incubated for various times up to 90 min. The measured acid-soluble radioactivity was not greater than in the controls incubated without enzyme for the same time.

In the second experiment, 25-µl aliquots of the diluted enzyme solution were mixed with 25 µl of nicked [^{3}H]DNA labelled with ^{32}P at the 5′-phosphate ends (3.8 µg) solution and incubated for various times up to 90 min. The ^{32}P released in the acid-soluble fraction was the same as in the controls incubated without enzyme for the same time.

Position of the Phosphodiester Bridge Cut by the 0.3 M Isozyme of AP Endodeoxyribonuclease

Uracil-containing poly(dA-dT) labelled with ^{3}H in the adenine residues and with ^{32}P 5′ to the uracil residues was used [6]. The details of the procedure which was followed can be found in [3]. Table 3 (unpublished results of P. Colson) shows that a treatment with uracil-DNA glycosylase or AP endodeoxyribonuclease alone did not enable the alkaline phosphatase to release much of the ^{32}P. When however, there were successive treatments with uracil-DNA glycosylase and AP endodeoxyribonuclease followed by an incubation with phosphatase (sample 5), there was a large increase in the amount of ^{32}P not adsorbed to Norit (i.e. inorganic phosphate) whereas the acid-soluble ^{3}H was still very low. The results indicate that the phosphodiester bridge which is cut by the 0.3 M isozyme of AP endodeoxyribonuclease is the immediate neighbour of the AP site on its 5′ side.

In sample 6, the incubation with the AP endodeoxyribonuclease was followed by NaOH treatment instead of phosphatase treatment. NaOH is known to hydrolyze a phosphoester bond 3′ to the AP site by a mechanism of β-elimination, leaving a 3′-hydroxyl on the base-free deoxyribose [10]. The release of ^{32}P unadsorbed on Norit indicates that the AP endodeoxyribonuclease cut 5′ to the AP site leaving a 5′-[^{32}P]phosphate end and that the NaOH treatment subsequently released the radioactive phosphate bound to the base-free deoxyribose.

Table 3. *Localization of the phosphodiester bridge cut by the 0.3 M isozyme of AP endodeoxyribonuclease*
Uracil-containing poly(dA-dT), labelled with ^{3}H in the adenine residues and with ^{32}P in the phosphate on the 5′ side of the uracil residues, was successively treated with *E. coli* uracil-DNA glycosylase, 0.3 M isozyme of AP endodeoxyribonuclease and alkaline phosphatase. The acid-soluble ^{3}H and ^{32}P radioactivities and the ^{32}P not adsorbed to Norit were measured; the results are expressed as percentages of the total ^{3}H or ^{32}P radioactivity in the sample. Controls were carried out where one or several enzymes were omitted. With sample 6, the phosphatase treatment was replaced by an incubation with 0.2 M NaOH (final concentration) for 30 min at 65 °C (P. Colson, unpublished)

Sample	Enzyme treatment			NaOH	Acid-soluble		Unadsorbed ^{32}P
	uracil-DNA glycosylase	AP endodeoxyribonuclease	phosphatase		^{3}H	^{32}P	
					% total		
1	–	–	–	–	2.0	6.8	4.8
2	–	+	+	–	2.4	8.3	8.2
3	+	–	+	–	2.7	12.1	8.7
4	+	+	–	–	2.5	39	29
5	+	+	+	–	2.9		52
6	+	+	–	+	9.5	56	44

Table 4. *Nature of the 3′ ends left by the hydrolysis of a phosphoester bond near the AP sites by the 0.3 M isozyme of AP endodeoxyribonuclease*
Acid-depurinated [^{3}H]DNA was incubated with the AP endodeoxyribonuclease (AP endo) and the solution was subsequently heated at 100 °C for 2 min. After another treatment with alkaline phosphatase (Pase), the denaturated DNA was treated with snake venom phosphodiesterase (PDE) (see details in the text). Controls were carried out in which one or several enzymes were omitted. The table indicates the acid-soluble fraction (as a percentage of total radioactivity) found after different times of the incubation with the snake venom phosphodiesterase

Sample	Enzyme treatment			Acid-soluble fraction after					
	AP endo	Pase	PDE	3 min	7 min	10 min	20 min	30 min	60 min
				% total					
1	+	−	−	1.1					1.3
2	−	+	+			6.8	7.6	9.0	11.6
3	−	−	+			5.7	9.1	10.5	10.8
4	+	+	+	19	19	24	27	28	31
5	+	−	+	18	24	26	29	30	35
4 − 2						17	19	19	19
5 − 3						20	20	20	24

The high value of the ^{32}P unadsorbed by Norit when there was no alkaline phosphatase during the last incubation at pH 8.8 and 65 °C (sample 4) is probably due to some β-elimination occurring in those conditions.

Nature of the Ends Resulting from the Hydrolysis of a Phosphoester Bond 5′ to the AP Site

Acid-depurinated [^{3}H]DNA (10 µg) in 200 µl of 0.1 NaCl/Cit, 20 mM $MgCl_2$, pH 8.0, and 200 µl of Prep V (400 enzyme units) in buffer E were incubated at 37 °C for 30 min; the mixture was then heated 2 min at 100 °C to inactivate the enzyme and denature the DNA. Addition of alkaline phosphatase (Sigma; 4 units) in 100 µl of 0.3 M glycine/NaOH buffer, pH 8.8, was followed by a 30-min incubation at 37 °C. To 20-µl aliquots of this solution were added 20 µl of NaCl/Cit, 10 mM $MgCl_2$, pH 7.0, containing 0.2 mU of snake venom phosphodiesterase (Sigma); the acid-soluble radioactivity was measured after different times of incubation at 37 °C. Controls without one or several enzymes were carried out in parallel. Table 4 shows that a treatment with the AP endodeoxyribonuclease increased the rate of appearance of the radioactivity in the acid-soluble fraction. This increase was not changed by previous treatment with alkaline phosphatase, indicating that the breaks introduced by the AP endodeoxyribonuclease are limited by 3′-hydroxyl ends.

In another experiment, it was shown that the labelling of the 5′ ends with ^{32}P from [γ-^{32}P]ATP using polynucleotide 5′-hydroxylkinase depended on a previous treatment with alkaline phosphatase. When there was no incubation of the acid-depurinated DNA with the AP endodeoxyribonuclease, but a treatment with alkaline phosphatase followed by incubation with [γ-^{32}P]ATP and kinase, the incorporated ^{32}P amounted to 26400 dis. min^{-1} µg DNA^{-1}. When there was an incubation with the AP endodeoxyribonuclease, this value rose to 204000 dis · min^{-1} · µg^{-1}. If, however, the phosphatase treatment was omitted, only 76300 dis · ^{32}P min^{-1} µg^{-1} were incorporated (G. Zocchi, unpublished). These results show that the breaks produced by the AP endodeoxyribonuclease are limited by 5′-phosphate ends.

Miscellaneous Properties

Prep V was diluted 400-fold in buffer E, 0.2 % bovine serum albumin. Portions of 300 µl of the diluted enzyme were prewarmed at 40 °C or 45 °C for various times; 20-µl aliquots were taken, mixed with 20 µl of alkylated-depurinated [^{3}H]DNA (0.4 µg) and incubated 10 min at 30 °C before measuring the acid-soluble radioactivities from which the break frequencies were calculated. The logarithm of the number of breaks due to the enzyme activity against time of prewarming yielded straight lines, the slopes of which indicated half-lives of about 2 h at 40 °C and 90 s at 45 °C.

Portions of 25 mM Tris, 25 mM Hepes, 10 mM $MgCl_2$ solution were adjusted to pH values from 6.5 to 9.5 by addition of HCl or KOH. Aliquots of Prep V were 800-fold diluted with these buffers. The diluted enzyme (20 µl) and the alkylated-depurinated [^{3}H]DNA solution (20 µl) were incubated for 15 min at 37 °C before measuring the acid-soluble radioactivity. The optimum pH is around 8.0.

Prep V was diluted 100-fold with buffer D; 20 µl of the diluted enzyme solution were mixed with 20 µl of alkylated-depurinated [^{3}H]DNA solution and 10 µl of water containing NaCl to produce a final concentration varying between 0 and 500 mM. The mixture was incubated at 37 °C for 15 min before measuring the acid-soluble radioactivity. The optimum NaCl concentration was found between 30 mM and 50 mM; the enzyme is eightfold inhibited at 500 mM NaCl.

The alkylated-depurinated [^{3}H]DNA solution was dialyzed successively against 10 mM EDTA and 0.1 NaCl/Cit, pH 8.0. Aliquots of 20 µl were mixed with 20 µl of Prep V 200-fold diluted with buffer E (2U enzyme) and 10 µl of water containing $MgCl_2$, $MnCl_2$ or $CaCl_2$ to reach final concentrations from 0 to 100 mM, then incubated 15 min at 37 °C. The enzyme had little action without divalent cations. The optimum concentration for $MgCl_2$ was between 5 mM and 10 mM; Mg^{2+} can be replaced by Mn^{2+}, but not by Ca^{2+}.

Some AP Sites in Methylated-Depurinated DNA Are Resistant to the AP Endodeoxyribonuclease

[^{3}H]DNA alkylated for 1 h with 0.3 M methyl methanesulfonate, then partially depurinated by heating 6 h at 50 °C [4], was used in three parallel experiments.

In the first one, equal volumes (350 µl) of diluted Prep V (15 U) and methylated-depurinated [^{3}H]DNA solutions were mixed and incubated at 37 °C. Aliquots were taken at different times to follow the appearance of breaks. The break frequency was found to reach a plateau after 60 min when the enzyme had nicked the DNA strands near 70 % of the alkali-labile sites

Table 5. *Protection of AP sites in alkylated-depurinated DNA against the AP endodeoxyribonuclease is not due to enzyme inactivation*
A mixture of diluted Prep V (180 µl; 140 units) and solution of [^{3}H]DNA alkylated with 0.3 M methyl methanesulfonate and partially depurinated (180 µl; 3.6 µg) was incubated at 37 °C. Aliquots (40 µl) were taken at different times to measure the acid-soluble fraction and the break frequency was calculated (as breaks/10^3 nucleotides). After 40 min, another portion of the same alkylated-depurinated [^{3}H]DNA solution diluted twice (200 µl; 2 µg) was added and the incubation carried on at the same temperature; aliquots (40 µl) were again taken to follow the break frequency. The indicated amount of DNA is that before taking the aliquot. The last column shows the increase in break frequency during the preceding interval. NaOH treatment of the alkylated-depurinated [^{3}H]DNA yielded 72 breaks/10^3 nucleotides

Time	DNA	Break frequency	Increase
min	µg		
0	3.6	6	—
5	3.2	36	30
10	2.8	43	7
15	2.4	45	2
20	2.0	47	2
30	1.6	47	0
40	1.2	47	0
40	2.8	13[a]	—
45	2.4	27	14
50	2.0	35	12
60	1.6	39	4
70	1.2	43	4
80	0.8	44	1

[a] Calculated value to take account of the dilution resulting from the second addition of alkylated-depurinated [^{3}H]DNA.

(results not shown). In the second experiment, starting with the same mixture also incubated at 37 °C, two additions of more concentrated enzyme solution (30 µl; 15 U) were made, one after 30 min, the other after 60 min. These supplementations of fresh enzyme did not change the plateau value of break frequency (not shown). In the third experiment, again starting with the same mixture incubated at 37 °C, another amount of methylated-depurinated [^{3}H]DNA was added after 40 min. The enzyme resumed its activity until it had produced a break near about 70 % of the alkali-labile sites (Table 5). The results of the second and third experiments indicate that is it not because the AP endodeoxyribonuclease was inactivated that it was unable to introduce nicks near more than 70 % of the alkali-labile sites.

In another set of experiments, different levels of [^{3}H]DNA methylation were carried out before a 6-h partial depurination at 50 °C. Equal volumes (270 µl) of diluted Prep V (62 U) and methylated-depurinated [^{3}H]DNA solutions were incubated together at 37 °C and the break frequency was followed. Fig. 3 shows that, when 0.075 M methyl methanesulfate was used for methylation, the AP endodeoxyribonuclease produced breaks near close to 100 % of the alkali-labile sites; when the concentration was raised, this percentage decreased: 80 % for 0.15 M, 70 % for 0.30 M and 35 % for 0.60 M methyl methanesulfonate. It was checked that a treatment with NaOH at the end of the incubation with the enzyme yielded, in every case, a break frequency equal to that obtained with the substrate before incubation with the enzyme. Fig. 4 shows a fair proportionality between the fraction of AP sites protected against the enzyme action and the concentration of methyl methanesulfonate used to alkylate the DNA.

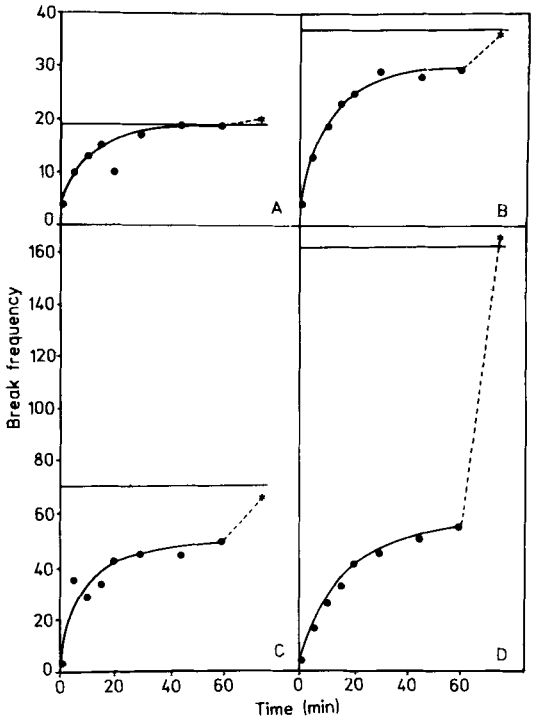

Fig. 3. *Activity of AP endodeoxyribonuclease on partially depurinated DNAs which had been submitted to different levels of methylation.* [^{3}H]DNA was alkylated either with 0.075 M (A), 0.15 M (B), 0.30 M (C), or 0.60 M (D) methyl methanesulfonate for 1 h at 37 °C, then heated for 6 h at 50 °C. Equal volumes of methylated-depurinated [^{3}H]DNA and AP endodeoxyribonuclease solutions were incubated at 37 °C; aliquots were taken at different times (min), the acid-soluble fraction was measured and the break frequency was calculated; it is given in number of breaks per 1000 nucleotides. The horizontal lines indicate break frequencies when the substrates were treated with NaOH; dashed lines leading to asterisks show the increase in break frequencies when the incubation with the enzyme was followed by treatment with NaOH

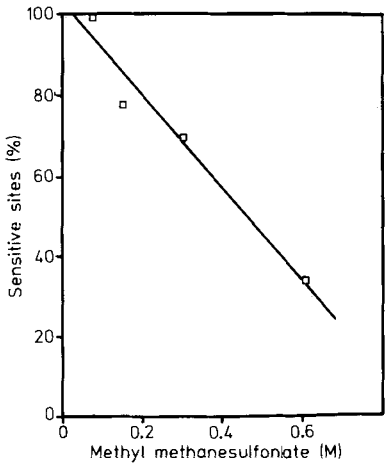

Fig. 4. *Relationship between fraction of AP sites sensitive to the AP endodeoxyribonuclease and the level of methylation.* The percentage of AP sites sensitive to the AP endodeoxyribonuclease as found in experiments described in Fig. 3 is plotted against the methyl methanesulfonate concentration used to alkylate the DNA

DISCUSSION

When the proteins of rat liver chromatin are chromatographed on hydroxyapatite, two AP endodeoxyribonucleases are separated: one is eluted with 0.2 M, the other with 0.3 M

potassium phosphate. They are called the 0.2 M and 0.3 M isozymes respectively. Our colleagues have shown (see miniprint supplement) that most of the 0.2 M isozyme is an artifact and that the important AP endodeoxyribonuclease in the chromatin of the living cells is the 0.3 M isozyme. Unhappily, we had purified previously the 0.2 M isozyme [2]. In this paper, we present the purification of the 0.3 M isozyme and a study of some of its properties.

The 0.3 M isozyme of AP endodeoxyribonuclease of rat liver chromatin was partially purified by successive chromatographies on hydroxyapatite, phosphocellulose, heparin-Sepharose and alkylated-depurinated DNA-cellulose.

The endonucleolytic activity of the final preparation (Prep V) was restricted to the AP sites; there was no action on intact DNA strands or on alkylated sites. No associated exonuclease activity could be demonstrated. A very sensitive assay for 5′–3′ exonuclease working from nicks on double-stranded DNA was used since Bose et al. [11] could not separate such an exonucleolytic activity from the AP endodeoxyribonuclease they found in whole extracts from human lymphoblasts. No such activity was present in Prep V.

The 0.3 M isozyme cuts the phosphodiester bridge which is the immediate neighbour of the AP site on its 5′ side leaving 3′-hydroxyl and 5′-phosphate ends. The same phosphoester bond is thus hydrolyzed by the 0.2 M [3] and 0.3 M isozymes; so far, we have been unable to find an AP endodeoxyribonuclease in rat liver chromatin cutting 3′ to the AP sites as Mosbaugh and Linn [12] did in whole extracts of human fibroblasts.

Our colleagues have determined a molecular weight of 42000 for the 0.3 M AP endodeoxyribonuclease isozyme of rat liver chromatin (see miniprint supplement); our value is closer 39000, but with a large error so that the two results are not really different. The 0.3 M isozyme has an optimum pH around 8.0; it needs Mg^{2+} or Mn^{2+} for activity; it is inhibited by high ionic strength and is rather thermolabile.

The question of the parenthood of the 0.2 M and 0.3 M isozymes of the AP endodeoxyribonuclease in rat liver chromatin extracts remains open. We presently know that they have approximately the same molecular weight (see miniprint supplement) and that they hydrolyze the same phosphoester bond 5′ to the AP site.

We started to investigate how the AP endodeoxyribonuclease places itself on the DNA near the AP site to hydrolyze a phosphoester bond. Like Springgate and Liu [13], we found that some alkali-labile sites in alkylated-depurinated DNA were not sensitive to the enzyme. However, when a low level of methylation was used (1 h; 0.075 M methyl methanesulfonate) before the standard partial depurination procedure, the AP endodeoxyribonuclease made a break next to nearly all the alkali-labile sites; this suggests that the alkali-labile sites in alkylated-depurinated DNA are indeed only AP sites. But, when the level of methylation increased and then the same partial depurination step was used, some AP sites could no longer be substrates for the AP endodeoxyribonuclease, probably because methyl groups hindered the formation of the enzyme-substrate complex.

Methyl methanesulfonate mainly alkylates the N-7 of guanine (82 % of total methylation) and the N-3 of adenine (10 % of total methylation) [14]. 3-Methyladenine is lost faster than 7-methylguanine; Paquette et al. [4] showed that the loss of methylated purines from methylated DNA during heating the aqueous solution at 50 °C had two components: a quickly released methylated adduct which had been practically eliminated from DNA after 2 h and a much slower one which had only decreased by 20 – 30 % after 24 h. Thus it seems that, after the standard partial depurination procedure (6 h at 50 °C), nearly all the remaining methyl groups are in 7-methylguanine residues. Calculation indicates that, after alkylation with 0.3 M methyl methanesulfonate followed by standard partial depurination, the alkylated-depurinated DNA has approximately 10 residues of 7-methylguanine/100 nucleotides.

The methyl group attached to the N-7 of a guanine residue sits in the large groove of the DNA molecule. The conformation of the DNA molecule at an AP site is unknown; in particular, we ignore whether there is a rotation of the base-free deoxyribose to expose the aldehyde function on the surface of the macromolecule. We also ignore any change in this structure which is brought about by the AP endodeoxyribonuclease when it forms an enzyme-substrate complex. If, to approach the AP site, the enzyme must have close contact with neighbouring bases in the large groove, the presence of methyl groups might prevent its action.

Ignoring base sequence and using only statistical averages, when 0.3 M methyl methanesulfonate is used for alkylation before the standard partial depurination, the probability of having a 7-methylguanine in any nucleotide position is around 10 %. Supposing that AP sites and 7-methylguanine residues have independent random distributions, this level of methylation would protect 10 % of the AP sites against the AP endodeoxyribonuclease if the contact with a single base in the large groove were critical. But the protection determined experimentally is 30 %; it thus seems that the enzyme must establish a contact with three bases in the large groove in order to cleave the phosphoester bond 5′ to the AP site. We cannot guess the position of these three bases. Are they on the same strand as the AP site? Are they only on one side (3′ or 5′) or rather straddling the AP site? Are some critical bases on the opposite strand?

If the presence of a methyl group in the large groove on any of three specified bases in the neighbourhood of the AP site stops the action of the AP endodeoxyribonuclease, the fraction of protected sites must be a linear function of the 7-methylguanine abundance in the methylated-depurinated DNA. Fig. 4 shows that there is indeed a fair proportionality between the fraction of AP sites protected against the enzyme action and the concentration of methyl methanesulfonate used to alkylate the DNA. In this discussion, no account was taken of the possible role of minor methyl adducts; further studies will be needed to ascertain that their action is negligible.

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Supplementary Material

to

APURINIC/APYRIMIDINIC ENDODEOXYRIBONUCLEASE OF RAT LIVER CHROMATIN

by Suzanne Bricteux-Grégoire, Yvette Habraken and Walter G. Verly

Summary

Non-histone proteins extracted from rat liver chromatin contain two different AP endonucleases : one is eluted with 0.2 M, the other with 0.3 M potassium phosphate. The two isozymes have molecular weights of about 42 000. The 0.3 M species seems to be the true chromatin enzyme as it exists in the living cell. Most of the 0.2 M species is an artifact; it is not known whether it derives from the 0.3 M species or not.

Introduction

AP endodeoxyribonucleases are DNA repair enzymes which hydrolyze a phosphoester bond near AP (apurinic or apyrimidinic) sites.

Chromatography of an extract of rat liver chromatin on DEAE-cellulose separates two AP endodeoxyribonucleases. The species which emerges at the lower ionic strength has been completely purified; it is eluted from hydroxyapatite with 0.2 M K phosphate whereas the other species is eluted with 0.3 M K phosphate [1]. The two isozymes can readily be distinguished by analyzing the chromatin extract directly on hydroxyapatite; their ratio varies from one experiment to another and it would be interesting to know whether the two AP endodeoxyribonucleases are chemically related.

A serine-protease, inactive in native chromatin, attacks histones and non-histone proteins after dissociation of the nucleoprotein [2]. One wonders whether the two AP endodeoxyribonucleases are present in the chromatin of the living cell or if one of the two species in the chromatin extract might not be a proteolytic degradation product. The following experiments were carried out to answer these questions.

Materials and Methods

1. Preparation of the chromatin extract

The rat liver is perfused in situ with ice-cold Locke's solution; after removal, it is homogenized in buffer A and the nuclei are collected by centrifugation; the nuclei are purified in a discontinuous sucrose gradient in diluted buffer A. The purified nuclei are made to swell in water, the nuclear membranes are disrupted in a tight-fitting Potter-Elvehjem apparatus and the chromatin is collected by centrifugation. The composition of buffer A and the details of the successive procedures can be found in [3].

Chromatin is sheared in a tight-fitting Potter-Elvehjem apparatus in buffer B (10 mM K phosphate, 10 mM Tris.HCl, pH 8.0) to have about 300 µg DNA per ml. Heparin-Ultrogel (LKB) is then added to have the same weight of heparin and chromatin DNA. The mixture is stirred for 30 min at 0°; the heparin-Ultrogel-DNA-protein complex is collected by centrifugation, the pellet is washed with buffer B, then extracted twice with 0.5 M KCl in buffer B. The pooled extracts contain no DNA and very little histones.

2. Hydroxyapatite chromatography

The chromatin extract is dialyzed against 5 mM K phosphate, pH 6.8. The solution is poured onto a 1.6 x 23 cm column of hydroxyapatite equilibrated with 5 mM K phosphate, pH 6.8. The elution is carried out, at a rate of 10 ml.h^{-1} and 2°, with 120-ml portions of K phosphate, pH 6.8 buffers, successively at 0.1 M, 0.2 M and 0.3 M concentrations. The AP endodeoxyribonuclease activity is measured on the collected 10-ml fractions.

3. Filtration on Sephadex G-75

A 1.6 x 90 cm column of Sephadex G-75 is equilibrated with buffer C (50 mM Tris.HCl, 0.3 M NaCl, 0.1 mM EDTA, 0.02 % NaN_3, pH 8.0). The protein in 2 ml of buffer C is applied on the column and the elution is carried out with the same buffer at a rate of 10 ml.h^{-1}; 3.2-ml fractions are collected. The void volume is measured with blue dextran and the column is calibrated with bovine serum albumin (67 000), ovalbumin (45 000), chymotrypsin (25 000), myoglobin (18 000) and cytochrome c (12 500).

4. Enzyme assay and unit

The AP endodeoxyribonuclease is assayed as described in [1] and the number of breaks introduced by the enzyme near AP sites is calculated. One unit of enzyme activity hydrolyzes phosphoester bonds near 1 pmol AP sites per min [4].

Experiments and Results

The two AP endodeoxyribonucleases in chromatin proteins from rat liver will be called 0.2 M and 0.3 M isozymes depending on the K phosphate concentration necessary to elute them from the hydroxyapatite column.

1. Effect of storage on the isozyme activities

Chromatin proteins in buffer B, 0.5 M KCl, containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF) were divided in three parts. One part was dialyzed overnight against 5 mM K phosphate, pH 6.8, and chromatographed the next day on hydroxyapatite. The second and third parts, kept at 2° for 6 and 10 days respectively, were subsequently treated in an identical way.

Table I shows that : (1) the enzyme activity, before the chromatography, increased with time of storage; (2) the activity eluted with 0.3 M K phosphate decreased whereas the activity eluted with 0.2 M K phosphate increased with storage time; (3) the overall yields of the hydroxyapatite chromatographies were around 40 %.

The fractions of the 0.3 M peaks of the three chromatographies were pooled and, after dialysis against 5 mM K phosphate, pH 6.8, rechromatographed on hydroxyapatite : no 0.2 M isozyme was found, all the activity was eluted with 0.3 M K phosphate. Conversely, when the fractions of the 0.2 M peaks were similarly treated, all the activity was eluted with 0.2 M K phosphate and none appeared in the 0.3 M eluate. The yields of these second chromatographies were excellent, exceeding 90 %.

2. Effect of PMSF on the isozyme relative activities

The livers of two rats were cut in small pieces which were distributed in two stocks from which chromatin proteins were prepared. For one preparation, the solutions did not contain PMSF; for the other, 0.1 mM PMSF was added in buffer A and 0.5 mM PMSF in the solution in which the swollen nuclei were sheared. Table I shows that, without PMSF, the proportion of 0.2 M isozyme found in the hydroxyapatite chromatography was 52 % (see also figure 1,A); it decreased to 30 % when PMSF was used.

When, in another experiment, 0.5 mM PMSF was moreover added to the 0.5 M KCl used to extract the complex resulting from the dissociation of chromatin with heparin-Ultrogel, the proportion of 0.2 M isozyme further decreased. The results were highly reproducible : 16 % in duplicates prepared from pieces of the same livers.

3. Conditions to have the lowest percentage of 0.2 M isozyme

0.5 mM PMSF was added to all solutions, including Locke's fluid used to perfuse the liver. The dialysis before the chromatography was eliminated when it was observed that 0.5 M KCl in the chromatin extract did not prevent the adsorption of the AP endodeoxyribonucleases on hydroxyapatite. Finally, to speed up the chromatography, a shorter column was used (1.6 x 13 cm), the first washing with 0.1 M K phosphate was suppressed and the volumes of 0.2 M and 0.3 M K phosphate were reduced to 60 ml while keeping the elution rate at 10 ml.h^{-1}. In these conditions, the 0.2 M and 0.3 M isozymes found in the hydroxyapatite chromatography represented 10 and 90 % respectively of the recovered activity (figure 1,B; Table I). These modifications did not however increase the low yield of the hydroxyapatite chromatography.

4. Molecular weight determinations

The pooled fractions of the two AP endodeoxyribonuclease peaks emerging from the hydroxyapatite column were separately dialyzed against buffer C and concentrated on Millipore filters; 2-ml portions were then analyzed on Sephadex G-75 as described in Materials and Methods. The elution volumes of both isozymes indicated molecular weights of about 42 000. A mixture of the two enzymes chromatographed on the same column gave a single symmetrical peak.

Discussion

The proportions of the 0.2 M and 0.3 M AP endodeoxyribonuclease isozymes separated by hydroxyapatite chromatography of the chromatin proteins prepared from rat liver vary widely depending on the preparation method that is used. Might one isozyme be a degradation product of the other ?

A conversion could not be demonstrated directly since, after a first chromatography on hydroxyapatite, the two isozymes were stable : on rechromatography, the 0.2 M isozyme was completely eluted with 0.2 M K phosphate and the 0.3 M isozyme was eluted only with 0.3 M K phosphate.

The situation is more complex with the chromatin extract : the total AP endodeoxyribonuclease activity increases with the time of storage at 2°. Several interpretations can be considered : disappearance of an inhibitor; formation of an enzyme from an inactive precursor; transformation of an isozyme into the other associated with an increased V_{max}. The distribution of the isozymes after different times of storage is remarkable : the 0.3 M activity decreases with time whereas the 0.2 M activity increases, suggesting that the 0.3 M isozyme might be transformed into the 0.2 M isozyme. But there is a great loss of the total activity during the chromatography (the overall yield is only about 40 %) so that no final conclusion can be reached. It looks however as if some 0.2 M isozyme might be a degradation product.

Table I : Isozyme activities and yields of the hydroxyapatite chromatographies

PMSF	delay (days)	dialysis	HA column	Enzyme activity			Recovery		yield
				P.C.	0.2 M	0.3 M	0.2 M	0.3 M	
				units	units	units	%	%	%
buffer B	0	+	L	48 300	7 000	15 300	31	69	46
	6	+	L	54 000	9 300	14 600	39	61	44
	12	+	L	60 000	10 200	12 900	44	56	39
buffer B	0	+	L	65 300	6 900	16 500	30	70	36
none	0	+	L	87 000	17 000	15 600	52	48	37
buffer B + 0.5 M KCl	0	+	L	29 600	1 400	7 400	16	84	30
	0	+	L	30 000	1 480	7 750	16	84	30
all the solutions	0	-	S	84 000	3 200	26 600	10	90	35

The nuclei are prepared in buffer A; chromatin is dissociated in buffer B; the heparin-Ultrogel-DNA-protein complex is extracted with 0.5 M KCl (in buffer B); the extract is dialyzed or not before a chromatography on a 1.6 x 23 cm (L) or 1.6 x 13 cm (S) column of hydroxyapatite. PMSF is added to part or all of the solutions used since the perfusion of the liver until the elution of the hydroxyapatite column; the Table specifically indicates the solutions containing 0.5 mM PMSF. Different experiments are separated by horizontal lines. Details can be found in the text.

P.C. are the chromatin proteins after dialysis (when there is one) and before the chromatography; 0.2 M and 0.3 M are the corresponding isozymes separated by the chromatography. The results are given in enzyme units and percents of the recovered activity. The last column indicates the overall yield of the chromatography.

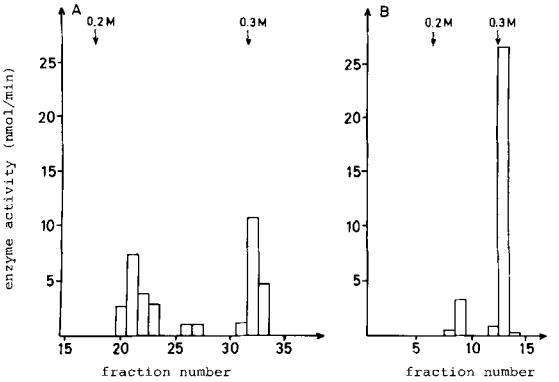

Figure 1 : Hydroxyapatite chromatographies.

In both cases, the elution rate was 10 ml/min. The graphs give the enzyme activity (nmol/min) per 10-ml fraction. The arrows indicate when the eluent emerging from the column reached the indicated concentration (refractory index determination).

A. The chromatin proteins were prepared without PMSF; the solution was dialyzed before the hydroxyapatite chromatography performed as described in Materials and Methods, using 120-ml portions of 0.1 M, 0.2 M and 0.3 M K phosphate. The fraction of recovered activity in the 0.2 M isozyme is 52 %.

B. All the solutions contained 0.5 mM PMSF, the dialysis step was suppressed and the chromatography made on a shorter column, skipping the 0.1 M K phosphate elution and decreasing to 60 ml the volumes of the 0.2 M and 0.3 M eluents. The fraction of the recovered activity in the 0.2 M isozyme is 10 %.

The absence of PMSF, a serine-protease inhibitor, in the solutions used to prepare the chromatin proteins also increases the amount of the 0.2 M isozyme. To have the lowest proportion of this species, PMSF must be present in all the solutions and the time necessary to reach the end of the hydroxyapatite chromatography must be kept to a minimum. This observation further strengthens the conclusion that most of the 0.2 M isozyme is a degradation product. It cannot however be concluded that the 0.2 M isozyme is just an artifact and that the 0.3 M isozyme is the only chromatin AP endodeoxyribonuclease in the living cell since we were unable to bring to zero the former species; the lowest figure ever obtained was 5 %. Some 0.2 M isozyme might thus be present in native chromatin, but there is no doubt that the 0.3 M isozyme is the major AP endodeoxyribonuclease of rat liver chromatin and not the 0.2 M as we previously thought [1].

The inhibition by PMSF of the appearance of the 0.2 M isozyme suggests that this AP endodeoxyribonuclease might result from proteolysis, but it is difficult to decide whether it comes from an inactive precursor or from the 0.3 M isozyme. The similarity of the molecular weights of the two isozymes (about 42 000) seems to reject the second hypothesis unless the proteolytic clivage releases only a very small peptide. But it cannot be excluded that the degradation leading to the 0.2 M isozyme is not a proteolysis but another modification like a dephosphorylation; the proteolysis might have an indirect effect, for instance the activation of a phosphoprotein phosphatase.

We must wait for the complete purification of the two AP endodeoxyribonuclease isozymes found in extracts of rat liver chromatin and the comparison of their primary structures to know whether there is any relationship between them and to learn the nature of this relationship.

Acknowledgments

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