Gelonin Is an Unusual DNA Glycosylase That Removes Adenine from Single-stranded DNA, Normal Base Pairs and Mismatches*

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We reported that plant ribosome inactivating proteins (RIP) have a unique DNA glycosylase activity that removes adenine from single-stranded DNA (Nicolas, E., Beggs, J. M., Haltiwanger, B. M., and Taraschi, T. F. (1998) J. Biol. Chem. 273, 17216–17220). In this investigation, we further characterized the interaction of the RIP gelonin with single-stranded oligonucleotides and investigated its activity on double-stranded oligonucleotides. At physiological pH, zinc and β-mercaptoethanol stimulated the adenine DNA glycosylase activity of gelonin. Under these conditions, gelonin catalytically removed adenine from single-stranded DNA and, albeit to a lesser extent, from normal base pairs and mismatches in duplex DNA. Also unprecedented was the finding that a lesser extent, from normal base pairs and mismatches in duplex DNA. Also unprecedented was the finding that a lesser extent, from normal base pairs and mismatches in duplex DNA. Also unprecedented was the finding that

Several lines of evidence suggest that the anti-tumor, anti-viral, and anti-parasitic effects of the plant proteins such as gelonin or pokeweed antiviral protein (PAP),1 well known as ribosome inactivating proteins (RIPs) for their ability to remove an invariant adenine in a conserved loop in the 28 S rRNA (1), are not solely due to ribosome inactivation (2–5). In vitro studies in search of alternative substrates that may be damaged by these enzymes revealed that they possess a single-stranded adenine DNA glycosylase activity (6). While there is still no direct evidence that this activity is physiologically relevant in plants or contributes to cytotoxicity, the ability of RIPs to damage DNA by removal of normal, non-mispaired bases in vitro distinguished them from the other members of the DNA glycosylase family, which protect the genome by removing potentially cytotoxic or mutagenic bases (7, 8). If the number of DNA lesions produced overwhelmed the DNA repair capacity of the cell or organism, the adenine glycosylase activity of the RIPs could be mutagenic or lethal. Recognition of the adenine DNA glycosylase activity of RIPs has been somewhat slow due to confusion in the literature, issues of possible contamination by nucleases, and the requirement of high protein/DNA ratio for activity (9, 10). Stirpe and co-workers (11, 12) reported that over 50 plant RIPs and the ricin-homologue, shiga-like-toxin found in Shigella dysenteria (13) removed adenine from various substrates, including DNA. In search of an enzyme classification that encompassed the removal of adenine from RNA and DNA, these investigators redefined RIPs as polynucleotide:adenosine nucleosidases (11) or polynucleotide:adenosine glycosidases (12). Gelonin, PAP, and ricin were demonstrated to have an adenine DNA glycosylase activity on single-stranded DNA (6). We suggested that this new classification was more appropriate than those used in Refs. 11 and 12. Recently, Wang et al. (14) suggested that the anti-HIV-1 and anti-tumor activity of the RIP MAP30 from Momordica charantia was a consequence of its adenine DNA glycosylase/AP lyase activity. This conclusion was made based on an extrapolation from the study with related proteins (6). Direct evidence for the adenine DNA glycosylase or the AP lyase activity of MAP30 was not provided in Ref. 14. The novelty of the biochemical activity of this group of naturally occurring enzymes was recognized in a commentary by Putman and Tainer (15). The classification of RIPs as AP lyases needed to be re-examined, however, since the conclusions drawn in Ref. 6 were partly based on results obtained using a borohydride trapping assay (16), the accuracy of which was recently questioned (17–20).

Issues of the possible contamination of RIPs by nucleases were addressed by zymography using naturally occurring RIPs and purified bacterial recombinant forms (21). The requirement for high protein/DNA ratios for activity on DNA may be a property of these proteins or could be due to the fact that, due to the newness of the discovery, the experimental conditions for the assay are not optimal. Barbieri et al. (12) reported that the removal of adenine from DNA proceeded without cofactors, at low ionic strength, in the absence of Mg2+, and with an optimal pH value of 4.0 (22). Our laboratory found that the adenine DNA glycosylase of gelonin, PAP, and ricin was stimulated by zinc at physiological pH (6). Kinetic studies undertaken to date to measure the rate of adenine removal from DNA utilized macromolecular substrates only (22), rather than the

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The abbreviations are: PAP, pokeweed antiviral protein; RIP, ribosome inactivating protein; rGel, recombinant gelonin; ss, single-stranded; ds, double-stranded; ODN, oligonucleotide; AP, apurinic/apyrimidinonic; βME, β-mercaptoethanol; MES 4-morpholinolthanesulfonic acid; MAP, Mirabilis antiviral protein.
single target oligonucleotides classically used for the character-ization of DNA glycosylases. The simultaneous or consecu-tive splitting of many N-glycosidic bonds that occurred using the macromolecular substrates resulted in complicated kinetics (22) and precluded any comparison with the DNA glycosylases. Many fundamental questions about the DNA glycosylase activity of the RIPs also require further investigation. These include whether the removal of adenines is the primary event that leads to DNA breakage, whether the breakage is RIP-mediated, and whether the activity is limited to the single-stranded regions of supercoiled DNA or also affects double-stranded DNA.

In this investigation, we characterize the adenine DNA glycosylase activity of gelonin using assays and substrates (e.g. a single-target-containing oligonucleotide) that are routinely used to characterize classical DNA glycosylases. We now characterize the kinetics of the glycosylase activity of gelonin on these substrates under different buffer conditions and revisit our previous conclusion that gelonin has an associated AP lyase activity. In addition, we address the question of an unusual specificity for a widely available target (e.g. adenine) by studying the activity of gelonin on single-stranded and double-stranded oligonucleotides containing multiple adenines. The results clarify some of the confusing data described above and reveal more features that make the RIPs unusual glycosylases. Insight into the molecular mechanisms of adenine removal from DNA and the DNA cleavage that can accompany it is also provided.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Substrates**—Plant gelonin was purchased from Sigma and resuspended in 10 mM HEPES, pH 7.0, and used within 3 weeks post-hydration. The wild type recombinant gelonin (rGel) and the mutants (rGel(C44A) and rGel(C50A)) were gifts from Drs. Stephen Carroll and Mark Better from the XOMA Corp. The production and characterization of these proteins has been previously described (23). Protein concentrations were measured using the BCA™ protein assay reagent from Pierce (Rockford, IL) using bovine serum albumin as a standard. The oligodeoxynucleotides (ODN) were prepared by the Nucleic Acid Facility at Thomas Jefferson University and further purified by preparative gel electrophoresis before use. After gel purification, the ODN were stored in 10 mM HEPES, pH 7.0, 100 mM NaCl, 2% b-mercaptoethanol (βME) (buffer I) or 10 mM MES, pH 5.0, 1 mM EDTA (buffer III) also supported the glycosylase activity. The DNA glycosylase activity obtained with these different conditions as a function of substrate concentration is shown in the autoradiogram of the denaturing polyacrylamide gel (Fig. 1A). The slower electrophoretic mobility of the product that was observed in buffer II was due to inhibition of β-elimination by βME (24). The difference in the processing of the substrate in the three conditions was so remarkable that it could not be ascertained without processing of the autoradiogram. The most striking difference was observed in conditions of multiple turnover ([protein] < [DNA]): at pH 7.0, in the presence of zinc, the addition of βME allowed more facile turnover so that, at a 1:50 protein/DNA ratio (mol:mol), the degradation of substrate increased from <5 to ~60%. To avoid experimental artifacts, which can be associated with measurement of activity after a set time, the time course of the reaction was studied. Two enzyme/ODN ratios (1 or 0.02) were used (Fig. 1B). At equimolar ratio, both buffers II and III allowed a rapid, total conversion of substrate to product. In buffer I and III at a 1:50 ratio, the low but steady progression of a degradation product suggested that the poor processing of the substrate by gelonin was not due to a single turnover mechanism as observed for the DNA glycosylase TDG (25). Due to the extremely inefficient turnover, buffer I and III could not be used for the determination of standard Michaelis-Menten kinetic parameters.

**RESULTS**

**Investigation of the Reaction Conditions for the Adenine DNA Glycosylase Activity of Gelonin**—We previously described the design of an oligonucleotide substrate and a method to study qualitatively the adenine DNA glycosylase activity of gelonin (6). The same 28-mer ODN containing a single adenine at position 25 was used to determine the enzyme kinetics of gelonin. The effect of different buffering conditions on gelonin’s DNA glycosylase activity was investigated. In addition to the buffer conditions we previously used (10 mM HEPES, pH 7.0, 100 μM ZnCl2 (buffer I)) in Ref. 6, we found that 10 mM HEPES, pH 7.0, 2 mM ZnCl2, 2% β-mercaptoethanol (βME) (buffer II) or 10 mM MES, pH 5.0, 1 mM EDTA (buffer III) also supported the glycosylase activity. The DNA glycosylase activity obtained using these different conditions as a function of substrate concentration is shown in the autoradiogram of the denaturing polyacrylamide gel (Fig. 1A). The slower electrophoretic mobility of the product that was observed in buffer II was due to inhibition of β-elimination by βME (24). The difference in the processing of the substrate in the three conditions was so remarkable that it could not be ascertained without processing of the autoradiogram. The most striking difference was observed in conditions of multiple turnover ([protein] < [DNA]): at pH 7.0, in the presence of zinc, the addition of βME allowed more facile turnover so that, at a 1:50 protein/DNA ratio (mol:mol), the degradation of substrate increased from <5 to ~60%. To avoid experimental artifacts, which can be associated with measurement of activity after a set time, the time course of the reaction was studied. Two enzyme/ODN ratios (1 or 0.02) were used (Fig. 1B). At equimolar ratio, both buffers II and III allowed a rapid, total conversion of substrate to product. In buffer I and III at a 1:50 ratio, the low but steady progression of a degradation product suggested that the poor processing of the substrate by gelonin was not due to a single turnover mechanism as observed for the DNA glycosylase TDG (25). Due to the extremely inefficient turnover, buffer I and III could not be used for the determination of standard Michaelis-Menten kinetic parameters.

**Determination of the Kinetic Parameters**—Buffer II was used to determine the enzyme kinetics. Reactions, done in triplicate, contained the enzyme at 0.1 μM and the substrate ssA25 at concentrations varying from 0.5 to 10.0 μM and were stopped after 2 min. A double-reciprocal plot of the initial rate versus ODN concentration allowed the determination of Vₗₒₒₙ and Kₘ. The catalytic constant (kₐ) was calculated as the ratio of Vₗₒₒₙ to the enzyme concentration used (10⁻⁷ M).

**Trapping Assay**—The glycosylase reaction buffer used above was supplemented with the desired concentration of NaCl, NaCNBH₃, or NaBH₄. After various times as indicated in the figure caption, the assays were terminated by addition of SDS-polyacrylamide gel electrophoresis loading buffer. The samples were boiled for 5 min and analyzed on a 7.5% polyacrylamide gel. The electrophoresis. After elution of the lower part of the gel that contained the free oligonucleotide, the distribution of the isotope on the gel was determined by PhosphorImage analysis. All autoradiograms were processed with Adobe Photoshop 5.0.

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strate, we measured the kinetic parameters $k_{\text{cat}}$ and $K_m$ of the reaction. Fig. 2 shows the concentration of abasic DNA produced in 2 min by $10^{-7}$ M gelonin plotted against the concentration of the substrate ssA25. Adenine excision by gelonin is shown to follow Michaelis-Menten kinetics. From the double-reciprocal plots of initial velocity versus substrate concentration, $k_{\text{cat}}$ and $K_m$ were estimated at 8.3 min$^{-1}$ and 1.7 mM, respectively.

Role of the Intradisulfide Bridge in Gelonin and Enzyme Turnover—Gelonin possesses two cysteines in positions 44 and 50 linked by a disulfide bridge (23). The stimulatory effect of βME described above suggested that the low turnover efficiency observed in buffer I could be due to the disulfide bridge and that it might be possible to increase the glycosylase activity by eliminating the bridge by genetic mutation. Such an approach has been used with the RIP MAP from Mirabilis: the inhibitory activity on protein synthesis of the mutant C36S/C220S, in which the disulfide bridge was eliminated was approximately 22 times higher than that of native MAP (26). To investigate the role of the disulfide bridge between cysteines 44 and 50 in the low efficiency turnover of gelonin in buffer I, we compared the activity of the gelonin mutants C44A and C50A to that of the wild type recombinant enzyme (Fig. 3). The results with the recombinant wild type protein indicated that the low turnover in buffer I and the stimulation of the activity by βME were intrinsic properties of gelonin, since they were observed with both the native (Fig. 1) and recombinant (Fig. 3) proteins. The elimination of the disulfide bridge had no effect on the gelonin turnover in buffer I, suggesting that the mechanism of stimulation by βME was not through modification of the protein. The elimination of these cysteines also had no effect on gelonin’s ability to inhibit protein synthesis (23).

Borohydride Trapping Assay and the Classification of DNA Glycosylase/AP Lyases—DNA glycosylase/AP lyases are glycosylases with an associated β-elimination activity that results in DNA strand breakage. A unifying hypothesis that rationalizes the apparent distinction between the two classes of glycosylases (glycosylase or glycosylase/AP lyase) has been proposed (16). The bifurcation is in the catalytic mechanism, i.e. the type of nucleophile that attacks C-1’ of the damaged base nucleoside. Bifunctional glycosylases utilize an amine nucleophile from the enzyme, while monofunctional glycosylases use a nucleophile derived from the medium. The hypothesis of a unified catalytic mechanism seemed to be supported by the borohydride-trapping assay, the principle of which is that only bifunctional glycosylases form a trappable complex with their substrate (16). In our previous paper (6), the trapping assay with an oligonucleotide substrate was used to investigate the possibility that gelonin had an associated AP lyase activity, which was suggested by the cleavage of a single-stranded DNA fragment (~800 bases) by gelonin, PAP, or ricin. We suggested that RIPs appeared unusual in that, while they formed a borohydride-trappable complex classifying them as DNA glycosylase/AP lyases (24), they could not be distinguished from monofunctional glycosylases in a strand cleavage assay using a short

**Fig. 1.** Adenine DNA glycosylase activity of gelonin in three different buffers. A, reaction mixtures contained the indicated concentrations (in μM) of substrate ssA25 and $10^{-7}$ M gelonin in the indicated buffer (I, II, or III) at 37 °C. After a 30-min incubation, samples were withdrawn and analyzed as described under “Experimental Procedures.” S and P are substrate and product, respectively. β and β6 mark the positions of β and β6 elimination products, respectively. B, reaction mixtures contained ssA25 and gelonin at a ratio of enzyme/substrate (E/S) of 1 or 0.02. Samples were withdrawn at different times and analyzed as in A. The data were plotted after quantification of the intensity of the autoradiogram. C, reactions were performed at equimolar enzyme/substrate ratio at enzyme concentrations varying from $10^{-8}$ to $10^{-7}$ M for 2 min in buffer II.

**Fig. 2.** kinetics of the removal of adenine from ssA25 by gelonin. Reactions contained the indicated concentrations of ssA25 in buffer II at 37 °C. Gelonin was present at a concentration of $10^{-7}$ M. The samples were withdrawn after 2 min and assayed as described under “Experimental Procedures.” The data were plotted and fitted to a Michaelis-Menten curve using KaleidaGraph.
Complex formation was progressively inhibited by the addition of increasing concentrations of EDTA (lanes 5 and 6).

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Activity of Gelonin on a Single-stranded Oligonucleotide with Multiple Adenines—Fig. 5 shows the glycosylase activity, as a function of time, of gelonin on the ODN CsaA25 that contained multiple adenines. Despite the fact that the samples were analyzed without alkali post-treatment (lanes 2–6), smears indicative of extensive DNA degradation were observed even at the shortest time of incubation (5 min). Electrophoresis in Tris buffer can cause breakage of the backbone of DNA containing abasic sites (27, 28); this can be prevented by stabilization of the abasic sites with NaBH₄. To verify that the smears were due to creation of abasic sites by gelonin and determine whether the degradation was produced by gelonin during the incubation or occurred during electrophoresis, the samples were incubated with NaBH₄ (100 mM) at the end of the treatment with gelonin. Fig. 5 (lanes 7–11) showed that this treatment resulted in the appearance of multiple degradation products. The same profile was observed when the concentration of NaBH₄ was increased to 250 mM (data not shown), suggesting that the DNA fragments were not due to incomplete stabilization, but rather to cleavage during the incubation. After a 15-min incubation, only one degradation product was detected (lane 8), which migrated slightly faster than the unmodified substrate (lane 1). CsaA25 contains adenines at positions 26 and 27 from the ³²P-labeled 5'-end. Removal of one or both of these adenines and cleavage at these sites could have generated the band observed in lane 8. To test this hypothesis, the

**Fig. 3. Activity of rGel, rGel(C44A), and rGel(C50A) on ssA25.**

Reactions, in the indicated buffer, contained 10⁻⁷ M of the indicated enzyme and ssA25 at the indicated enzyme/substrate (E/S) molar ratio. After 30 min, samples were withdrawn and assayed as described under “Experimental Procedures.”

(28-mer) ODN substrate, since post-treatment was necessary to break the DNA at the resulting abasic site. Similar behavior was subsequently reported for the adenine DNA glycosylase, MutY, whose classification has long been a matter of controversy (17, 18, 20). It was concluded from a kinetic analysis that the slow dissociation rate of MutY from its product was suggestive of specific contacts with DNA that persisted after base removal. These contacts may result in borohydride-trappable complex formation independent of a lyase activity.

These results and the observations reported in Fig. 1 prompted us to reinvestigate the origin of the gelonin-ODN complex observed in the presence of NaBH₄. In particular, we investigated the formation of a trappable complex in buffer II, which supported a more efficient enzyme turnover. We (6) and others (17) have noted that the experimental conditions for the trapping assay may not be as straightforward as proposed in the initial paper (16), mainly because of the hypersensitivity of some glycosylases to salt. In Fig. 4, we compared the effect of NaCl on the glycosylase activity of gelonin in buffers I and II. The interaction of gelonin with the abasic site was further investigated when the concentration of NaCNBH₃ was increased over the 30-min time course of the assay. No complex formation was observed when 25 mM NaCNBH₃ was replaced by 25 mM NaBH₄. A stronger reducing agent was reduced to 10 mM, which is consistent with the results shown in Fig. 4A, complex formation was detectable, although highly reduced, in the absence of zinc and βME.
adenine DNA glycosylase activity of gelonin on a single-stranded oligonucleotide containing multiple adenines. Gelonin and CsaA25 (lanes 2–11) or CsaA25-G26T27 (lane 13) were mixed at equimolar ratio in buffer II. Lanes 1 and 12 were controls (c) (no protein) for CsaA25 or CsaA25-G26T27, respectively. At the indicated times, samples were withdrawn and analyzed without post-treatment or after post-treatment with NaBH4, as indicated under “Experimental Procedures.”

The experiment was repeated with an ODN that contained GT in place of AA in positions 26 and 27 from the 3′-end. The appearance of a product with increased mobility following gelonin treatment (compare lanes 12 and 13) suggested that this band, and the product in lane 8, arose from DNA containing multiple (NaBH4-stabilized) abasic sites. The observation that the abasic product in lane 8 migrated faster than the abasic product in lane 13 suggested that CsaA25 had broken after removal of adenines in positions 26 and 27 from the 3′-end. Consistent with this interpretation was the finding that the product in lane 8 migrated faster than the untreated substrate after electrophoresis in native conditions (data not shown).

The disappearance of the product in lane 8 and the appearance of faster migrating bands with increasing incubation time (lanes 9–11), despite the post-treatment with NaBH4, suggested that breakage of the substrate occurred during the reaction. The experiment did not allow us to distinguish whether the breakage was directly protein-mediated or was a consequence of the instability created by multiple base removal. The banding pattern of the products was consistent with the removal of adenines from CsaA25 based on its sequence.

Activity of Gelonin on Adenine in a Double-stranded ODN—To study the activity of gelonin when adenine was in a Watson-Crick base pair or in a mismatch in double-stranded (ds) DNA, ssA25 was annealed to CsaA25 or CsaA25-G, which contained G in place of T opposite A25. The tracer DNA was 5′-32P-end-labeled either on ssA25 (Fig. 6, A and C) or CsaA25 (Fig. 6, B and D). Post-treatment with hot alkali or NaBH4 was performed to determine the glycosylase activity and amount of cleavage during the reaction, respectively. Fig. 6A showed that incubation of the duplexes labeled on ssA25 with gelonin at equimolar ratio, followed by post-treatment with alkali and electrophoresis under denaturing conditions, resulted in the appearance of products that co-migrated with the products obtained using ssA25 as the substrate, indicating that gelonin removed adenine from ds-ODN. Gelonin had similar adenine glycosylase activity on A in a normal base pair (A-T) or in a mismatch (A-G). The removal of A from the A-T base pairs was remarkable, as no DNA glycosylase has been shown directly to have such a property. Only a minor product was observed when the post-treatment was performed after treatment with NaBH4, indicating that, similar to what was observed with ss-ODN (6), the glycosylase activity on a DNA strand with a single target was not accompanied by cleavage. When the activity was monitored with the label in the bottom strand of the duplex, which contained multiple adenines (Fig. 6B), multiple products were formed. This suggested that the glycosylase activity was not restricted to a specific base pair. Following a 60-min incubation, none of the substrate was intact. After stabilization with NaBH4, a mobility shift characteristic of the presence of abasic sites was observed (lane 6). The band had a higher mobility than the one observed when ss-ODN was used as a substrate (lane 8), which we assigned to multiple adenine removal and cleavage at the 3′ end (Fig. 5). This suggested that residual base pairing prevented strand cleavage of the DNA.

Thermodynamic studies have shown that abasic sites impact the stability, conformation, and melting behavior of a DNA duplex (29). To determine the consequences of the removal of adenines on the integrity of the duplexes, the NaBH4-stabilized samples were also analyzed under native conditions (Fig. 6, C and D). The duplex substrates were confirmed to be free of single-stranded DNA (Fig. 6, C, lanes 3 and 6, and D, lane 2). With all three duplexes, a band of higher mobility was detected upon incubation with gelonin. Its intensity increased with increasing incubation time. To identify its origin, its migration was compared with the migration of ssA25 (lanes 1 and 9) and ssA25-damaged with gelonin (lane 2). It was found to co-migrate with the damaged ssA25. The product of the activity on the duplex labeled on CsaA25 was found to migrate close to the position of the ss-ODN. Due to the cleavage of Csa25 by gelonin, a marker for abasic-CsaA25 could not be generated. However, while it could not be demonstrated as clearly as for the other duplexes that the signal originated from damaged ss-ODN, this conclusion was reached from the analysis of the denaturing gel (Fig. 6B, lane 3), which indicated that none of the substrate was left intact. This also suggested that the band that co-migrated with intact ds-ODN in the gelonin-treated samples arose from a damaged (abasic) duplex.

Binding of Gelonin to an Adenine-free Oligonucleotide—According to Ischenko et al. (30), the current ideas concerning the ability of DNA repair enzymes to recognize an individual damaged base pair are in many respects erroneous, since they do not consider the relative contribution of specific and nonspecific interactions and their role in protein-nucleic acid recognition. These authors have shown that the DNA glycosylase Fpg can interact effectively not only with oligonucleotides containing a specific lesion, but also nonspecifically with single-stranded and double-stranded oligonucleotides, which can act as competitive enzyme inhibitors.

To determine if gelonin had affinity for non-target DNA, we analyzed the extent of inhibition of the adenine-glycosylase activity by an excess of adenine-free oligonucleotide. This competitor assay was chosen over an electromobility shift assay due to the experimental problems associated with the high pi of gelonin already reported in Ref. 31. Fig. 7 shows the modulation of the glycosylase activity of gelonin on ssA25 in the presence of increasing concentrations of unlabeled oligonucleotide containing either A or U in position 25; both oligonucleotides were found to similarly inhibit the degradation of labeled ssA25.
Base excision repair of DNA is initiated by DNA glycosylases, which catalyze the hydrolysis of the N-glycosyl bond linking particular damaged bases to the sugar-phosphate backbone. The base excision repair system includes several types of DNA glycosylases that recognize and remove many types of modified bases to leave an AP site. Our characterization of the plant ribosome inactivating protein, gelonin, revealed it to be a peculiar DNA glycosylase, removing normal adenine from single-stranded and double-stranded DNA. Removal of multiple adenines from DNA by gelonin produced unstable DNA, resulting in the melting of duplex DNA. To our knowledge, removal of normal bases by DNA glycosylases has only been described for site-specific mutants of UDG made to accommodate cytosine and thymine in their active site (32) and various 3-methyladenine glycosylases (AlkA, AAG, and Mag1), known for their unusual broad specificity toward a wide variety of damaged DNA bases (33, 34). None of these studies addressed the questions of the distribution of the abasic sites when the target is widely available and the consequences for the stability of the DNA.

The recent kinetics analyses of MutY (17–20) and TDG (25), which suggested that the assays routinely used to characterize glycosylases can provide misleading results under some conditions, caused us to re-examine the interpretation of previous results and expand our biochemical characterization of gelonin. In this investigation, we have described two buffer conditions (10 mM HEPES, pH 7.0, 100 μM ZnCl₂ or 10 mM MES, pH 5.0, 1.0 mM EDTA) which support adenine DNA glycosylase activity, but which do not promote efficient enzyme turnover. The first set of conditions was utilized in our initial description of...
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Gelonin’s DNA glycosylase activity (6). The choice for the low pH buffer was guided by the study of the influence of pH on the catalytic activity of rickin on oligoribonucleotides containing a base paired strand and a GAGA tetraloop motif, which revealed a pH optimum of 4.0 (35). Barbieri et al. (22) reported a similar acidic pH value as optimal for the removal of adenines from herring sperm DNA by the RIP saporin. At neutral pH, the addition of βME to the reaction buffer greatly enhanced gelonin’s DNA glycosylase activity, conferring higher turnover and lower salt sensitivity. The stimulation of the activity by zinc and βME is unusual and not fully understood. The analysis of the solution structure of MAP30 has indicated that Zn$^{2+}$ preferentially interacts with certain negatively charged regions near the active site and likely facilitates DNA binding by shielding the negative charges on the DNA backbone from the negatively charged protein surface (14). The results of the borohydride-trapping assay using an oligonucleotide with a preformed abasic site (Fig. 4) also argued that zinc facilitated substrate binding rather than catalysis. Zinc binding is lost upon protonation of the histidine imidazole below pH 6.5. This may explain the lack of zinc dependence at low pH.

The $k_{cat}$ value that we measured (8.3 min$^{-1}$) is close to the $k_{cat}$ value (3.75 min$^{-1}$) that was reported for the activity of rickin on an oligoribonucleotide that mimics the structure of the site of action on ribosomes (36). It was remarked that this value was small compared with the value obtained for the reaction on ribosomes (1777 min$^{-1}$) (36). We note, however, that it is in the same order of magnitude as the $k_{cat}$ obtained with multisubstrate DNA glycosylases such as 3-methyladenine glycosylases (7, 34). Gelonin belongs to a new class of DNA glycosylases, whose activity of adenine removal would be expected to produce damage, rather than be part of a DNA repair process. It is difficult to predict, based on our in vitro assays, the physiological relevance of the adenine DNA glycosylase activity of gelonin and related plant proteins. The excessive production of abasic sites could create an imbalance in the base excision repair pathway. Despite the fact that the site-specific mutants of UDG made to accommodate cytosine and thymine in their active site have a $k_{cat}$ value at least 3 orders of magnitude lower than that of a highly selective and efficient UDG, transformation of Escherichia coli with plasmids expressing these proteins produced an increase in mutation frequency and DNA degradation in the presence of an inducer of expression, indicating that excision occurred at a biologically significant rate (32). Transformation of E. coli with plasmids expressing AlkA also produced an increased spontaneous mutation frequency (33). There is also genetic evidence that the mutator effect of Mag1 expression in yeast depends on the production of abasic sites and that these abasic sites are converted into mutations by the REV1/REV3/REV7 lesion bypass system (37). Glycosylases that were seen as defenses against potentially injurious modifications of the DNA are now suspected to play a role in carcinogenesis due to this potential to generate a mutator phenotype (37). Interestingly, unlike Mag1, Tag does not remove normal bases from DNA and has no mutator activity (37). To our knowledge, there is no report of a similar activity for RIPs. When considering the lethality of DNA damage, it is necessary not just to consider the type of lesion but also the distribution of the lesions. Studies of the effects of ionizing radiation have shown that closely spaced abasic sites generated within a few base pairs of each other are a challenging damage (38). Attempts to repair multiply damaged sites can convert non-lethal or mutagenic lesions into lethal double stranded breaks (39, 40). Adenine is a widely available target in DNA. The ability of gelonin to create multiple abasic sites in close proximity (Fig. 5) and to induce melting of the duplexes (Fig. 6) might well be more detrimental than the creation of dispersed abasic sites. Similarly, the classical DNA glycosylases that have been described to remove normal bases and are suspected to be mutagenic could also be responsible for similar damage.

The mechanism that allows RIPs to remove normal bases is unknown. Several of the modified bases that are efficiently excised by the human 3-methyladenine AAG are known to cause little or no distortion of the double stranded helix (Ref. 34 and references therein). As already discussed above, AlkA, Mag1, and AAG also remove normal bases from DNA (33, 34). Similarly to gelonin, the 3-methyladenine glycosylases Tag and AlkA remove their target from both single- and double-stranded DNA (41). Verdin and Bruner (42) hypothesized that these enzymes might move along the DNA by flipping or attempting to flip successive nucleotides out of the helix and into the active site where substrate recognition occurs. Other dedicated glycosylases have been shown to promote flipping of their target or base opposite to their target (43). The hypothesis raised in Ref. 42 seems to be confirmed by the analysis of the crystal structure of human AAG in a complex with DNA containing a modified abasic site (44). The RIPs might use a similar mechanism. A prerequisite of the sliding mechanism is the ability to interact with non-target DNA. The results of the competition experiment with an adenine-free oligonucleotide (Fig. 7) suggest that gelonin effectively interacts with a target-free ODN. Using an active site mutant of the RIP PAP, which does not depurinate rRNA, Wang and Tumer (45) presented evidence that PAP damages supercoiled DNA using the same active site that is required for depurination of rRNA. Evidence that the sites for RNA and DNA glycosylase activities of MAP30 are identical was provided by the observation that the DNA-induced NMR chemical shift changes were localized to the adenine-binding pocket that is important for RNA glycosylase activity (14). When they act on rRNA, RIPs selectively cleave an N-glycosidic bond located within a stem loop (1). Crystallographic studies of the geometry of the active center have concluded that the reactive residue must be a loop-out residue (46). As pointed out by Putman and Tainer (15), a RIP-facilitated nucleotide flipping of bases from DNA would force a rethinking of the natural role of the RIPs as protectors from viral and fungal invasions. Alternatively, capture of the extra-helical base could be fortuitous depending on the transient flipping from the DNA helix by thermal motion (15). The set of adenines that are specifically removed by a specific RIP (6) would be determined by the quality of the fit of the adenine in the binding pocket of each protein. The order of relative activity (ss $>$ ds) could be understood in terms of energy required to destack the adenine and make it available to the binding pocket. Circular dichroism studies have shown that the binding of the complexes (Zn$^{2+}$-polypeptides containing Gha and Tyr residues) to poly(A) induced an unstacking of adenine bases (47). In the absence of Zn$^{2+}$, there was no evidence for the binding of the polypeptides to poly(A). These results suggest that zinc could have an unstacking function in the adenine DNA glycosylase activity of gelonin. This would support the proposal by McFail-Isom et al. (48) that cations have mechanistic roles in DNA bending, strand separation, DNA-protein recognition, base flipping, RNA folding, and catalysis through $\pi$-interactions with the faces of DNA and RNA bases.

The behavior of gelonin in the borohydride-trapping assay clearly resembles the behavior of MutY, which is now understood after years of controversy. Lys$^{142}$ has been shown to be the residue responsible for complex formation between MutY and DNA (18, 20). Cross-linking results from an unspecific interaction between the aldehydic abasic site and the nearby Lys$^{142}$ rather than from the trapping of the intermediate in a
glycosylase/AP lyase reaction. High-resolution crystallography (49) and site-directed mutagenesis (50) of MutY have confirmed that Lys142 is not involved in the base removal. According to Ref. 14, the active site of MAP30 and other RIPs is not suitable as an AP lyase site because there is no amino group available nearby to serve as a nucleophile. However, following depurination, the AP site would be brought close to a lysine chain (195 in MAP, 232 in PAP, 200 in gelonin). Our results are consistent with this model. The use of the trapping assay for the distinction of two classes of glycosylases has brought much confusion in the literature because the possibility that monofunctional glycosylases could form a Schiff base independently of the catalytic step of base removal was not considered. We suggest that whether or not a monofunctional glycosylase (as determined by a strand cleavage assay) forms a complex with its substrate upon addition of borohydride could be part of its characterization, with the caveat that complex formation does not imply that base removal involves an enzyme amino group. An oligonucleotide with a single target would be used to avoid the possibility of strand breakage associated with the instability created by multiple abasic sites (Fig. 5). Only glycosylases that catalyze both the glycosylase and the lyase reactions with equal stoichiometry would be classified as bifunctional. Gelonin, similar to MutY, would be characterized as a monofunctional glycosylase that forms a covalent complex with its substrate upon addition of borohydride could be part of its characterization, with the caveat that complex formation does not imply that base removal involves an enzyme amino group. An oligonucleotide with a single target would be used to avoid the possibility of strand breakage associated with the instability created by multiple abasic sites (Fig. 5). Only glycosylases that catalyze both the glycosylase and the lyase reactions with equal stoichiometry would be classified as bifunctional. Gelonin, similar to MutY, would be characterized as a monofunctional glycosylase that forms a covalent complex with its substrate upon addition of borohydride could be part of its characterization, with the caveat that complex formation does not imply that base removal involves an enzyme amino group. An oligonucleotide with a single target would be used to avoid the possibility of strand breakage associated with the instability created by multiple abasic sites (Fig. 5).

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