
Selective hydrolysis by exo- and endonucleases of phosphodiester bonds adjacent to an apurinic site

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ABSTRACT

Partial depurination of d-APA produced two UV_{260nm}-absorbing isomers, d-SpA and d-APs (where S represents the depurinated deoxyribose sugar), that provided simple model compounds with which to examine, by HPLC, the response of nucleases to phosphodiester bonds flanked 3' or 5' by an apurinic site. The structural identity of each compound was established by (i) reaction with methoxyamine to confirm the presence of an abasic deoxyribose group, and (ii) degradation of d-SpA under mild alkaline conditions to distinguish it from d-APs. At an enzyme concentration which led to complete hydrolysis of d-APA, snake venom phosphodiesterase readily cleaved d-SpA to 5'-dAMP but had no discernible effect on d-APs. Calf spleen phosphodiesterase also failed to act on one isomer, in this instance d-SpA, but additionally reacted at a much slower rate (~100 fold) with d-APs than with d-APA. Three single-strand specific endonucleases, nuclease P1, nuclease S1 and mung bean nuclease, all responded in an identical manner, hydrolysing d-APs but not d-SpA. The possibility that the aldehyde group at the AP sites might be responsible for some of these observations was rejected after repeating the enzyme digestions with the methoxyamine-capped molecules and observing no differences from the reactions with d-SpA and d-APs.

INTRODUCTION

Cleavage of the N-glycosylic bond between a base and its associated deoxyribose group results in the formation of an apurinic/aprimidinic (AP) site. Although base loss occurs 'spontaneously' at neutral pH, the process is greatly enhanced by low pH, elevated temperature or alkylation of the N-3 and N-7 positions in purines and the O² atom in pyrimidines (1-3). In living cells, AP sites also appear as transient intermediates in enzyme-mediated repair of base damage in DNA (4). These sites are themselves acted upon by a series of enzymes, starting with an AP endonuclease, that culminates in their replacement by bonafide nucleotides (5). If unrepaired, AP sites have been found to be potentially mutagenic lesions (6).

Most in vitro biochemical studies of AP sites have concentrated either on reaction mechanisms of AP endonucleases or on the fidelity of DNA and RNA polymerases acting on templates containing AP sites. A few reports have dealt with the responses of other DNA processing enzymes to AP sites, notably the 3'-5' exonuclease activities of the DNA polymerases from *Escherichia coli* (7) and phage T4 (8), snake venom phosphodiesterase (9), calf spleen phosphodiesterase (10), deoxyribonuclease IV from rat liver (11) and T4 DNA ligase (12).

In this study, we have examined how the activities of several exo- and endonucleases, which are employed in a wide variety of molecular biological techniques, are affected by the loss of a base in their nucleic acid substrates. The enzymes under review include

the exonucleases, snake venom phosphodiesterase and calf spleen phosphodiesterase, as well as three single-strand specific endonucleases, nuclease P1, nuclease S1 and mung bean nuclease. As our initial substrates, we chose the two compounds arising from the removal of a single base from d-ApA, namely, d-SpA and d-ApS (where S represents the sugar residue at the apurinic site). These molecules represent the simplest nucleic acid species with which to demonstrate any differences in the reactivity of the nucleases towards phosphodiester groups located either 3' or 5' to an AP site. All of the enzymes examined were observed to be selective in their reactivity towards the two monodepurinated compounds—snake venom phosphodiesterase only cleaving d-SpA while the other enzymes only hydrolysed d-ApS—indicating that each of these nucleases interacts with at least one of the two bases flanking a target phosphodiester bond. The substrate requirements of the enzymes, as well as the bearing our observations have on the use of these enzymes in techniques involving the digestion and analysis of carcinogen-damaged DNA, are discussed.

MATERIALS AND METHODS

Chemicals

The nucleosides, 2'-deoxyadenosine (dA), 2'-deoxycytosine (dC) and 2'-deoxyinosine (dI); the 3'- and 5'- monophosphates of deoxyadenosine (dAMP), deoxycytosine (dCMP) and thymidine (TMP); and the oligodeoxyribonucleotides d-ApA, d-pApTpC, d-ApT and oligo(dA)₁₂₋₁₈ were all purchased from Pharmacia Canada (Dorval, PQ). Methoxyamine hydrochloride was supplied by Sigma Chemical Co. (St. Louis, MO) and [8-³H]dATP (specific activity, 22 Ci/mmol) by ICN (St. Laurent, PQ).

Enzymes

Snake venom phosphodiesterase (*Crotalus adamanteus*, type II, 4.5 mg/ml, 1 unit/ml), mung bean nuclease (0.16 mg/ml, 5000 units/ml) and bacterial alkaline phosphatase (6.5 mg/ml, 227 units/ml) were obtained from Sigma Chemical Co. Nuclease P1 (1 mg/ml, 300 units/ml), nuclease S1 (1.5 mg/ml, 4 × 10⁵ units/ml), and calf intestinal phosphatase (22,000 units/ml) were purchased from Boehringer Mannheim Canada (Dorval, PQ) and calf spleen phosphodiesterase (20 units/ml) from Pharmacia Canada. Unit definitions of the nucleases are as follows: snake venom phosphodiesterase—1 unit hydrolyzes 1.0 μl of bis(p-nitrophenyl) phosphate per min at pH 8.8 at 37°C; calf spleen phosphodiesterase—1 unit produces 16 AU_{260nm} of nucleotides from RNA in 30 min at 37°C at pH 6.5 in a 2 ml reaction mixture (13); mung bean nuclease and nuclease S1—1 unit causes 1.0 μg of heat denatured DNA to become acid soluble per min at 37°C at pH 5.0 and 4.5, respectively; nuclease P1—1 unit hydrolyzes 1.0 μmole equivalent of RNA phosphodiester linkages per min at pH 5.3 at 37°C.

HPLC Chromatography

The instrumentation consisted of a computer-based Waters 840 system coupled to a Waters 490 multiwavelength detector (Waters Associates, Mississauga, ON). Peaks of UV-absorbing material were digitally integrated by the 840 system. Reverse phase HPLC was performed on a Whatman Partisil 10 ODS-2 column (250 × 4.6 mm i.d., Whatman Inc., Clifton, NJ). Elution conditions were as follows: 100% buffer A [50 mM NaH₂PO₄, pH 4.5] and 0% buffer B [100 mM NaH₂PO₄, pH 4.5/methanol (1:1 v/v)] for 1 min followed by a linear gradient to 0% buffer A and 100% buffer B over 30 min. The flow rate was 1 ml/min.

Use of SEP-PAK cartridges for desalting samples

Before loading a sample, the C₁₈ SEP-PAK cartridge (Waters) was activated by methanol (2 ml) and washed with distilled water (5 ml). The oligonucleotide sample was dissolved

in distilled water and applied to the cartridge. Salt was removed from the sample by elution with 3 ml distilled water and then the oligonucleotides were eluted in 2–3 ml 50% aqueous methanol (v/v).

Preparation of d-SpA and d-ApS

Nine μl of 1 M HCl were incubated with 81 μl of an aqueous solution of d-ApA (25 $\text{AU}_{260\text{nm}}/\text{ml}$) at 37°C for 40 min. At this time the combined concentration of these two compounds was equal to ~40% of the initial concentration of d-ApA. (Since each of the monodepurinated products contained a single adenine base, their extinction coefficients at 260 nm were assumed to be half that of d-ApA). After the addition of 0.5 ml of buffer A (see above), the reaction mixture was applied to the reverse phase HPLC column, and eluted as described above. The two desired products, d-SpA and d-ApS, were collected, taken to dryness under vacuum, redissolved in 0.5 ml distilled water, desalted by passage through a C₁₈ SEP-PAK cartridge (see above) and evaporated, before being finally dissolved in distilled water at a concentration of 1.5 $\text{AU}_{260\text{nm}}/\text{ml}$ (~0.1 mM). d-SpA and d-ApS were distinguished by their differential sensitivity to hydrolysis under mild alkaline conditions (see below).

Preparation of d-SpTpC

Starting with d-pApTpC, the first step was the removal of the terminal phosphate group by treatment with alkaline phosphatase. A 55- μl solution containing 0.5 $\text{AU}_{260\text{nm}}$ of d-pApTpC and 0.27 units of bacterial alkaline phosphatase in 40 mM Tris-HCl (pH 7.5) and 8 mM MgCl₂ was heated at 37°C for 2 h. The resulting d-ApTpC was depurinated by the addition of 10 μl of 1 M HCl and incubation at 37°C for a further 2 h. The mixture was neutralized with 10 μl 1 M NaOH and immediately loaded onto the reverse phase column. d-SpTpC was collected, desalted, evaporated and dissolved in distilled water at a concentration of 1.5 $\text{AU}_{260\text{nm}}/\text{ml}$ (~0.1 mM).

Reaction of d-SpA, d-ApS and d-SpTpC with methoxyamine

To one volume of a solution containing 75 mM Tris-HCl (pH 7.0) and 0.007–0.07 $\text{AU}_{260\text{nm}}$ (~0.5–5 nmol) of the depurinated oligonucleotide was added half a volume of 1 M methoxyamine (dissolved in water and brought to pH 7.0 with NaOH). The reaction mixture was incubated for 1 h at 37°C, and the products analyzed by HPLC. Larger quantities (5 nmol) of d-MpA and d-ApM (where M indicates a methoxyamine-modified deoxyribose residue) were isolated by reverse phase HPLC and desalted as described for the other prepared compounds.

Alkaline hydrolysis of depurinated compounds

Samples (~0.4 nmol), undergoing mild alkali treatment, were incubated with an equal volume of 0.4 M NaOH at 37°C for 15 min. They were then neutralized by addition of 0.2 M HCl and analyzed by HPLC.

The more vigorous alkali treatment necessitated heating the samples in 0.2 M NaOH at 65°C for 30 min.

Nuclease Treatment

(a) Snake venom phosphodiesterase—Digestion was carried out in a total volume of 50 μl containing 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.5–1.0 nmol oligonucleotide, and 0.001 unit of the phosphodiesterase. Each reaction mixture was incubated for 30 min before the products were analyzed by reverse phase HPLC. d-ApS (0.5 nmol) was also incubated with a higher concentration of enzyme (0.01 unit) for 2 h under the same conditions.

(b) Calf spleen phosphodiesterase—In the initial experiments the oligonucleotides (~0.5–1.0 nmol) were each incubated in 100 mM Tris-HCl (pH 7.5) with 0.004 unit

calf spleen phosphodiesterase in a total volume of 20 μ l at 37°C for 1 h. In subsequent reactions, the oligonucleotides were subjected to 0.2 unit of enzyme in the same buffer for 6 min in the case of d-ApA and for 2 h with the other oligonucleotides.

(c) Nuclease P1 – Each reaction with this enzyme was performed in a total volume of 50 μ l containing 0.5–1.0 nmol oligonucleotide, 10 mM sodium acetate (pH 5.3), 1 mM ZnSO₄ and 0.3 unit nuclease P1. The samples were incubated at 37°C for 30 min.

(d) Nuclease S1 – Initial treatment was carried out in a buffer of 10 mM sodium acetate (pH 4.3), 50 mM NaCl, 1 mM ZnCl₂ with 0.5–1.0 nmol oligonucleotide and 100 units S1 nuclease in a total volume of 20 μ l. The reaction mixtures were incubated at 37°C for 1 h. In follow-up experiments, d-SpA was treated with 400 units of enzyme, and d-ApA and d-ApS each with 50 units.

(e) Mung bean nuclease – Digestions with this enzyme were conducted in the same buffer and with the same oligonucleotide concentrations as the nuclease S1 reactions. Ten units of mung bean nuclease were employed in each reaction.

Enzymatic Hydrolysis of partially depurinated [8-³H]poly(dA)

The radiolabelled polymer (specific activity, ~1.8 Ci/mmol adenine) was prepared by terminal transferase-catalysed polymerisation according to the procedure of Nelson and Brutlag (14) using oligo(dA)_{12–18} as primer and [8-³H]dATP (specific activity, 2.2 Ci/mmol). To generate apurinic sites, 50 μ l of polymer (10⁶ dpm) were incubated with an equal volume of 20 mM HCl at 37°C for 1 h. The polymer was precipitated by addition of 2 μ g of unlabelled poly(dA) (1 μ g/ μ l), 10 μ l 2.5 M sodium acetate (pH 5.5) and 224 μ l ice-cold ethanol. After centrifugation (10⁴g for 10 min), the precipitate was resuspended in 100 μ l distilled water.

For digestion with snake venom phosphodiesterase and calf intestinal phosphatase, 10⁵ dpm of polymer (parental or depurinated) were incubated in 100 μ l containing 100 mM Tris-HCl (pH 7.5), 20 mM MgCl₂, 0.005 unit exonuclease and 1 unit phosphatase for 2 h at 37°C. The solution was then loaded directly onto the HPLC column and eluted as described above. Fractions (0.5 ml) were collected and their radioactive content measured by liquid scintillation counting.

Nuclease P1 digestion of 10⁵ dpm of polymer was carried out for 1 h at 37°C in 10 mM sodium acetate (pH 5.3) and 1 mM ZnSO₄ with 0.3 unit endonuclease in a total volume of 100 μ l. The buffer was then altered by addition of 5 μ l 1 M Tris-HCl (pH 8.0) and 1 μ l 1 M MgCl₂ to permit dephosphorylation by calf intestinal phosphatase (1 unit) for 1 h at 37°C. The samples were fractionated and counted as described above.

RESULTS

Characterization of d-SpA and d-ApS

To produce substrates containing an apurinic site, d-ApA was hydrolysed in 0.1 M HCl at 37°C and the reaction products resolved by reverse phase HPLC (Fig. 1). The two early eluting products, presumed to be the monodepurinated isomers, were analysed in order to (i) ensure that both contained a depurinated sugar moiety and (ii) determine which compound was d-SpA and which was d-ApS.

In DNA, the only sites of addition for methoxyamine (CH₃ONH₂), at neutral pH, are the carbonyl groups of ring-opened deoxyribose moieties at AP sites (15,16). When the acid-modified dinucleoside monophosphates were treated with methoxyamine their retention on the reverse phase column increased by ~5 min (compare panels a and b in Fig. 2), consistent with the addition to these compounds of a hydrophobic group, and indicated that both contained an AP site.

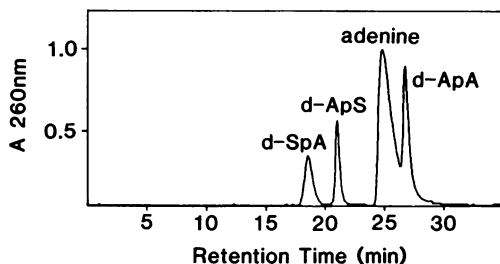


Figure 1. HPLC analysis of the products following incubation of d-ApA in 0.1 M HCl at 37°C for 70 min. Elution conditions are detailed in Materials and Methods.

Differential susceptibility towards alkaline hydrolysis was used to distinguish between d-SpA and d-ApS. In the former molecule the denuded sugar is bound to the phosphate group at its 3'-position and can undergo a base-catalyzed β -elimination reaction that results in cleavage of the sugar-phosphate bond (2). This mechanism is not possible in d-ApS and alkaline hydrolysis of its depurinated sugar-phosphate bond requires more severe conditions (17). Figure 2c shows the result of mild alkali treatment (0.2 M NaOH for 15 min at 37°C) of d-SpA and d-ApS. While the second of the two monodepurinated compounds remained unaffected, the first was converted to a new molecule having the same retention time as 5'-dAMP. Thus, the earliest eluting detectable product following acid hydrolysis of d-ApA was deemed to be d-SpA and the second was d-ApS. In keeping with these designations, we observed that (i) the material from the second peak was

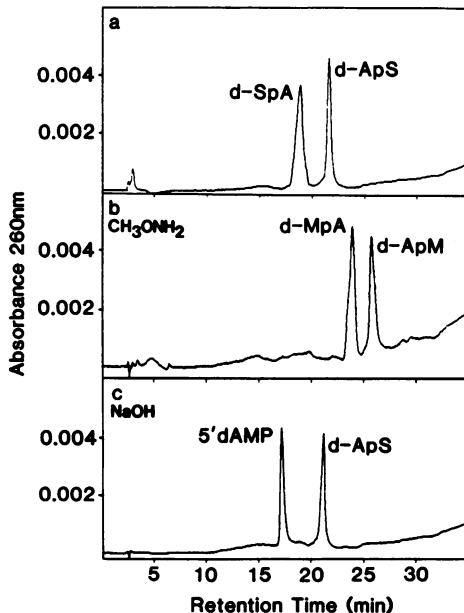


Figure 2. Characterization of partially depurinated products of d-ApA. HPLC analysis of (a) d-SpA and d-ApS, (b) methoxyamine treatment of d-SpA and d-ApS, and (c) mild alkali treatment of d-SpA and d-ApS.

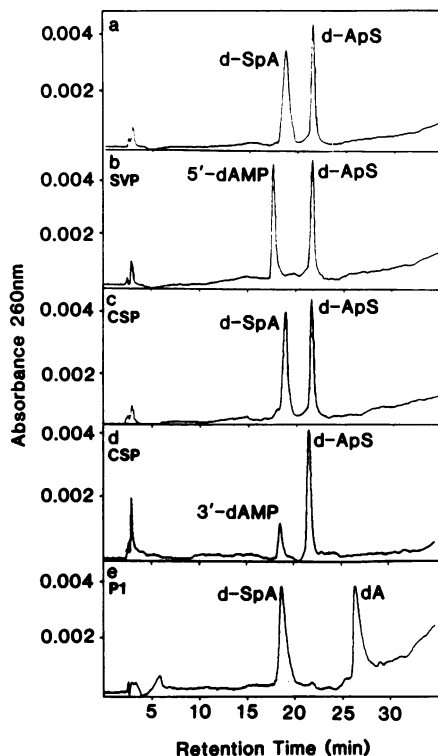


Figure 3. Chromatographic analysis of nuclease action on d-SpA and d-ApS: (a) unreacted substrates, (b) snake venom phosphodiesterase digest, (c) calf spleen phosphodiesterase digest, (d) calf spleen phosphodiesterase digest of d-ApS using a 50 fold higher concentration of enzyme, and (e) nuclease P1 digest. Under the conditions used d-ApA was completely hydrolyzed.

hydrolysed to 3'-dAMP by incubation in 0.2 M NaOH at 65°C for 1 h (data not shown), and (ii) d-MpA (the product of methoxyamine addition to d-SpA) was resistant to mild alkaline hydrolysis since, in this modified form, d-SpA can no longer undergo a β -elimination reaction (18).

Digestion of d-SpA and d-ApS by exonucleases

Reactions were first performed with d-ApA to ensure that the concentration of enzyme employed was sufficient for complete hydrolysis of the parent substrate within the reaction time. The treatment was then repeated with d-SpA and d-ApS individually and combined; the latter in order to show that failure to react with one of the isomers was not due to an artifact.

The results of a 30-min incubation of the two depurinated substrates with 0.001 unit of snake venom phosphodiesterase are shown in Fig. 3a and b. It can be seen that d-SpA was fully hydrolysed to 5'-dAMP while d-ApS remained totally unchanged. Increasing the enzyme concentration ten-fold and extending the time of incubation to 2 h did not alter the outcome with d-ApS.

The results of treatment of d-SpA and d-ApS with calf spleen phosphodiesterase proved less straightforward than the digestion with the venom exonuclease. HPLC analysis (Fig.

3c) following incubation with calf spleen phosphodiesterase, under conditions that resulted in complete hydrolysis of an equimolar concentration of d-ApA, suggested that d-SpA was unaffected by the treatment). However, the presence of contaminating adenosine deaminase in the commercial preparation of the exonuclease, identified from the digestion of d-ApA (data not shown), rendered the result with d-SpA inconclusive. If calf spleen phosphodiesterase had reacted with d-SpA, the visible product should have been deoxyadenosine, but the contaminating enzyme activity would have converted this to deoxyinosine (dI). Unfortunately, under our elution conditions, dI has almost the same retention time as d-SpA, making it impossible to discern the fate of the reaction. This was one reason why an alternative depurinated molecule was employed as a potential substrate for the exonuclease (see below).

The chromatogram (Fig. 3c) also indicated negligible hydrolysis (< 5%) of d-ApS to 3'-dAMP (partially obscured by the peak of d-SpA). The extent of hydrolysis to 3'-dAMP was elevated to 22% when the concentration of enzyme was increased 50-fold and the reaction time doubled to 2 h (Fig. 3d). In contrast, an equimolar concentration of d-ApA was totally digested within 6 min. (After 2 h incubation the enzyme had lost some of its potency but was still capable of hydrolysing within 6 min almost half of the d-ApA added at this time.) Thus, calf spleen phosphodiesterase reacts approximately 100 times faster with d-ApA than with d-ApS.

Endonucleolytic Treatment of d-SpA and d-ApS

The three endonucleases under study degrade DNA to 5'-mononucleotides (19). Again, initial experiments with d-SpA and d-ApS utilized enzyme concentrations previously found to fully digest d-ApA. The results of incubation with nuclease P1 are given in Fig. 3e. It is evident that this enzyme readily converted d-ApS to dA (and presumably deoxyribose-5-phosphate) while failing completely to react with d-SpA. The same phenomenon was observed with nuclease S1 and mung bean nuclease. Raising the concentration of the endonucleases 4–10 fold had no effect on d-SpA. On the other hand, halving the amount of S1 nuclease in each reaction to 50 units indicated that this enzyme acts on d-ApS and d-ApA at similar rates; after a 1-h incubation, HPLC analysis revealed that these substrates were 68% and 65% hydrolyzed, respectively.

Exo- and endonuclease reactions with d-SpTpC

There were two reasons for investigating the action of the nucleases, in particular calf spleen phosphodiesterase, on this molecule. First, as mentioned above, was the need to avoid the problem of contaminating adenosine deaminase in the calf spleen phosphodiesterase preparation. If this exonuclease was capable of digesting d-SpTpC then it would generate UV-detectable products (3'-TMP and deoxycytidine) that could be easily distinguished by HPLC from the starting material. Second, it was important to establish whether the inhibitory action of an AP site was restricted to the phosphodiester group(s) immediately adjacent to the AP site, or if it blocks nuclease activity at phosphodiester groups further removed from the AP site.

d-SpTpC was prepared from d-ApTpC by acid-catalyzed depurination in 0.1 M HCl and characterized by reaction with methoxyamine and base-catalyzed β -elimination as described above for d-SpA. It was then subjected to enzyme treatment under the same conditions as the other depurinated substrates—conditions that allowed complete hydrolysis of d-ApTpC. Figure 4 shows the products of d-SpTpC following incubation with three of the enzymes. As expected, snake venom phosphodiesterase hydrolyzed both phosphodiester groups to give, as visible products, 5'-TMP and 5'-dCMP (Fig. 4b).

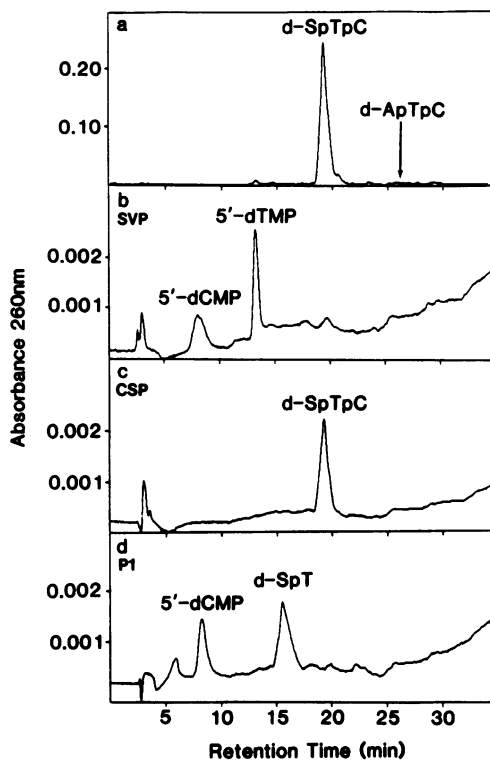


Figure 4. Chromatographic analysis of nuclease action on d-SpTpC: (a) unreacted substrate, (b) snake venom phosphodiesterase digest, (c) calf spleen phosphodiesterase digest, and (d) nuclease P1 digest. The retention time of d-SpT produced in the reaction with nuclease P1 was identical to the product resulting from acid-catalyzed depurination of d-ApT (data not shown). Under the conditions employed for each enzyme reaction, d-ApTpC was fully hydrolyzed.

Nuclease P1 cleaved the phosphate group flanked by the unmodified nucleosides but, as with d-SpA, the phosphodiester linkage immediately 3' to the AP site was refractory to the enzyme (Fig. 4d). By contrast, calf spleen phosphodiesterase (0.004 unit) was unable to hydrolyze either phosphodiester group (Fig. 4c), even under more rigorous conditions (0.2 unit). This last result implies that the exonuclease had also failed to act on d-SpA.

Enzyme Reactions with Methoxyamine-Modified Depurinated Molecules

The possibility existed that the presence of the carbonyl group of the ring-opened form of the depurinated deoxyribose function (20) exerted an influence on the course of the reactions examined. We have already demonstrated that a base-catalyzed β -elimination reaction of d-SpA gives rise to 5'-dAMP. Others have shown that certain small peptides (21) and class I AP endonucleases (8) cleave DNA at AP sites via this β -elimination process. Since snake venom phosphodiesterase also produced 5'-dAMP from d-SpA, the possibility that this hydrolysis occurred by such a mechanism could not be ignored. On the other hand, in cases where a nuclease failed to react with a depurinated substrate, the potential existed for inactivation or interference of the enzyme's activity by reaction of amino groups of the protein with the deoxyribose aldehyde function (22,23). We therefore repeated several of the enzyme reactions with the methoxyamine-capped molecules, d-MpA and d-ApM.

Incubation of these compounds produced identical results to those with the uncapped molecules, i.e. snake venom phosphodiesterase hydrolyzed d-MpA but not d-ApM; calf spleen phosphodiesterase slowly cleaved d-ApM but failed to act on d-MpA and; nuclease P1 hydrolyzed d-ApM but not d-MpA. We thus conclude that the deoxyribose carbonyl function does not influence the interaction of these nucleases with phosphodiester groups adjacent to AP sites.

Digestion of lightly depurinated [8-³H]poly(dA)

Stuart and Chambers (9) recently demonstrated that an apurinic site in the middle of a long oligonucleotide is released by snake venom phosphodiesterase as a dinucleotide with the general formula d-pNpS. Based on our results with the di- and trinucleotides, it would be predicted that digestion by nuclease P1 and alkaline phosphatase of polynucleotides containing AP sites would liberate these sites in association with their 3'-neighbouring nucleotide, i.e. as d-SpN. This prediction was borne out when ³H-labelled poly(dA), lightly depurinated by treatment with 10 mM HCl at 37°C for 1 h, was used as a substrate for nuclease hydrolysis. Taking advantage of Stuart and Chambers' observation, we measured the number of AP sites in the polymer by digestion with venom phosphodiesterase and alkaline phosphatase. This resulted in a large peak of radioactivity eluting with dA and a small peak, constituting 0.65% of the total counts, of d-ApS. Hydrolysis with nuclease P1 and phosphatase gave a peak of d-SpA containing 0.72% of the total counts, the remainder eluting with dA.

DISCUSSION

The information reported here can be added to a growing body of data obtained with other modified nucleic acid molecules in order to determine substrate elements required for full enzyme activity. That snake venom phosphodiesterase can readily hydrolyze d-SpA and d-SpTpC is not surprising since the enzyme can act on many 'artificial' substrates lacking a 5'-nucleoside (e.g. the nitrophenyl ester of 5'-TMP) at rates similar to, or even greater than, a natural substrate like d-TpT (24). On the other hand, the inability of the nuclease to cleave d-ApS and phosphodiester bonds attached to other altered 3'-nucleosides (25–27) adds further weight to a model for enzyme-substrate interaction involving non-covalent binding to the base component of the 3'-nucleoside (28).

Calf spleen phosphodiesterase, unlike the venom nuclease, was severely impeded by an AP site on *either* side of the phosphate linkage. A similar observation, albeit less well delineated, was made by Margison and coworkers (10) using random oligonucleotides obtained by DNase II digestion of DNA. The ~ 100 fold difference in the rate of hydrolysis of d-ApA compared to d-ApS is also reminiscent of our recent finding that calf spleen phosphodiesterase releases the 5'-nucleotide from d-TpT<p>T [the UV product of oligo(dT)₃ bearing a cyclobutane pyrimidine dimer between the middle and 3' bases] approximately 20 times slower than from the unirradiated trinucleotide (29). As with other poorly reacting substrates, the question arises as to whether the low reactivity toward d-SpA and d-ApS is due to weak binding of the substrate by the enzyme or, once bound, to a slow rate of reaction (i.e. K_m vs V_{max}). Margison and coworkers provided compelling kinetic evidence that their depurinated DNase II fragments did not bind significantly to calf spleen phosphodiesterase. Since a high percentage of the products of DNase II digestion are dinucleotides (30), their substrate must have contained a mixture of molecules with 3' or 5' AP sites. Taken together, their and our results strongly suggest that this exonuclease binds *both* nucleosides flanking a phosphodiester bond, although a 3'-nucleoside is not an absolute requirement for enzyme function. The conclusion that the protein may

simultaneously bind two nucleosides has been drawn by others (31) from studies of the nucleotidyltransferase activity of calf spleen phosphodiesterase.

It is probable that nuclease P1, nuclease S1 and mung bean nuclease share a common mechanism of nucleic acid hydrolysis with similar binding sites (19). It has previously been shown that they are each capable of releasing the 5'-nucleoside from d-TpT <p>T but not the 3'-nucleotide from d-T <p>TpT (29). Similarly, dinucleoside monophosphates bearing a mitomycin C-modified 5'-base have been reported to be refractory to P1 nuclease (32). To this list can now be added the observation that the nucleases hydrolyse d-APs but not d-SpA. These results with base-modified nucleotides indicate that the three endonucleases bind to the base 5' to an internucleotide phosphate group. Our proposal is somewhat at variance with that put forward by Drew (33) who suggested that nuclease S1 may simply recognize a string of three to four phosphates. He accounted for the single-strand specificity of the enzyme by arguing that the sugar-phosphate strands in native DNA protect one another from attack merely by their proximity. We would argue that the enzyme interacts with bases in single-stranded DNA and this interaction is precluded with double-stranded DNA.

The practical implications of our observations stem from the wide use made of these five enzymes for the complete digestion of DNA, particularly in the examination of carcinogen, or otherwise, modified nucleosides (e.g. 34–36). If the nucleases are used in isolation, AP sites will be recovered as dimers, d-pNpS species from a snake venom phosphodiesterase digest and d-pSpN species from a nuclease P1, S1 or mung bean nuclease digest. In this regard, these enzymes differ from the 3' → 5' exonuclease activity of *E. coli* DNA polymerase I, which releases AP sites as deoxyribose-5-monophosphate (7). Calf spleen phosphodiesterase lacks any endonuclease activity (37), and it would appear from our observation with d-SpTpC that an AP site will completely block the progress of this exonuclease.

In the postlabelling analysis of carcinogen-DNA adducts devised by Randerath and coworkers (36), DNA is first digested by micrococcal nuclease (an endonuclease producing short oligonucleotides) and calf spleen phosphodiesterase to give nucleoside 3'-monophosphates (normal and modified) that are subsequently radiolabelled by incubation with [γ -³²P]ATP and T4 polynucleotide kinase. The procedure has the advantage of not requiring labelled carcinogen and is, therefore, particularly useful for the study of DNA lesions in tissues. One of the major problems, however, is trying to identify all the labelled products. As is evident with d-APs, calf spleen phosphodiesterase-catalyzed removal of the nucleotide 5' to an apurinic site requires higher concentrations of enzyme than are routinely used. Thus, as a result of natural and carcinogen-induced depurination, we feel it is feasible for some of the labelled lesions to be AP site-containing dinucleotides with the general formula d-pNpSp or d-pNpS, or even longer oligonucleotides. In a recent modification to the protocol (38), the products of nuclease digestion are treated with nuclease P1, in order to selectively remove 3'-phosphate groups from the normal nucleotides, prior to the labelling reaction. As our results with P1 nuclease indicate, an added advantage of this procedure is the hydrolysis of any molecules of d-NpS present in the digest, thereby eliminating AP site-containing species from the pool of labelled products. It is important to bear in mind, however, that while P1 nuclease fails to remove 3'-phosphate groups from nucleotide adducts of bulky carcinogens, it may not display the same inability towards smaller lesions such as alkylated nucleotides, and so this particular modification to the protocol may not always be applicable.

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