Mung bean (*Phaseolus aureus*) nuclease

A mechanistic investigation of the DNA-cleavage reaction using a dinucleoside phosphorothioate

Michael R. HAMBLIN, Jane H. CUMMINS and Barry V. L. POTTER*

Department of Chemistry, University of Leicester, Leicester LE1 7RH, U.K.

Mung-bean (*Phaseolus aureus*) nuclease has been found to cleave the S_p diastereoisomer of 5'-O-thymidyl 3'-O-(2'-deoxyadenosyl) phosphorothioate, (S_p)-d[Ap(S)T], in ^{18}O -labelled water with inversion of configuration at phosphorus to give (S_p)-thymidine 5'-[^{18}O , ^{18}O]phosphorothioate, the stereochemistry of which was deduced by methylation to (R_p, S_p)-thymidine 5'-S-methyl-O-methyl-[^{18}O , ^{18}O]phosphorothioate and ^{31}P -n.m.r. analysis. This result is consistent with a mechanism involving a direct 'in-line' attack of water on DNA for the nuclease-catalysed reaction without the involvement of a covalent nucleotidylated-enzyme intermediate.

INTRODUCTION

Single-strand-specific nucleases that hydrolyse both DNA and RNA have been isolated from diverse sources (Shishido & Ando, 1982). These enzymes exhibit high selectivity for single-stranded nucleic acids and produce mono- and oligo-nucleotides terminating in 5'-phosphate and 3'-hydroxy groups. The presence of a single-strand-specific endonuclease activity from *Phaseolus aureus*, commonly known as 'mung-bean nuclease', has been reported (Johnson & Laskowski, 1968). The enzyme is a glycoprotein of M_r 39000 requiring Zn^{2+} and Mg^{2+} ions for activity. With denatured DNA as substrate the enzyme is a typical endonuclease, the products of cleavage being oligonucleotides and mononucleotides. With large amounts of enzyme, mononucleotides are the sole products. Initially, interest in mung-bean nuclease centred on its ability to preferentially hydrolyse single-stranded DNA, leaving double-stranded DNA intact, and its use in the 'tailoring' of DNA for genetic manipulation. Subsequently, interest has shifted to its use as a reagent for probing the structure of native viral DNA and chromatin. The purification and properties of this enzyme have been reviewed (Laskowski, 1980). However, little is known about the mechanism of phosphodiester cleavage catalysed by this enzyme.

A mechanistic study of these enzymes should take into account the protein–nucleic acid interactions inherent in their selectivity, and also the catalytic mechanism of phosphodiester hydrolysis. A first step in understanding the latter is the determination of whether the enzymic reaction proceeds by direct attack of water on the phosphodiester bridge or whether a double-displacement mechanism is operative, whereby a covalent nucleotidylated-enzyme intermediate is formed and subsequently hydrolysed. A stereochemical experiment permits distinction between these two possibilities (Knowles, 1980; Frey, 1982; Gerlt *et al.*, 1983; Eckstein, 1983, 1985). Methods whereby this question can be addressed for nucleases have been developed recently and rely, *inter*

alia, on the use of dinucleoside phosphate analogues stereospecifically labelled with sulphur (Potter *et al.*, 1983a; Connolly *et al.*, 1984) or ^{18}O isotope (Potter *et al.*, 1983b,c; Seela *et al.*, 1983). We have used these analogues recently to study two members of the single-strand-specific nuclease family, nuclease S1 (Potter *et al.*, 1983a) and nuclease P1 (Potter *et al.*, 1983b) respectively.

We report here a stereochemical investigation of phosphodiester cleavage catalysed by mung-bean nuclease, using a deoxydinucleoside phosphorothioate, d[Ap(S)T] (Fig. 1).

A preliminary account of this work has been presented by one of us (B.V.L.P.) at the 617th Meeting of the Biochemical Society held in Dundee, Scotland, 19–21 March 1986 (Hamblin *et al.*, 1986).

MATERIALS AND METHODS

Mung-bean nuclease (EC 3.1.30.1) was obtained from Sigma (London) Chemical Co. as a freeze-dried powder of specific activity 58000 units/mg of protein (1 unit solubilizes 1 μg of denatured calf thymus DNA/min at pH 5.0 and 37 °C). Nuclease P1 was obtained from Sigma (London) as a freeze-dried powder of specific activity 320 units/mg of protein (1 unit liberates 1 μmol of acid-soluble nucleotides from RNA/min at pH 5.3 and at 37 °C). Snake (*Crotalus durissus terrificus*)-venom phosphodiesterase was obtained from Boehringer Mannheim as a suspension in 50% (v/v) glycerol, pH 6.0, with a specific activity of 52 units/mg of protein (with UDP-glucose as substrate).

Thymidine was obtained from Pharma Waldhof G.m.b.H. (Düsseldorf, Germany). Thymidine 5'-O-phosphorothioate was synthesized as described by Potter & Eckstein (1984). ^{18}O -enriched water (98 atom%) was obtained from Amersham International. N^6 -benzoyl-5'-O-monomethoxytrityl-2'-deoxyadenosine was synthesized as described by Schaller *et al.* (1963) as modified by

Abbreviations used: R_p - and S_p -d[Ap(S)T], the R_p and S_p diastereoisomers of 5'-O-thymidyl 3'-O-(2'-deoxyadenosyl) phosphorothioate; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate; TEAA, triethylammonium acetate; TMPS, thymidine 5'-O-phosphorothioate.

* To whom correspondence and reprint requests should be sent.

Gait *et al.* (1982) and phosphitylated to *N*⁶-benzoyl-5'-*O*-monomethoxytrityl-2'-deoxyadenosine-3'-*O*-di-isopropylaminomethoxyphosphoramidite by using bis(di-isopropylaminomethoxyphosphine) as described by Barone *et al.* (1984). 3'-Dimethoxytritylthymidine was synthesized as described by Matteucci & Caruthers (1980). Diazomethane was synthesized from Diazald (Aldrich) as described by de Boer & Backer (1963). DMF was dried and purified by azeotropic distillation of benzene, followed by treatment with activated alumina and MgSO₄ and subsequent distillation *in vacuo*. Methanol was dried and purified by distillation from iodine and magnesium turnings. Acetonitrile was dried by refluxing over CaH₂ and distilled from P₂O₅. 1-*H*-Tetrazole was resublimed before use.

H.p.l.c. was performed on a Shimadzu LC4A chromatograph using an Apex 5 μ C₁₈ ODS reverse-phase column (Jones Chromatography, Llanbradach, Caerphilly, Mid Glam., Wales, U.K.), with 6% (v/v) acetonitrile in 50 mM-TEAA, pH 6.8, as eluant. Nucleotides eluted from the column were detected by a Shimadzu SPD-2AS spectrophotometric detector operating at 257 nm. Ion-exchange chromatography was performed using columns of Sephadex A-25 resin and simple linear gradients derived from gravity mixing of TEAB buffers from two chambers. Fractions were collected on an LKB Ultrarac 2070 fraction collector with a Uvicord II detector attached to an LKB 2210 chart recorder. Silica-gel flash column chromatography was performed on silica-gel 60H (Merck).

³¹P-n.m.r. spectra were recorded either on a Bruker AM300 spectrometer operating at 121.5 MHz or on a Jeol FX60 spectrometer at 24.15 MHz with broad-band proton decoupling. Samples, dissolved in the appropriate solvent, were contained in 5 mm precision tubes. Chemical shifts (δ) are given in p.p.m. and are referenced to 85% H₃PO₄, being positive when downfield from this reference.

d[Ap(S)T]

*N*⁶-Benzoyl-5'-*O*-monomethoxytrityl-2'-deoxyadenosine-3'-*O*-di-isopropylaminomethoxy phosphoramidite (330 mg, 420 μ mol), 3'-dimethoxytritylthymidine (200 mg, 380 μ mol) and tetrazole (120 mg, 1.7 mmol) were dissolved in dry acetonitrile (10 ml) and stirred together under N₂ for 2 h. Pyridine (10 ml) and sulphur (200 mg) were then added and stirring was continued for a further 2 h. The mixture was filtered and the filtrate evaporated to dryness and dissolved in chloroform (50 ml). The chloroform solution was washed with saturated NaHCO₃, followed by brine (saturated NaCl), dried (Na₂SO₄) and evaporated. The residue was dissolved in methanol and filtered to remove traces of sulphur. T.l.c. (methanol/chloroform, 3:97, v/v) showed the product as a pair of spots with *R*_F values 0.40 and 0.42. ³¹P n.m.r. showed, among other peaks, a peak at 71.8 p.p.m. which split into two peaks at 72.0 and 71.6 p.p.m. at lower sweep width, corresponding to the two diastereoisomers of the fully blocked title compound, 5'-*O*-(3'-dimethoxytrityl)-3'-*O*-(*N*⁶-benzoyl-5'-*O*-monomethoxytrityl-2'-deoxyadenosyl)-*O*-methyl phosphorothioate.

Flash column chromatography on a 2 cm $\times$ 30 cm column run in chloroform with a gradually increasing concentration of methanol (to 5%) gave two sets of combined fractions. The first (fraction A) was almost

Fig. 1. Diastereoisomers of d[Ap(S)T]

pure 'fast' diastereoisomer (*R*_F, 0.42; ³¹P, 72.0 p.p.m.), and the second (fraction B) was an approx. 5:3 mixture of 'slow' and 'fast' diastereoisomers. The two fractions were separately treated with a mixture of thiophenol, triethylamine and dioxan (2 ml, 1:1:2, by vol.) for 1 h at ambient temperature, followed by evaporation of solvent and trituration of the resulting gums with petrol. These gums were then treated with conc. NH₃ at 50 °C in sealed vessels overnight, followed by evaporation of solvent, dissolution in water and extraction of the solutions with diethyl ether. The resulting two solutions were then treated with 80% (v/v) acetic acid at ambient temperature for 2 h to remove the trityl groups, the solvent evaporated and the residues redissolved in water and these solutions extracted with diethyl ether. ³¹P n.m.r. now showed two peaks at 58.1 and 57.5 p.p.m. for the deblocked diastereoisomers. Fraction A showed these peaks in a ratio of about 9:1 respectively, whereas fraction B showed them in a ratio of about 3:5. H.p.l.c. (Apex 5 μ ODS, 6% isocratic acetonitrile/50 mM-TEAA, pH 6.8) showed the two diastereoisomers of d[Ap(S)T] as well-separated peaks of retention times 16.1 and 24.7 min. The mixtures of diastereoisomers were further purified by ion-exchange chromatography on a 2 cm $\times$ 20 cm column of DEAE-Sephadex A-25 run in a linear gradient of 20–250 mM-TEAB pH 7.0. After pooling of product-containing fractions and evaporation of solvent, followed by successive co-evaporations with methanol, pure samples of diastereoisomeric mixtures of d[Ap(S)T] were obtained: Fraction A, *R*_p/*S*_p 9:1, 47 μ mol; fraction B, *R*_p/*S*_p 3:5, 62 μ mol. The total yield was 109 μ mol (29%) (for configurational assignment, see below).

Configurational assignments of the diastereoisomers of d[Ap(S)T]

A sample of fraction B (2 μ mol) was incubated with nuclease P1 (20 units) in 30 mM-acetate buffer, pH 5.3, for 24 h at 37 °C. H.p.l.c. showed that the peak at 24.7 min had completely disappeared, producing peaks at 3.4 min (TMPS, assigned by co-injection with authentic material) and 4.8 min (dA, assigned by co-injection). The peak at 16.1 min remained unchanged.

A sample of fraction B (2 μ mol) was incubated with snake-venom phosphodiesterase (~30 μ l) in 200 mM-Tris/HCl, pH 8.75, containing 2 mM-MgCl₂ at 37 °C for 48 h. H.p.l.c. showed that the material giving rise to the peak at 16.1 min had been partly digested to compounds exhibiting peaks at 4.2 min, identified as thymidine, and 4.8 min, identified as 2-deoxyadenosine. That thymine

Scheme 1. Synthesis of d[Ap(S)T]

Abbreviations used: MMTr, monomethoxytrityl; DMTr, dimethoxytrityl; Me, methyl; bzA, *N*-benzoyladenine; *i*Pr₂, di-isopropyl; S₈, cyclo-octasulphur.

rather than TMPS had been produced was attributed to desulphurization and phosphatase action by impurities in the enzyme preparation (a common occurrence with commercial preparations of this enzyme).

The h.p.l.c. peak at 16.1 min can thus be assigned to the *R*_p diastereoisomer and that at 24.7 min to the *S*_p diastereoisomer.

Hydrolysis of d[Ap(S)T] by mung-bean nuclease

A mixture of *R*_p- and *S*_p-d[Ap(S)T] (0.5 μmol; *R*/*S*, 3:5) was incubated with mung-bean nuclease (200 units) in a

buffer comprising 50 mM-sodium acetate, 10 mM-zinc acetate, 1 mM-β-mercaptoethanol and Triton X-100 (0.001%), pH 4.8 at 37 °C, for 24 h. H.p.l.c. showed that the *S*_p diastereoisomer had been digested to the extent of about 25% giving TMPS and 2'-deoxyadenosine as products (confirmed by co-injection of authentic standards). Addition of further nuclease (200 units) and incubation for 24 h led to a total of 33% hydrolysis of *S*_p-d[Ap(S)T]. Small-scale experiments using diastereoisomers separated by h.p.l.c. confirmed that only the *S*_p diastereoisomer is a substrate.

Hydrolysis of d[Ap(S)T] in H₂¹⁸O by mung-bean nuclease

A mixture of the diastereoisomers of d[Ap(S)T] (*R*_p/*S*_p, 3:5; 8.5 μmol) was evaporated to dryness and dissolved in H₂¹⁸O (250 μl) to which was added 10-times-concentrated buffer to give the final concentrations of buffer and additives as in the 'unlabelled' experiment above; mung-bean nuclease was added (~1500 units) and the mixture incubated at 37 °C for 24 h. H.p.l.c. showed that ~30% of the *S*_p isomer had reacted. A further similar portion of enzyme was added and the mixture incubated a further 24 h. H.p.l.c. now showed ~60% reaction of *S*_p-d[Ap(S)T]. The solution was then applied to a column (1 cm × 30 cm) of Sephadex A-25 equilibrated with 30 mM-TEAB, pH 7.0, and the column was run with a linear gradient of 30–300 mM-TEAB (total volume 1200 ml). Three peaks were eluted: the first, eluted at ~40 mM-TEAB, was 2'-deoxyadenosine (~2 μmol), the second, eluted at ~100 mM-TEAB, was a mixture of unchanged *R*_p- and *S*_p-d[Ap(S)T] (~5 μmol) and the third, eluted at ~180 mM-TEAB, was [¹⁸O,¹⁸O]TMPS (~1.5 μmol), which was identical with authentic TMPS by h.p.l.c. and u.v.

Methylation of [¹⁸O,¹⁸O]TMPS

[¹⁸O,¹⁸O]TMPS (~1.5 μmol), to which had been added [¹⁸O₂]TMPS (~1 μmol) was dried by three evaporations of dry methanol, and the residue was dissolved in dry DMF (200 μl). A solution of ethereal diazomethane (1–1.5 ml, ~200–300 μmol) was then added and the resulting solution stirred at ambient temperature for 36 h in a sealed flask. After this time, excess diazomethane was removed with a stream of N₂, the solution was evaporated to dryness and the residual diastereoisomers of thymidine *S*-methyl-*O*-methyl-[¹⁸O,¹⁸O] phosphorothioate dissolved in DMF/[²H]methanol (3:1, v/v; 500 μl) for ³¹P-n.m.r. analysis.

Table 1. ³¹P n.m.r., chromatographic and h.p.l.c. data for the diastereoisomers of d[Ap(S)T]

The solvent for the protected diastereoisomers was chloroform; for the deprotected diastereoisomers it was methanol; 'fast' and 'slow' refer to chromatographic mobilities on silica-gel 60H for the protected diastereoisomers and mobility on h.p.l.c. for the deprotected diastereoisomers. H.p.l.c. was performed as described in the Materials and methods section.

Diastereoisomer	³¹ P n.m.r. shift (p.p.m.)		H.p.l.c. retention time (min) (deprotected)
	Protected	Deprotected	
<i>R</i> _p -d[Ap(S)T]	'Fast' 72.0 <i>R</i> _F 0.42	'Fast' 58.1	16.1
<i>S</i> _p -d[Ap(S)T]	'Slow' 71.6 <i>R</i> _F 0.40	'Slow' 57.5	24.7

Fig. 2. H.p.l.c. analysis of the hydrolysis of S_p -d[Ap(S)T] in $H_2^{18}O$ by mung-bean nuclease

H.p.l.c. was performed on an Apex ODS 5μ column run isocratically in 50 mM-TEAA (pH 6.8)/acetonitrile (47:3, v/v). Peaks are: 1, $[^{18}O]$ TMPS; 2, 2'-deoxyadenosine; 3, R_p -d[Ap(S)T]; 4, S_p -d[Ap(S)T].

RESULTS

Synthesis

The diastereoisomers of d[Ap(S)T] have been synthesized by coupling N^8 -benzoyl-5'- O -monomethoxytrityl-2'-deoxyadenosine-3'- O -di-isopropylaminomethoxyphosphoramidite with 3'- O -dimethoxytritylthymidine, and subsequent oxidation to the fully blocked dinucleoside phosphorothioate triester (Scheme 1). Partial separation of diastereoisomers by silica-gel flash chromatography and subsequent deblocking gave 90% diastereoisomerically pure R_p -d[Ap(S)T]. (Small samples of $\geq 95\%$ pure diastereoisomers could be obtained by h.p.l.c. of the deblocked compound). Absolute configurations were assigned by differential digestions of R_p - and S_p -d[Ap(S)T] by snake-venom phosphodiesterase, which

Fig. 3. ^{31}P -n.m.r. spectrum of the diastereoisomers of N^8 -methylthymidine 5'- S -methyl- O -methyl- $[^{18}O]$ phosphorothioate [~ 50 mM solution in DMF/ $[^2H]$ methanol (3:1, v/v)]

Chemical shifts: R_p , 29.9 p.p.m.; S_p , 30.7 p.p.m. ^{31}P -n.m.r. parameters were: sweep width, 3649 Hz; pulse width, 5 μ s; acquisition time, 2.25 s; block size, 32K; no of transients, 200. Abbreviations used: Me, methyl; T' , N^8 -methylthymine.

digests the R_p -diastereoisomer (Eckstein, 1983, 1985), and nuclease P1, which digests the S_p -diastereoisomer (Potter *et al.*, 1983b; Eckstein, 1983, 1985).

Chromatographic mobilities and ^{31}P -n.m.r. data are summarized in Table 1.

Scheme 2. Cleavage of S_p -d[Ap(S)T] by mung-bean nuclease and stereochemical analysis of $[^{18}O, ^{18}O]$ TMPS

Abbreviations used: Me, methyl; $\bullet$, ^{18}O ; A, adenine; T' , N^8 -methylthymine.

Fig. 4. ^{31}P -n.m.r. spectrum of the diastereoisomers of N^3 -methylthymidine 5'-S-methyl-O-methyl- $^{16}\text{O}, ^{18}\text{O}$]phosphorothioate derived from the mung-bean-nuclease-catalysed cleavage of S_P -d[Ap(S)T] in H_2^{18}O

N^3 -Methylthymidine 5'-S-methyl-O-methyl- $^{16}\text{O}_2$]phosphorothioate was added as reference. The total concn. was ~ 5 mM in DMF/ ^2H]methanol (3:1, v/v). ^{31}P -n.m.r. parameters were: sweep width, 4854 Hz; pulse width, 5 μs ; acquisition time, 1.69 s; block size, 32K; no. of transients, 12000; isotope shifts: on S_P , 2.5 Hz; on R_P , 5.7 Hz. Abbreviations are as for Fig. 3; $\bullet$, ^{18}O .

Stereochemistry

S_P -d[Ap(S)T] was found to be a substrate for mung-bean nuclease, being digested to 2'-deoxyadenosine and TMPS. R_P -d[Ap(S)T] is not a substrate for this enzyme. S_P -d[Ap(S)T] was digested in ^{18}O -labelled water to $^{16}\text{O}, ^{18}\text{O}$]TMPS (as monitored by h.p.l.c.; Fig. 2), which was methylated by diazomethane to thymidine 5'-S-methyl-O-methyl- $^{16}\text{O}, ^{18}\text{O}$]phosphorothioate (Scheme 2). The diastereoisomers of the unlabelled compound have been assigned (J. H. Cummins & B. V. L. Potter, unpublished work) in an analogous fashion to those derived from 2'-deoxyadenosine 5'-phosphorothioate (Cummins & Potter, 1985), and are shown in Fig. 3. The S_P diastereoisomer resonates at low field.

When the ^{31}P -n.m.r. spectrum of the methylated $^{16}\text{O}, ^{18}\text{O}$]TMPS was examined (after addition of an approximately equal amount of unlabelled material), the spectrum shown in Fig. 4 was obtained. Two $^{31}\text{P}(^{18}\text{O})$ isotope shifts were observed, a small one (2.5 Hz) on the S_P diastereoisomer, and a larger one (5.7 Hz) on the R_P diastereoisomer. Since ^{18}O isotope shifts are dependent on bond order (Lowe *et al.*, 1979;

Cohn & Hu, 1980), being larger in doubly bonded systems compared with singly-bonded systems, the ^{18}O atom could be located in the bridging P-OCH₃ system in the former, and in the doubly bonded P=O system in the latter. Consequently, the precursor $^{16}\text{O}, ^{18}\text{O}$]TMPS must have had the S_P configuration. Since the S_P diastereoisomer of d[Ap(S)T] was digested, the cleavage reaction is therefore shown to proceed with *inversion* of the configuration at the phosphorus atom.

DISCUSSION

We describe here a synthesis of the diastereoisomers of d[Ap(S)T]. Reference to this compound has been made previously in relation to its use in characterizing phosphorothioate-containing poly[d(A-T)] (Eckstein & Jovin, 1983); however, no synthetic details have been published. Our findings (Table 1), that the diastereoisomers of d[Ap(S)T] are chromatographically separable, are in line with previously published reports for the diastereoisomers of d[Tp(S)A] (Romaniuk & Eckstein, 1982), Tp(S)T (Bartlett & Eckstein, 1982; Uznanski *et al.*, 1982; Potter *et al.*, 1983b), d[Gp(S)T] and d[Cp(S)T] (Eckstein, 1983), d[Gp(S)A] (Connolly *et al.*, 1984), d[Gp(S)C] and d[Cp(S)G] (Cosstick & Eckstein, 1985) and d[Gp(S)G] (M. R. Hamblin, A. Holland & B. V. L. Potter, unpublished work), which demonstrate that the 'fast' diastereoisomer on reversed-phase h.p.l.c. has the R_P configuration and resonates at low field in the ^{31}P -n.m.r. spectrum, and correspondingly the 'slow' diastereoisomer has the S_P configuration and resonates at high field.

We have used S_P -d[Ap(S)T] as a substrate for mung-bean nuclease and have shown here that cleavage of this dinucleoside phosphorothioate occurs with inversion of the configuration at the phosphorus atom.

More than 40 enzymic phospho- and nucleotidyl-transfer reactions have now been investigated using either chiral phosphorothioates or purely oxygen-isotope-labelled substrates. Evidence has been accumulated which indicates that reactions proceeding with a single displacement at phosphorus have the stereochemical consequence of inversion of configuration at phosphorus, and a reaction requiring the formation and breakdown of a covalent enzyme-bound intermediate, thus necessitating two displacements at phosphorus, will proceed with retention of configuration at phosphorus (Knowles, 1980; Frey, 1982; Eckstein, 1983; Gerlt *et al.*, 1983). Although, in principle, more complicated mechanisms are possible, such as those involving pseudorotation, triple displacements at phosphorus, or the possible attack of an active-site carboxylate on the phosphodiester linkage with inversion of configuration at phosphorus and the formation of an acyl-phosphate-enzyme intermediate [where subsequent attack of water on the carboxy-group carbon of this intermediate would lead to a net stereochemical course of inversion of configuration at phosphorus, although an enzyme intermediate had been involved (Gerlt *et al.*, 1983)], there is no evidence compelling consideration of such mechanisms for any enzyme yet studied.

We have carried out this investigation with a dinucleoside phosphorothioate rather than with a dinucleoside phosphate isotopically chiral at phosphorus (Potter *et al.*, 1983b; Gerlt *et al.*, 1983; Seela *et al.*, 1983). However, in those cases where both chiral phosphates

Scheme 3. Proposed mechanism of action of mung-bean nuclease

B represents a general base in the active site of the enzyme.

and phosphorothioates have been used for the same enzyme, the stereochemical outcomes of the reactions have been the same (Gerlt *et al.*, 1983).

Our configurational analysis involves an extension (J. H. Cummins & B. V. L. Potter, unpublished work) of a method which we have recently developed (Cummins & Potter, 1985) whereby a chiral nucleoside 5'-[^{18}O , ^{18}O]phosphorothioate is methylated with diazomethane and the resulting diastereoisomeric triesters examined by ^{31}P -n.m.r spectroscopy for ^{31}P (^{18}O) isotope shifts. We have used d[Ap(S)T] as our substrate because of the known preference of mung-bean nuclease for cleavage in AT-rich regions at d[A]pN] and d[T]pN] sites (Johnson & Laskowski, 1970). Although methods exist for the configurational analysis of deoxyadenosine 5'-[^{18}O , ^{18}O]phosphorothioate (Frey, 1982), these are dependent on the stereospecificity of adenylate kinase, and for other labelled nucleotides, including the thymidine 5'-phosphorothioate obtained here, no other methods are available except ours.

The simplest interpretation of our finding that mung-bean nuclease catalyses cleavage with inversion of configuration at phosphorus is that the hydrolysis occurs with a direct 'in-line' attack of water opposite the nucleotide-3'-O-linkage in our substrate (and presumably, of course, in natural single-stranded DNA) as shown in Scheme 3, rather than via the intermediacy of a covalent nucleotidyl-enzyme intermediate. This finding is in contrast with what has been found for snake-venom phosphodiesterase (Bryant & Benkovic, 1979; Burgers

et al., 1979; Mehdi & Gerlt, 1981b) and spleen phosphodiesterase (Mehdi & Gerlt, 1981a), where retention of configuration has been observed and consequently nucleotidyl-enzyme intermediates are implicated. However, it should be discussed in the light of other recent stereochemical results for members of the single-stranded nuclease family, nuclease S1 from *Aspergillus oryzae* (Potter *et al.*, 1983a) and nuclease P1 from *Penicillium citrinum* (Potter *et al.*, 1983b).

Both of these latter enzymes also catalyse phosphodiester cleavage with inversion of configuration at phosphorus, and presumably via the 'in-line' mechanism. Additionally, they too use the S_p diastereoisomer of a dinucleoside phosphorothioate as substrate. Like P1 and S1 nucleases, the mung-bean enzyme is a glycoprotein and a zinc metalloenzyme with multiple metal-ion-binding sites. Effectively, all three enzymes catalyse the same reaction, and although many similarities exist between these nucleases, there are quantitative differences between their individual catalytic properties. The determination of the stereochemical course of phosphodiester cleavage catalysed by these enzymes, however, allows mechanistic comparisons to be made at a very fundamental level in the absence of other information.

Although it is not generally understood why nucleases and phosphodiesterases use both types of mechanism in the reactions they catalyse, it appears at least that, in the small subgroup of single-strand-specific nucleases, of which mung-bean nuclease is a member, some mechanistic uniformity is emerging.

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