J. Biochem. 128, 883–889 (2000)

Polynucleotide:Adenosine Glycosidase Is the Sole Activity of Ribosome-Inactivating Proteins on DNA

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Received July 31, 2000; accepted September 18, 2000

Polynucleotide: adenosine glycosidases (PNAG) are a class of plant and bacterial enzymes commonly known as ribosome-inactivating proteins (RIP). They are presently classified as rRNA *N*-glycosidases in the enzyme nomenclature [EC 3.2.2.22]. Several activities on nucleic acids, other than depurination, have been attributed to PNAG: in particular modifications induced in circular plasmids, including linearisation and topological changes, and cleavage of guanidinic residues. Here we describe a chromatographic procedure to obtain nuclease-free PNAG by dye-chromatography onto Procion Red derivatized Sepharose®. Highly purified enzymes depurinate extensively pBR322 circular, supercoiled DNA at neutral pH and exhibit neither DNase nor DNA glycolyase activities, do not cause topological changes, and adenine is the only base released from DNA and rRNA, even at very high enzyme concentrations. A scanning force microscopy (SFM) study of pBR322 treated with saporin-S6 confirmed that (i) this PNAG binds extensively to the plasmid, (ii) the distribution of the bound saporin-S6 molecules along the DNA chain is markedly variable, (iii) plasmids already digested with saporin-S6 do not appear fragmented or topologically modified. The observations here described demonstrate that polynucleotide:adenosine glycosidase is the sole enzymatic activity of the four ribosome-inactivating proteins gelonin, momordin I, pokeweed antiviral protein from seeds and saporin-S6. These proteins belong to different families, suggesting that the findings here described may be generalized to all PNAG.

Key words: atomic force microscopy, polynucleotide:adenosine glycosidase, Red-Sepharose®, ribosome-inactivating protein, scanning force microscopy.

Gelonin, momordin I, PAP-S, and saporin-S6 are enzymes belonging to the class of PNAG (*1*), plant, and bacterial (*2*) enzymes known as ribosome-inactivating proteins (RIP), due to their property of irreversibly damaging eukaryotic ribosomes (reviewed in Refs. *3* and *4*). They can be divided into two groups: type 1, consisting of a single polypeptide chain; and type 2, which has a lectin subunit linked to the enzymatic chain. PNAG have been previously identified as rRNA *N*-glycosidases in the enzyme classification [EC 3.2.2.22] because the larger rRNA molecule in intact eukaryotic ribosomes was the first substrate to be identified (*4*). These enzymes remove a single adenine (A^{4324}) from 28 S rRNA in rat ribosomes, or its equivalent in other species, located at a stem and loop region highly conserved throughout evolution. Since the early studies on their mechanism of action in whole cells, the existence of substrate(s) other than ribosomes was suspected, and evidence has increasingly accumulated. Among pertinent observations, the most significant are: (i) the pathology at cellular level in animals intoxicated with PNAG is very different from that produced by other ribosome-blocking inhibitors of protein synthesis, such as cycloheximide (*5*); (ii) a C-terminal deletion mutant recombinant of PAP retained its antiviral property but did not show depurination of the ribosomes of the host tobacco plant (*6*); (iii) gelonin from *Gelonium multiflorum* inhibited the growth of *Plasmodium falciparum*, one of the malaria parasites, in erythrocytes, causing the disappearance of the 6-kb mitochondrial DNA (*7*), (iv) several forms of PAP depurinate HIV genomic RNA and have anti-HIV activity, whereas ricin does not depurinate HIV RNA and has no anti-HIV activity, whilst being as active as PAP on host cell ribosomes (*8*), and finally (v) PAP can inhibit translation by a mechanism other than ribosome depurination, by recognizing the cap structure and specifically depurinating the capped RNAs (*9*).

DNA has been proposed as an additional target of PNAG, although its importance and the mechanism by which PNAG act on DNA are still unsettled questions. All ribosome-inactivating proteins tested depurinated deoxyadenosine residues of double-stranded DNA (*1*), at least at acidic pH, which was found to be optimal for PNAG activity (*10*, *11*). On the other hand, in the past few years various activities on single- and double-stranded, circular and linear DNA, other than removal of adenine, have been described by several authors (*7*, *12–20*). It was reported that trichosanthin (*12*) cleaved supercoiled double-stranded

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Abbreviations: hsDNA, DNA from herring sperm; PAP(-S), pokeweed antiviral protein (from seeds); PNAG, polynucleotide:adenosine glycosidase; RIP, ribosome-inactivating protein; SFM, scanning force microscopy.

DNA into relaxed and linear forms *in vitro*, observation later extended to dianthin, gelonin, luffin, cinnamomin, and saporin (*17–20*). Gelonin treatment of erythrocytes infected with *Plasmodium falciparum* (*7*) eliminated the 6-kb mitochondrial DNA of the protozoa. Recent reports indicate that gelonin, PAP and ricin damage single-stranded DNA by removing adenines and cleaving at the resulting abasic sites (*21*). Some of these findings are questionable because of two observations: (i) these proteins display a relatively simple three-dimensional structure with a single putative reactive site (reviewed in Ref. *22*), and thus the presence of two distinct enzymatic activities is unlikely; and (ii) at least in two cases, contaminant nucleases [DNase from ricin (*23*) and RNase from momordin II (*24*)] have been detected and removed from apparently pure PNAG. Furthermore, recombinant forms of ricin A chain and PAP, with full depurinating activity on ribosomes, did not have any detectable DNase activity (*23*). Additional indirect evidence has been presented that an isoform of PAP extracted from leaves, as well as other natural type 1 RIP, are contaminated by nucleases, and that DNase activity in PAP preparations can be selectively inhibited (*23*). On the other hand, very recently other authors reported that PAP cleaves double-stranded supercoiled DNA using the same active site required to depurinate rRNA (*25*), and that momordin has intrinsic RNase activity (*26*).

The determination of the polynucleotide:adenosine glycosidase activity on DNA is relevant to the studies on the mechanism of action at the molecular level, to studies on the antiviral (anti-HIV included) activity, and to the studies on the induction of apoptosis by PNAG (*27–33*). Furthermore, the removal of traces of nucleases may be important to the pharmaceutical preparations of these proteins intended for the use in the experimental therapy of neoplasia as immunotoxins (*34*).

Here we describe a simple dye-chromatography procedure, which produces highly purified PNAG devoid of contaminant traces of nucleases. Biochemical, electrophoretic, and morphological proofs (atomic force microscopy, alias scanning force microscopy) are given for the unicity of the polynucleotide:adenosine glycosidase activity of gelonin, momordin I, PAP-S, and saporin-S6 purified by dye-chromatography: they depurinate extensively circular double-stranded supercoiled pBR322 DNA at neutral pH, without cleaving single or double strands in DNA, and adenine is the sole base released.

EXPERIMENTAL PROCEDURES

Materials—From the confusing variety of denominations applied to the same PNAG (alias RIP) at different times, here we utilize the nomenclature originally adopted as referred in Ref. *3*: gelonin, momordin I, PAP-S, and saporin-S6 for the most abundant isoforms from the seeds of *Gelonium multiflorum* (Euphorbiaceae), *Momordica charantia* (Cucurbitaceae), *Phytolacca americana* (Phytolaccaceae), and *Saponaria officinalis* (Caryophyllaceae), respectively. Standard preparations of these enzymes were obtained following the method described in Ref. *35* for gelonin, PAP-S, and saporin-S6 and following (*24*) in the case of momordin I.

Poly(A), poly(U), rRNA from *Escherichia coli*, tRNA from *Saccharomyces cerevisiae*, pUC19 DNA, *Pst*I and *Eco*RI restriction enzymes were from Boehringer GmbH, Mannheim, Germany. DNA from herring sperm (hsDNA) from Sigma (St. Louis, MO, USA) was mechanically sheared and made RNA-free by treatment with DNase-free RNase A (Boehringer) for 2.5 h at 37°C. DNA was then repeatedly precipitated in ethanol to remove the enzyme. Bases used as standard were from Sigma. Materials and equipment for low-pressure chromatography, including Procion Red derivatized Sepharose® (Red-Sepharose®), were from Pharmacia (Uppsala, Sweden). All other reagents were of analytical or molecular biology grade and, when possible, RNase-free. Water was Milli-Q (Millipore). Chloroacetaldehyde was prepared according to Ref. *36*.

Dye-Chromatography onto Red-Sepharose®—Nuclease activities were removed from gelonin, momordin I, PAP-S, and saporin-S6 with an FPLC® apparatus essentially as described in Ref. *24* for momordin II. Briefly: all operations were at room temperature unless otherwise stated; standard protein preparations were dissolved at 2 mg/ml in 20 mM Tris/HCl, pH 8.0, applied to a column (25 cm height × 1.6 cm diameter) of Red Sepharose® washed following manufacturer's instructions and then equilibrated in 20 mM Tris/HCl, pH 8.0. Bound proteins were eluted with a gradient of NaCl (0–1 M) in the same buffer (total volume 500 ml). Elution was followed by absorbance at 280 nm. Fractions were collected as indicated in Fig. 1, dialysed extensively against water at 8°C, concentrated (Amicon Centriprep®) to approx. 1 mg/ml [by extinction coefficients at 280 nm (*3*)], then stored in aliquots at –20°C.

Preparation of pBR322 DNA—pBR322 plasmid was grown in DH5α cells and extracted as described in Ref. *37*. The crude plasmid preparation was then subjected to RNase treatment (100 μg/ml for 1 h at 37°C). Plasmidic DNA was purified by gel filtration onto Sephadex-G50 (31 cm height × 2.6 cm diameter) equilibrated and eluted at room temperature in 10 mM Tris/HCl, pH 8.0, containing 1 mM EDTA and 140 mM NaCl. Elution was followed by absorbance at 260 nm. Fractions containing plasmidic DNA were pooled, ethanol-precipitated, resuspended in 10 mM Tris/HCl, pH 8.0 containing 1 mM EDTA, and stored in aliquots at –20°C. When appropriate, pBR322 and pUC19 DNA were linearized with restriction enzymes *Pst*I and *Eco*RI, respectively, (1 U/μg of DNA) for 16 h at 37°C. Restriction enzymes were removed by phenol extraction and DNA was recovered by ethanol precipitation.

Electrophoresis—Electrophoresis of plasmidic DNA was in 1% agar in 40 mM Tris-acetate containing 1 mM EDTA, pH 8.0. Gels were stained with ethidium-bromide, and destained with mQ grade water. Pictures with UV illumination were taken either on film or by electronic imaging on a Kodak apparatus.

Determination of Nuclease Activity—Direct quantitative measurement of nuclease activities was attempted on poly(A), hsDNA, tRNA, and poly(U) essentially as described for RNase in Ref. *24*, a modification of the method described in Ref. *38*. Measurements of activity could be performed quantitatively only on poly(U), due to the presence of high amounts of adenine released by the highly active enzymes from adenosine-containing substrates. DNA strand–breaking activity was evaluated semi-quantitatively by gel electrophoresis on pBR322 plasmidic DNA, before and after dye-chromatography of the enzymes. Experimental conditions are described in the legend to Fig. 2.

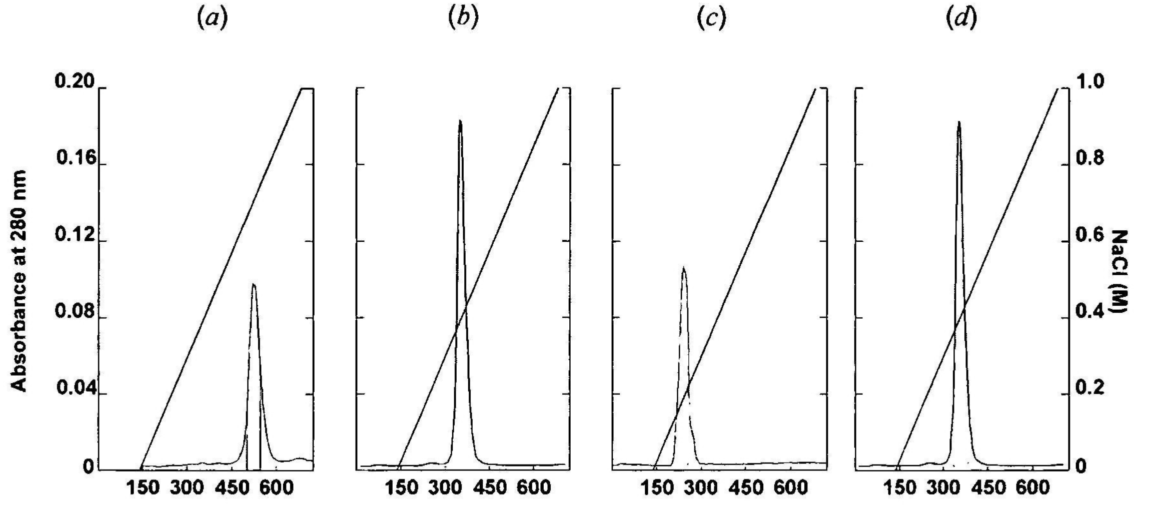

Fig. 1. **Dye-chromatography on Red-Sepharose® of polynucleotide:adenosine glycosidases.** (a) Gelonin, (b) momordin I, (c) PAP-S, (d) saporin-S6. Shading indicates pooled purified fractions. Other experimental conditions are described in the "EXPERIMENTAL PROCEDURES."

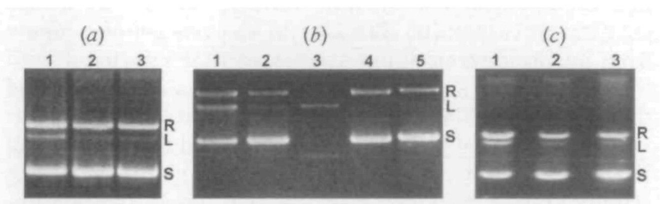

Fig. 2. **pBR322 DNA strand cleaving activity before and after dye-chromatography.** PNAG reaction conditions were: 1.5 pmol of pBR322 (corresponding to 3,150 pmol of adenosine residues potential substrate), a large excess of PNAG (150 pmol), 10 mM HEPES, pH 7.0, 2 mM $MgCl_2$, 10 mM NaCl, in a final volume of 37.5 µl, for 2 h at 37°C. Samples were subjected to phenol extraction to remove bound protein, and to electrophoresis. Other experimental conditions are described in "EXPERIMENTAL PROCEDURES." L stands for linearized form of pBR322, S for supercoiled form, and R relaxed form. (a) Momordin I: lane 1, before dye-chromatography; lane 2, after dye-chromatography; lane 3, circular pBR322 (as control). (b) PAP-S: lane 1, before dye-chromatography; lane 2, after dye-chromatography; saporin-S6: lane 3, before dye-chromatography; lane 4, after dye-chromatography; lane 5, circular pBR322 (as control). (c) Gelonin: lane 1, before dye-chromatography; lane 2, after dye-chromatography; lane 3, circular pBR322 (as control).

Determination of Polynucleotide:Adenosine Glycosidase Activity—Polynucleotide:adenosine glycosidase activity was determined by measuring adenine released from the macromolecular substrate. Acidic conditions were found to be optimal for PNAG activity (*10, 11,* and authors, unpublished observations), and thus were chosen for determination of PNAG activity on hsDNA before and after purification procedures. Neutral conditions for treatment of pBR322 DNA were chosen as optimal for analysis with atomic force microscopy and for conformational analysis. Enzymic reaction conditions are detailed in the legends to the specific figures and table.

Released adenine was measured after separation by HPLC. Reaction was stopped in ice by addition of 200 µl of cold buffer BA (50 mM Na acetate, pH 4.0, 20 mM KCl), then the reaction products were applied to sample preparation columns (Bond Elut® NH_2, from Varian, Harbor City CA, USA) previously equilibrated at 2°C with buffer BA. Adenine was eluted in 400 µl of buffer BA by briefly centrifuging at 200 ×*g*, whereas phosphate-containing nucleic acids were retained. Released adenine was measured after derivatisation to its fluorescent derivative ethenoadenine as previously described (*24*) according to Ref. *39*.

Determination of the Release of Bases Other than Adenine—Adenine, guanine, cytosine, thymine, and uracyl were determined by HPLC from samples containing 30 pmol of enzyme (300 pmol in the case of momordin I), 10 µg of substrate, corresponding to 30,000 pmol of nucleosidic residues, in 50 mM Na acetate, pH 4.0, 100 mM KCl in a final volume of 50 µl, at 37°C for 2 h. Controls were run without enzyme, and with standard bases added. Samples were prepared as described above for adenine determination and applied in portions of 100 µl to an ODS(3) column equilibrated in the same buffer described for ethenoadenine measurement. Gradient elution was slightly different (from 0 to 5 min 0% methanol, from 5 min to 20 min from 0 to 20% methanol, from 20 to 30 min from 20 to 40% methanol in the same buffer). Elution was followed by absorbance at 260 nm.

Scanning Force Microscopy (SFM) Analysis—For the SFM analysis of pBR322 depurinated by PNAG samples from the experiment described in Fig. 3 (containing 10 pmol of saporin-S6/pmol of pBR322) were diluted to a concentration of 1 µg/ml (as DNA) in 4 mM HEPES, pH 7.5, 10 mM NaCl, 2 mM $MgCl_2$. To study the complexes between PNAG and pBR322, pBR322 circular double-stranded DNA molecules were diluted as described above, saporin-S6 was then added to a stoichiometric ratio of 10:1 of protein to DNA molecules, and the mixture was incubated on ice for 2 min. Samples (10–15 µl) were deposited onto a 1.5 cm^2 disc of freshly cleaved ruby mica (Mica New York, New York, NY, USA) and left to adsorb for 2–3 min. The disc was then rinsed with a few ml of water and dried in a gentle flow of nitrogen gas. Tapping Mode SFM images were collected on a NanoScope IIIa system equipped with a multimode head

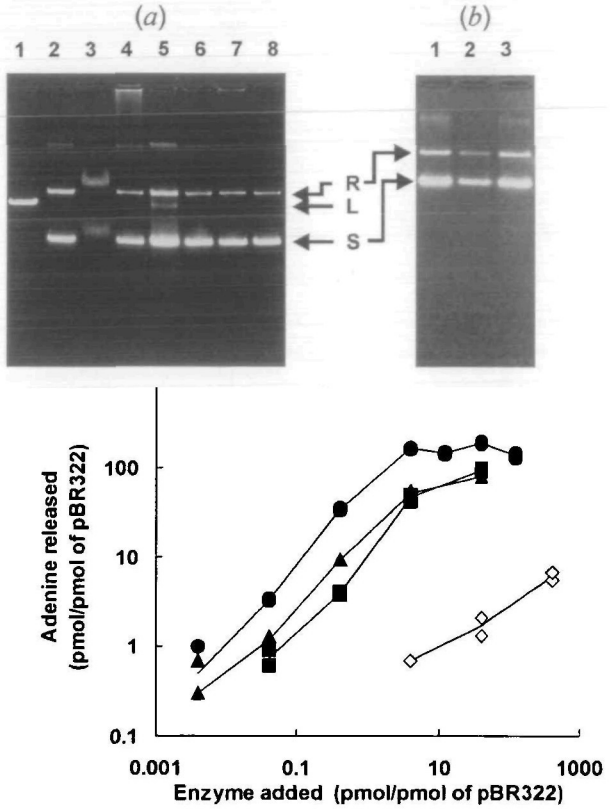

Fig. 3. **PNAG activity on supercoiled pBR322 plasmid.** PNAG reaction conditions were: 3 pmol of pBR322 (corresponding to 6,300 pmol of adenosine residues potential substrate), the appropriate amount of PNAG as indicated below, 10 mM HEPES, pH 7.0, 0.5 mM $MgCl_2$, in a final volume of 75 μl for 30 min at 37°C. Reaction was stopped and adenine measured as described in "EXPERIMENTAL PROCEDURES" on portions of the samples (1/3). The remaining sample portions were subjected to phenol extraction, when indicated, and to electrophoresis in 1% agar. L stands for linearized, R for relaxed, and S for supercoiled form. (a) Lane 1, *Pst*I linearized pBR322 (as control). circular pBR322: lane 2, no PNAG (control); lane 3, treated with saporin-S6 (120 pmol/pmol of pBR322); lane 4, as lane 3 but phenol extracted; lane 5 , treated with gelonin (40 pmol/pmol of pBR322); lane 6, as lane 5 but phenol extracted, lane 7, treated with PAP-S (40 pmol/pmol of pBR322); lane 8, as lane 7 but phenol extracted. (b) Lane I, circular pBR322 (as control); lane 2, treated with momordin I (400 pmol/pmol of pBR322); lane 3, as lane 2 but phenol extracted. (c) Adenine released from circular pBR322 (■) gelonin, (◇) momordin I, (▲) PAP-S, (●) Saporin-S6. Other experimental conditions are described in "EXPERIMENTAL PROCEDURES."

and a type-E piezoelectric scanner (Digital Instruments, S. Barbara, CA, USA) using silicon probes for non-contact/tapping-mode applications. The images were collected at ambient humidity with a sampling density of 15–30 nm^2/pixel at a linear speed of 10–15 μm/s.

RESULTS

DNA-Strand Cleaving Activity of Standard Preparations of Ribosome-Inactivating Proteins—Standard preparations of gelonin, momordin I, PAP-S, and saporin-S6 gave a single band on SDS-PAGE and a single peak on reverse-phase C4 chromatography, results compatible with a purity of >98–99%, beyond the sensitivity limits of the analytical procedures employed (results not shown). Direct measurement of nuclease activity [as successfully performed with momordin II, even though it was heavily contaminated and had an intrinsic low adenine release activity (*24*)], also gave negative results, due also to the release of adenine from adenosine-containing substrates that interfered with the assay (data not shown). Semi-quantitative analysis of the effect of standard preparations of PNAG on pBR322 at very high enzyme concentration, confirmed the presence of DNA strand–cleaving activity. The supercoiled DNA strands (Fig. 2, S bands) were cleaved giving rise to a linearized form (Fig. 2, L bands) and to relaxed forms (Fig. 2, R bands). In the case of saporin-S6 a new band of lower molecular mass appeared. The cleaving of pBR322 was undetectable at enzyme concentrations up to that producing maximal depurination (data not shown). Aspecific DNase activity was absent, as demonstrated by the digestion pattern shown in Fig. 2 and by the lack of appreciable modifications when the heterogeneous hsDNA was used as substrate (data not shown). The behaviour of standard preparations of PNAG thus substantially confirmed the reports by other authors (*12–20*).

TABLE I. **Activities of PNAG prior and after Red-Sepharose®.** DNA strand–cleaving activity was evaluated semi-quantitatively by gel electrophoresis (see Fig. 2) and adenine release was evaluated on hsDNA as a substrate. Reaction conditions for hsDNA were: 20 μg of substrate, corresponding to 15,000 pmol of adenosinic residues, in the presence of 50 mM sodium acetate buffer, pH 4.0, in a final volume of 50 μl, at 30°C for 40 min. Incubated samples contained 0.1 pmol of PNAG (10 pmol in the case of momordin I, which is far less active on DNA); experiments were run in duplicate; other experimental conditions are described in "EXPERIMENTAL PROCEDURE."

		Dye-chromatography purification			
		no	yes	no	yes
		DNA strand-cleaving activity		PNAG activity	
				Adenine released (pmol)	
PNAG:	Gelonin	+	–	6–8	9–13
	Momordin I	+/–	–	17–22	15–16
	PAP-S	++	–	13–16	18–22
	Saporin-S6	++++	–	18–22	16–20

Highly Purified PNAG Do Not Show Nuclease Activity While Retaining Their Deadenylating Activity—Dye chromatography on Red-Sepharose® was applied to purify four different type 1 PNAG. Elution profiles are given in Fig. 1. Single, fairly broad peaks, indicating a complex interaction with the chromatographic medium, were eluted together with only faint traces of other components. Peaks were collected as indicated in Fig. 1, in which the leading and trailing components were discarded. Gelonin, momordin I, PAP-S, and saporin-S6 were then assayed for nuclease and PNAG activities. In all cases DNA strand–cleaving activity was lost, as indicated in Fig. 2, whereas PNAG activity on hsDNA in optimal conditions at pH 4.0 (*1*, *10*) was fully retained (Table I). No activity was detected on poly(U) (results not shown).

Dye-purified PNAG were then assayed for deadenylating activity, also at neutral pH, in conditions similar to those employed by authors describing nuclease and topological activities, and that could be utilized in experiments with scanning force microscopy. All proteins depurinated deoxyadenosine residues in double-stranded circular pBR322

DNA at pH 7.0, in good agreement with previous observations at acidic pH with hsDNA. As shown in Fig. 3, saporin-S6 was the most active, releasing one mol of adenine per mol of substrate (as whole plasmid) at 0.2 nM enzyme concentration (equivalent to a PNAG/pBR322 mol ratio of 0.01). The same activity was obtained with approx. 2 nM PAP-S or gelonin. Much higher concentrations of momordin I (600 nM) were needed to obtain the same depurination, in agreement with observations at pH 4.0 with the other major isoenzyme from *Momordica charantia* seeds, momordin II (*24*), and all RIPs from the family of Cucurbitaceae (*1*). Differences in activity on DNA do not correlate to ribosomal inhibitory activity (*3*). It is noteworthy that with the most active proteins, namely, gelonin, PAP-S and saporin-S6, the depurination reached a plateau when about 10% of the adenine present in the substrate had been released. Furthermore, as shown in Fig. 4, no difference in depurination extent was observed when saporin-S6 was assayed at different concentrations on circular supercoiled or on *Pst*I linearized pBR322.

DNA Deadenylated by Highly Purified PNAG Is Neither Nicked nor Cut—Figure 4a shows the electrophoretic behaviour of *Pst*I linearized pBR322 samples treated with saporin-S6 and the corresponding deadenylating activity. The DNA band remained unchanged when up to 10 pmol of enzyme/pmol of pBR322 was added (lane 6). Even in the presence of heavily deadenylated DNA, no fragmentation of this linearized DNA was observed. When up to 120 pmol of enzyme/pmol of pBR322 was added (lanes 7 and 8), very large smears of low mobility appeared, most likely due to the highly variable distribution of the saporin-S6 molecules that were still bound along the DNA chains, as is well depicted by the SFM picture of Fig. 5b. In fact, when the sample was treated with phenol in order to remove the bound protein from the DNA chains (see Fig. 4b) the smear was lost and the electrophoretic pattern of the deadenylated DNA was revealed to be perfectly coincident with that of the starting control, which was not treated with saporin-S6. A similar behavior was found with native circular supercoiled pBR322, as shown in Fig. 3 (lanes 3–4), and partially also in the case of gelonin (lanes 5–6). This behavior was confirmed by experiments conducted with pUC19, another plasmid utilized by some authors to show DNA-modifying activities of PNAG. This plasmidic DNA was also depurinated extensively at neutral pH by saporin-S6, and double-strand cuts were not produced even at high PNAG

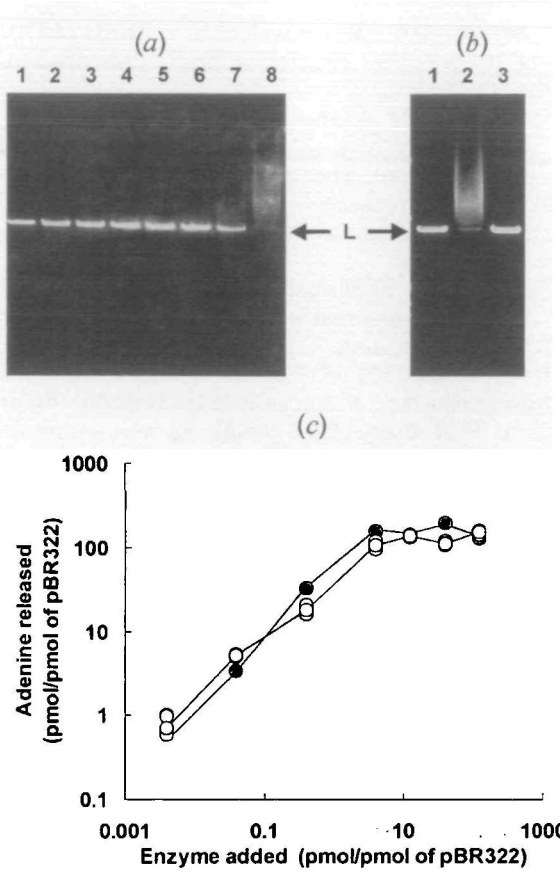

Fig. 4. **PNAG activity of purified saporin-S6 on linearized pBR322 plasmid.** PNAG reaction conditions were: 3 pmol of pBR322 (corresponding to 6,300 pmol of adenosine residues potential substrate), the appropriate amount of saporin-S6 as indicated below, 10 mM HEPES, pH 7.0, 0.5 mM $MgCl_2$, in a final volume of 75 μl for 30 min at 37°C. Reaction was stopped and adenine measured on portions of the samples (1/3) as described in "EXPERIMENTAL PROCEDURES." The remaining sample portions were subjected to phenol extraction, when indicated, and to electrophoresis in 1% agar. L stands for linearized. (a) *Pst*I linearized pBR322 incubated with saporin-S6: lane 1, no saporin-S6; lane 2, 0.004 pmol/pmol of pBR322; lane 3, 0.04 pmol; lane 4, 0.4 pmol; lane 5, 4 pmol; lane 6, 12 pmol; lane 7, 40 pmol; lane 8, 120 pmol. (b) *Pst*I linearized pBR322 incubated with saporin-S6: lane 1, no saporin-S6; lane 2, 120 pmol/pmol of pBR322; lane 3, as lane 2 after phenol extraction. (c) Adenine released from pBR322: (○) linearized, (●) circular supercoiled (as control). Other experimental conditions are described in "EXPERIMENTAL PROCEDURES."

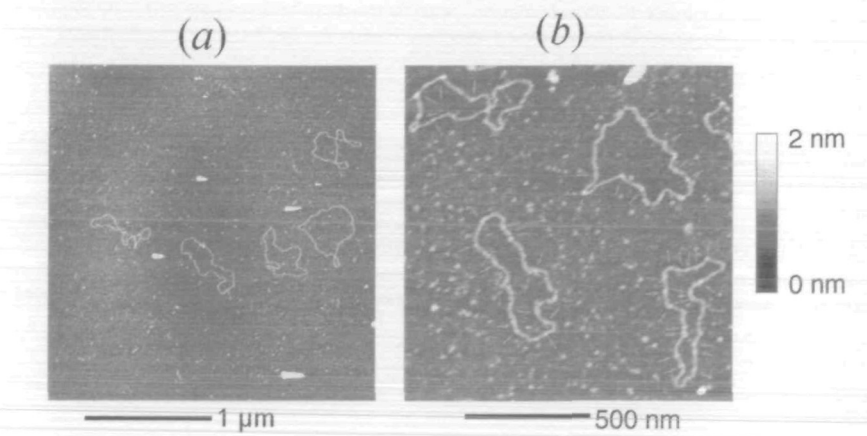

Fig. 5. **Tapping-mode SFM images of pBR322 treated with saporin-S6.** The height of the features on the surface in the SFM images are coded in shades of gray according to the attached look-up tables. (a) pBR322 treated with saporin-S6 (10 pmol) from experiment described in Fig. 4. Protein was removed by phenol extraction and deposition on mica layer was as described in the Experimental section. (b) Double stranded DNA complexes of saporin-S6 with pBR322 plasmid displaying a low number of proteins per DNA molecule. Deposition was in 4 mM HEPES, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.4.

concentrations 200 nM, equivalent to a PNAG/pUC19 mol ratio of 10) on either circular or *Eco*RI linearized pUC19 (results not shown).

Only Adenine Is Released from DNA and rRNA—Adenine was the only base released from hsDNA and rRNA (results not shown). The sensitivity limits of the assay would show the presence of bases at less than one hundredth the concentration of the adenine effectively released in the experiment.

SFM Analysis—SFM analysis of pBR322 after treatment with saporin-S6, in conditions in which each molecule of pBR322 was depurinated at an average of 100 sites, gives further morphological proof of the absence of DNase activity causing cleavage of double strands (Fig. 5a). Figure 5b shows an SFM image of the complexes between intact, not yet depurinated pBR322 and saporin-S6 obtained in the ionic conditions utilized to assay PNAG activity. Many protein molecules are bound at the same time to the plasmids, and the distribution along the chain seems to be markedly variable.

DISCUSSION

Several authors (*7*, *12–20*, *25*, *26*) concluded that topological and specific nuclease activities on DNA were additional intrinsic properties of ribosome-inactivating proteins (see "Introduction"), since they could not detect aspecific nucleolytic activity or appreciable contaminants in their enzyme preparations. To answer the question whether activities on DNA other than depurination were due to undetectable contaminants, we subjected four standard enzyme preparations of gelonin, momordin I, PAP-S, and saporin-S6 to dye-chromatography on Red Sepharose®. All nuclease activities depicted in Fig. 2 disappeared after chromatography on Red Sepharose®.

Here we report a simple chromatographic procedure to remove contaminating nucleases in preparations of four type 1 ribosome-inactivating proteins as different from each other as these proteins can be. The absence of DNA topological or nucleolytic activity in dye-purified PNAG was confirmed by electrophoresis and morphology with SFM, whereas full deadenylating activity was retained.

Experiments at low resolution conducted with cinnamomin indicated that SFM could give information on the interaction between PNAG and macromolecular substrates (*40*). Thus we used SFM to obtain morphological images of PNAG/pBR322 interaction. Many saporin-S6 molecules can most likely work at the same time until a plateau corresponding to depurination of 10% of deoxyadenosine residues is reached (see Figs. 3c and 4c). The fact that only a portion of deoxyadenosine is depurinated suggests that this enzymatic activity is somewhat specific. In most DNA-protein complexes, the binding specificity comes from the selective recognition of a well-matched complementarity between a local protein surface, the edges of the DNA bases and the sugar-phosphate backbone (*41*). Specific hydrogen bonds and van der Waals contacts are established: this is the so-called "direct read-out" mechanism. It is common knowledge that in other cases longer range characteristics of the DNA chain can also drive protein DNA recognition and interaction by a mechanism which was termed "indirect read-out." The proteins in this case recognize not-so-local DNA sequences and conformations. In Fig. 5b the proteins are located at numerous positions on the contour of the molecule. We speculate that specificity for a particular sequence would make the PNAG molecules spend most of their time at their specific binding locations, and there they would be found in the SFM experiments. The digestion of 10% of the adenines means that on the average one base in 40 is released from the DNA molecule: this is one base in approximately 7 nm of the molecule contour, in the case that the PNAG were uniformly distributed. If the sequence specificity of the enzymes were determined on this size scale, the SFM would not be able to reveal it, due to intrinsic limitations in the spatial resolution of its data. On the other hand, the existence of an end-point in the activity of PNAG does not necessarily prove some specificity: at some point during the digestion, some general DNA structural changes might make the substrate less appealing for PNAG, regardless of the sequences involved.

Furthermore, the structural analysis carried out by SFM suggests that indirect read-out mechanisms may drive recognition processes between PNAG and DNA chain segments.

The results discussed here demonstrate that the DNA modifications by PNAG other than depurination reported by various authors may well be attributed to contamination with nucleases, particularly when high amounts of enzymes were required. These contaminations are below the sensitivity limits of common analytical procedures and cannot be easily removed with conventional chromatographic procedures. We regret that the presence, at that time unknown, of traces of nucleases in our preparations might have led other authors to misleading results (*16*) in experiments on plasmidic DNA with gelonin, momordin I, and saporin-S6 and possibly other ribosome-inactivating proteins. Dye-chromatography on Red Sepharose® is hence proposed as a general method for the preparation of nuclease-free PNAG to be used in the ongoing studies in the fields of apoptosis, DNA conformation, immunotoxin preparation for experimental therapy, anti-HIV protocols.

Since gelonin, momordin I, PAP-S, and saporin-S6 do not cleave DNA strand(s), and the only base released from DNA is adenine, it may be concluded that polynucleotide: adenosine glycosidase is the sole enzymatic activity on DNA of these, and possibly all, ribosome-inactivating proteins.

We are indebted to Dr. Letizia Polito for standard purification of PNAG. This study was supported by grants from the Ministero dell'Università e della Ricerca Scientifica Programmi Biotecnologie legge 95/95 (MURST 5%), from the Università degli Studi di Bologna "*Quota ex 60%*," "*Special Funds for Selected Research Topics*," and Programmi di ricerca "*Quota ex 40%*" 1997–1999, 1999–2000; from the Associazione Nazionale per la Ricerca sul Cancro, and by the Pallotti's Legacy for Cancer Research.

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