Stereochemical Outcome of the Hydrolysis Reaction Catalyzed by the *Eco*RV Restriction Endonuclease†

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ABSTRACT: The stereochemical course of the reaction catalyzed by the *Eco*RV restriction endonuclease has been determined. This endonuclease recognizes GATATC sequences and cuts between the central T and dA bases. The R_p isomer of d(GACGATsATCGTC) (this dodecamer contains a phosphorothioate rather than the usual phosphate group between the central T and dA residues, indicated by the s) was a substrate for the endonuclease. Performing this reaction in $H_2{}^{18}O$ gave [^{18}O]dps(ATCGTC) (a pentamer containing an ^{18}O-labeled 5′-phosphorothioate) which was converted to [^{18}O]dAMPS with nuclease P1. This deoxynucleoside 5′-[^{18}O]phosphorothioate was stereospecifically converted to [^{18}O]dATPαS with adenylate kinase and pyruvate kinase [Brody, R. S., & Frey, P. A. (1981) *Biochemistry 20,* 1245–1251]. Analysis of the position of the ^{18}O in this product by ^{31}P NMR spectroscopy showed that it was in a bridging position between the α- and β-phosphorus atoms. This indicates that the *Eco*RV hydrolysis proceeds with inversion of configuration at phosphorus. The simplest interpretation is that the mechanism of this endonuclease involves a direct in-line attack at phosphorus by H_2O with a trigonal bipyramidal transition state. A covalent enzyme oligodeoxynucleotide species can be discounted as an intermediate. An identical result has been previously observed with the *Eco*R1 endonuclease [Connolly, B. A., Eckstein, F., & Pingoud, A. (1984) *J. Biol. Chem. 259,* 10760–10763]. X-ray crystallography has shown that both of these endonucleases contain a conserved array of amino acids at their active sites. Possible mechanistic roles for these conserved amino acids in the light of the stereochemical findings are discussed.

Type II restriction endonucleases cut double-stranded DNA at sequence-specific sites typically 4–6 base pairs in length. Progress in understanding how these enzymes achieve their high specificity has been greatly aided by the crystal structures of both the *Eco*RI (McClarin et al., 1986; Kim et al., 1990; Rosenberg, 1991) and the *Eco*RV (Winkler, 1992; Winkler et al., 1992) endonucleases. Cocrystals with oligodeoxynucleotides containing the cognate sequence (GAATTC for *Eco*RI, GATATC for *Eco*RV) have shown that direct protein contacts to the bases, DNA distortion on binding, and protein contacts to the phosphate groups of the distorted bound substrate all contribute to the recognition of the target sites. However, details of the catalytic site and possible hydrolysis mechanisms remain obscure. Both proteins contain two acidic side chains and a lysine residue in similar orientations near the scissile phosphodiester group (these are Lys92, Asp74, and Asp90 for *Eco*RV and correspondingly Lys113, Asp91, and Glu111 for *Eco*RI) as well as a third acidic group (Glu45 and Glu144 for RV and R1, respectively) more remote from the active site. It is thought that the two proximal acidic groups may form the binding site for Mg^{2+}, an essential cofactor for hydrolysis (all crystal structures were determined in the absence of Mg^{2+} to prevent hydrolysis). Some time ago it was shown that the reaction catalyzed by the *Eco*R1 endonuclease proceeded with inversion of configuration at phosphorus (Connolly et al., 1984a). This was interpreted as a direct in-line attack on the scissile phosphodiester by a water molecule with a trigonal bipyramidal transition state (Knowles, 1980; Eckstein, 1983, 1985; Gerlt et al., 1983; Frey, 1989). The role of the lysine residue may be to stabilize the negative charges on this phosphate group in the transition state. Problems still remain with this mechanism. In both crystal structures there is no appropriately placed amino acid which could act as a base to activate the attacking water molecule. In view of this difficulty, we have now decided to investigate the stereochemical course of the *Eco*RV hydrolysis. This will show if the two endonucleases, which have conserved amino acids at their active sites, use similar catalytic mechanisms. Furthermore, *Eco*RV is an intensely studied endonuclease (second only to *Eco*R1). Not only are excellent crystal structures available but a variety of studies involving kinetics with plasmids (Halford et al., 1986; Halford & Goodall, 1988; Taylor & Halford, 1989; Olsen et al., 1990; Taylor et al., 1990, 1991), oligodeoxynucleotides (Fliess et al., 1986, 1988; Mazarelli et al., 1989; Newman et al., 1990a,b), and also site-directed mutagenesis (Thielking et al., 1991, 1992; Selent et al., 1992) have helped in the delineation of its mode of action. Evaluation of the *Eco*RV endonuclease stereochemical course would add to this knowledge and may be helpful in answering the crucial question of how restriction endonucleases couple recognition of specific sequences to phosphodiester bond cleavage.

MATERIALS AND METHODS

The *Eco*RV restriction endonuclease was purified from an overproducing *Escherichia coli* strain (Bougueleret et al., 1985) using slight modifications to a previously published method (D'Arcy et al., 1985). The *E. coli* cell disruption, chromatography on phosphocellulose, and $(NH_4)_2SO_4$ precipitation of the phosphocellulose pool that contained the endonuclease were as in this paper. This $(NH_4)_2SO_4$ precipitate

† This paper is dedicated to Prof. F. Eckstein on the occasion of his 60th birthday.

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was then dissolved in 7 mL of 20 mM KH_2PO_4 (pH 7.4) containing 200 mM NaCl, 2 mM dithioerythreitol, and 1 mM EDTA. Final purification was by gel filtration (of 14 × 0.5 mL samples) using a Millipore-Waters protein purification system equipped with a Protein-Pak glass 300SW column (8 × 300 mm) and the above buffer at a flow rate of 1 mL min^{-1}. The eluate was monitored at 280 nm, and fractions that contained the endonuclease (retention time 11.4 min) were pooled and concentrated to about 2 mL using an Amicon Centriprep 10 spun concentrator at 4 °C. The resulting solution was rapidly frozen (dry ice/acetone) and water removed by freeze-drying to give a solid. This was redissolved in 700 μL of $H_2{}^{18}O$/glycerol (4:3 v/v) and stored at −20 °C. SDS–PAGE showed that the endonuclease was >95% pure. The endonuclease concentrated was determined by absorbance at 280 nm using an $E^{1\%}$ value of 18.0 (D'Arcy et al., 1985).

Snake venom phosphodiesterase and alkaline phosphatase were obtained from Promega (Southampton, Hants, U.K.). Nuclease P1, rabbit muscle adenylate kinase, and pyruvate kinase were purchased from Boehringer (Lewes, East Sussex, U.K.). The adenylate kinase was supplied as an $(NH_4)_2SO_4$ suspension and was dialyzed extensively against 50 mM Tris (pH 7.5), 0.1 mM EDTA, and 0.1 mM dithioerythreitol prior to use. All units refer to the definitions given by the manufacturers. Oxygen-18-containing water was obtained from Amersham International (Amersham, U.K.) and contained 91% ^{18}O. All other reagents were from the usual suppliers.

Synthesis of Phosphorothioate-Containing Oligodeoxynucleotides. The R_p and S_p diastereomers of d(GACGATsATCGTC) (this nomenclature with an s between the central T and dA bases indicates that a phosphorothioate rather than the usual phosphate group is present at this position) were synthesized on an Applied Biosystems 381A DNA synthesizer on a 10-μmol scale using the usual 10-μmol synthesis cycle. The phosphoramidite method was used, and the required deoxynucleoside phosphoramidites and ancillary reagents were purchased from Applied Biosystems (Warrington, Cheshire, U.K.). The synthesis cycle was interrupted during the addition of the seventh base (T) immediately prior to the iodine oxidation step. The intermediate phosphite triester was sulfurized to give the required phosphorothioate using a 0.05 M solution of 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Iyer et al., 1990a,b). This reagent was either kindly supplied by Dr. S. Beaucage (FDA, Bethesda, MD) or else bought from Cambio Ltd. (Cambridge, U.K.). The sulfurizing solution was allowed to flow to the column for 270 s. After a 45-s wait, the column was washed with acetonitrile and the synthesis resumed. Syntheses were performed trityl-off, and after ammonia deblocking in the usual manner, two such 10-μmol syntheses yielded 1352 A_{254nm} units (in a volume of 16 mL) of a mixture of the two diastereomers. The two diastereomers were separated by reversed-phase HPLC using Apex 1 octadecylsilyl (C-18) columns (5-μm particle size, 25 × 0.45 cm) bought from Jones Chromatography (Llanbradach, Wales). A linear gradient system consisting of t = 0, 100% HPLC buffer A, 0% HPLC buffer B, and t = 25 min 88% HPLC buffer A, 12% HPLC buffer B [A, 0.1 M triethylammonium acetate (pH 6.5) containing 5% acetonitrile; B, 0.1 M triethylammonium acetate (pH 6.5) containing 65% acetonitrile], at a flow rate of 1 mL min^{-1} and a column temperature of 55 °C was used. Aliquots of 120 μL were injected (necessitating a total of 135 injections) and the column eluate monitored at 254 nm [for a more detailed description of the HPLC apparatus used, see Connolly and Newman (1989)]. The more rapidly eluting diastereomer (which has the *R* configuration, see below) had a retention time of 19.2 min, whereas the later eluting diastereomer (of *S* configuration) had a retention time of 19.7 min. The fractions containing either the *R* or the *S* diastereomers were pooled and the volumes reduced to about 30 mL by rotary evaporation. These solutions were desalted by dialysis against 2 × 1 L of distilled water using benzoylated low molecular weight cutoff dialysis tubing (Sigma, Poole, Dorset, U.K.). This tubing retains the oligonucleotides but allows the triethylammonium acetate to pass through. The purity of the separated diastereomers was checked using the above HPLC conditions; 460 A_{254nm} units of the fast eluting isomer and 451 A_{254nm} units of the slow were obtained. This represents a yield of about 27% for each diastereomer beginning from 20 μmol of support-bound dC. The concentrations of these two dodecamers were determined using an E_{254nm} of 1.66×10^5 M^{-1} cm^{-1} for the double-stranded form, the true substrate for the endonuclease (Newman et al., 1990a).

Configurational Analysis of the Fast and Slow Diastereomers of d(GACGATsATCGTC). To a solution of purified diastereomer (1 OD_{254nm}) in 80 μL of H_2O was added 10 μL of a 1 mg mL^{-1} solution of nuclease P1. After a 3-h incubation at 30 °C, 100 μL of 200 mM Hepes (pH 7.5), 200 mM NaCl, 20 mM $MgCl_2$, and 5 μL of a 1 mg mL^{-1} solution of alkaline phosphatase were added, and the solution was left for a further 10 min. The deoxynucleosides produced were analyzed by HPLC (columns and HPLC buffers A and B as above) using a gradient consisting of t = 0, 97% A, 3% B, t = 25 min, 75% A, 25% B, and t = 35 min, 50% A, 50% B, at a flow rate of 1 mL min^{-1} and at room temperature. The identity of the deoxynucleosides was confirmed by coinjection of standard dC, dG, T, and dA (Sigma). A standard of dAMPS was kindly provided by Prof. F. Eckstein (Gottingen). Assignment of d(TsA) was tentative as no standard exists for this compound. To a solution of pure diastereomer (1 OD_{254nm}) in 100 μL of 50 mM Hepes (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ were added 10 μL of a 1 mg mL^{-1} solution of snake venom phosphodiesterase and, after a 3-h incubation at 37 °C, 5 μL of a 1 mg mL^{-1} solution of alkaline phosphatase. After a further 10 min, the deoxynucleosides were analyzed as for the nuclease P1 digestion.

Initial Investigation of the EcoRV-Catalyzed Hydrolysis of Phosphorothioate-Containing Oligodeoxynucleotides. To a 12 μM solution of either S_p or R_p d(GACGATsATCGTC) in 390 μL of 50 mM Hepes (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ was added 10 μL of a 22 μM *Eco*RV endonuclease stock solution (final enzyme concentration 0.9 μM). Aliquots were withdrawn at 1, 3, and 24 h and analyzed by HPLC using the systems given for the synthesis of the sulfur-containing dodecamers. Any hydrolysis products of this reaction were collected using this HPLC system and subject to deoxynucleoside composition analysis using nuclease P1 and alkaline phosphatase as above.

Comparison of the Rates of the EcoRV-Catalyzed Hydrolysis with d(GACGATATCGTC) and R_p-d(GACGATsATCGTC). To a 1-mL solution of 6 μM dodecamer in 50 mM Hepes (pH 7.5), 100 mM NaCl, and 10 mM $MgCl_2$ was added *Eco*RV endonuclease to give a final concentration of 0.2 μM. The samples were incubated at 25 °C and the rates determined by the increase in absorbance at 254 nm with time (Waters & Connolly, 1992).

Dependence of the Rate of EcoRV Hydrolysis of R_p-d(GACGATsATCGTC) with Mg^{2+} Concentration. To a 0.5-

mL solution containing 6 μM dodecamer in 50 mM Hepes (pH 7.5) and either 124 mM NaCl/2 mM $MgCl_2$, 115 M NaCl/5 mM $MgCl_2$, 100 mM NaCl/10 mM $MgCl_2$, 70 mM NaCl/20 mM $MgCl_2$, 40 mM NaCl/30 mM $MgCl_2$, or 10 mM NaCl/40 mM $MgCl_2$ was added *Eco*RV endonuclease to give a final concentration of 0.22 μM. The solutions were incubated at 25 °C and the rates of reaction monitored by the increase in 254-nm absorbance with time (Waters & Connolly, 1992).

Hydrolysis of R_p-d(GACGATsATCGTC) in $H_2{}^{18}O$ for Stereochemical Course Evaluation. R_p-d(GACGATsATCGTC) (340 A_{254nm} units), 2 μmol of double-stranded substrate, or 4 μmol of phosphorothioate equivalents was dissolved in 3 mL of 50 mM Hepes (pH 7.5), 10 mM NaCl, and 40 mM $MgCl_2$ in $H_2{}^{18}O$. To this solution was added 200 μL of a 650 μM *Eco*RV endonuclease stock (in $H_2{}^{18}O$, glycerol) (final enzyme concentration 43 μM) and the mixture incubated at room temperature. HPLC (conditions described under Synthesis of Phosphorothiate-Containing Oligodeoxynucleotides) showed that the reaction was complete after 195 min. The solution was heated at 70 °C for 1 h to denature the endonuclease and rapidly frozen using dry ice/acetone and the $H_2{}^{18}O$ removed by freeze-drying.

Preparation of [^{18}O]dAMPS from the [^{18}O]dps(ATCGTC) Produced by the Endonuclease. The lyophilized mixture produced above was redissolved in H_2O, and 500 μL of nuclease P1 (1 mg mL^{-1} stock solution) was added. The reaction was monitored by HPLC using the system given under Configurational Analysis of the Fast and Slow Diastereomers of d(GACGATsATCGTC). Small samples were removed, and 1 μL of a 1 mg mL^{-1} solution of alkaline phosphatase was added prior to analysis. After 6 h at 37 °C, the nuclease P1 digestion was complete and 100 μL of 1 mg mL^{-1} alkaline phosphatase was added to the main mixture. HPLC showed complete reaction after a further 30 min at 37 °C, and the mixture was heated at 70 °C for 1 h to destroy the enzymes. The [^{18}O]dAMPS was separated from the other deoxynucleosides using a Millipore-Waters protein purification system equipped with a Protein-Pak DEAE 5PW (7.5 × 75 mm) ion-exchange column. The buffers employed were 0.025 M triethylammonium bicarbonate (C) and 1 M triethylammonium bicarbonate (D), and a linear gradient of 10–40% D over 40 min was used at 1 mL min^{-1}. The [^{18}O]dAMPS eluted after 21.2 min, whereas all other deoxynucleosides eluted in the void volume (after 4–6 min). Ten injections were required to purify the [^{18}O]dAMPS, and all of the fractions that contained this product were pooled and evaporated to dryness. The residue was freed from triethylammonium bicarbonate by repeated coevaporations from methanol. A total of 24.5 A_{260nm} units (1.7 μmol, 42% yield from starting dodecamer) of [^{18}O]dAMPS was produced, which was pure by HPLC.

Phosphophorylation of [^{18}O]dAMPS to S_p-[^{18}O]dATPαS. The [^{18}O]dAMPS produced above was dissolved in 1.5 mL of 50 mM Tris (pH 7.5) containing 50 mM KCl, 10 mM $MgCl_2$, 0.8 mM ATP, and 10 mM phosphoenolpyruvate. Pyruvate kinase (120 units) and adenylate kinase (1500 units) were added, and the mixture was incubated at 37 °C for 2.5 h. HPLC [buffer conditions given under Configurational Analysis of the Fast and Slow Diastereomers of d(GACGATsATCGTC)] indicated complete reaction after this time. The S_p-[^{18}O]dATPαS produced was purified using a Millipore-Waters protein purification system/Protein-Pak DEAE 5PW column detailed above. A linear gradient of t = 0, 90% buffer C, 10% buffer D; and t = 40 min, 30% buffer C, 70% buffer D (buffers C and D as above), at 1 mL min^{-1} was used. The

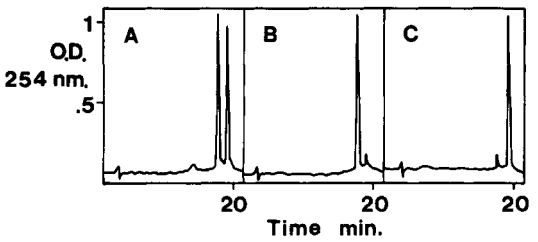

FIGURE 1: Reversed phase HPLC analysis of the phosphorothioate-containing dodecamers used in this paper. (A) Crude d(GACGATsATCGTC). The two diastereomers are clearly visible. As detailed in the text, the fast isomer has the R_p configuration and the slow the S_p. (B) Purified fast (R_p) d(GACGATsATCGTC). (C) Purified slow (S_p) d(GACGATsATCGTC).

S_p-[^{18}O]dATPαS eluted after 30.2 min, well-resolved from ATP (25 min); 15.6 A_{260nm} units of S_p-[^{18}O]dATPαS (1.1 μmol, 64% from [^{18}O]dAMPS) which was pure by HPLC was produced.

Determination of the ^{18}O Position in S_p-[^{18}O]dATPαS by ^{31}P NMR Spectroscopy. The S_p-[^{18}O]dATPαS produced above was dissolved in 0.5 mL of 10 mM Tris (pH 7) containing 100 mM EDTA in D_2O. The ^{31}P NMR spectrum was recorded on a Bruker WH 360 spectrometer operating at 145.79 MHz with ^1H broad band decoupling; 85% H_3PO_4 was employed as an external references. To measure the oxygen-18-induced shift in the ^{31}P resonances, S_p-dATPαS (7.5 A_{260nm} units) was added to the sample and a second spectrum recorded.

RESULTS AND DISCUSSION

To determine the stereochemical course of enzymatic reactions at phosphorus, it is necessary to render the substrates and products chiral and to determine the absolute configurations of both. This can be achieved using either sulfur (to give phosphorothioates) or the stable isotopes of oxygen (oxygen-17 and -18) (Knowles, 1980; Eckstein, 1983, 1985; Gerlt et al., 1983; Frey, 1989). In general, the phosphorothioate method is easier (both in terms of the preparation and in the analysis of substrate and products), and so we have selected this approach. Both methods have been demonstrated to give identical stereochemical outcomes. We have previously shown that d(GACGATATCGTC) is a substrate for the *Eco*RV endonuclease which cuts between the central T and dA bases giving d(GACGAT) and dp(ATCGTC) (Connolly & Newman, 1989; Newman et al., 1990a,b). Thus, we have decided to prepare the analogue of this dodecamer containing a phosphorothioate between the central T and dA bases. Oligodeoxynucleotides containing phosphorothioates are easily prepared by replacing the phosphite to phosphate conversion (using H_2O/I_2) with a sulfurization reaction (Connolly et al., 1984b; Stec et al., 1984; Ott & Eckstein, 1987; Zon & Stec, 1991). This has previously been performed using sulfur, but the reagent 3*H*-1,2-benzodithiol-3-one 1,1-dioxide (Iyer et al., 1990a,b) has been reported to be faster and give better yields. We have used this reagent and obtained excellent results. Figure 1 shows that after trityl-off synthesis and NH_3 deblocking, the crude d(GACGATsATCGTC) consisted of essentially two products which represent the two diastereomers arising because of the chiral phosphorothioate. These diastereomers could be separated by reversed-phase HPLC to give final products that were about 95% pure with 5% contamination by the other isomer (Figure 1). Studies using very many oligodeoxynucleotides that contain phosphorothioates have shown that the R_p isomer invariably elutes before the S_p on reversed-phase columns using triethylammonium acetate buffers (Connolly et al., 1984b; Stec et al., 1984, 1985).

Table I: Determination of the Absolute Configurations of the Fast and Slow Diastereomers of d(GACGATsATCGTC)[a]

digestion with	oligodeoxynucleotide	base composition		
		found	expected for R_p	expected for S_p
nuclease P1/alkaline phosphatase	d(GACGATsATCGTC) (fast)	T (2.1), dAMPS (0), d(TsA) (1.1)	T (2), dAMPS (0), d(TsA) (1)	T (3), dAMPS (1), d(TsA) (0)
	d(GACGATsATCGTC) (slow)	T (3), dAMPS (0.9), d(TsA) (0)	T (2), dAMPS (0), d(TsA) (1)	T (3), dAMPS (1), d(TsA) (0)
snake venom phosphodiesterase/ alkaline phosphatase	d(GACGATsATCGTC) (fast)	T (3.2), dAMPS (0.4), d(TsA) (0)	T (3), dAMPS (1), d(TsA) (0)	T (2), dAMPS (0), d(TsA) (1)
	d(GACGATsATCGTC) (slow)	T (1.9), dAMPS (0), d(TsA) (1.1)	T (3), dAMPS (1), d(TsA) (0)	T (2), dAMPS (0), d(TsA) (1)

[a] The method relies on the stereospecificities of nuclease P1 (which cleaves S_p diastereomers of nucleoside phosphorothioates) and snake venom phosphodiesterase (which cuts R_p isomers). The alkaline phosphate removes 5′-phosphate groups (but not 5′-phosphorothioates). In addition to the bases given above, all digests yielded the expected dG(3), dC(3) and dA(2). All figures in parentheses refer to equivalents expected or found. The low yield of dAMPS found using snake venom phosphodiesterase is due to desulfurization that is sometimes seen with this enzyme (Burgers et al., 1979).

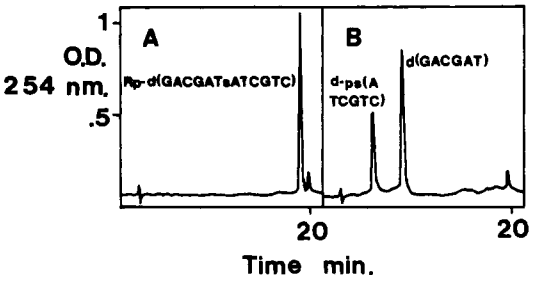

FIGURE 2: Cleavage of R_p-d(GACGATsATCGTC) by the *Eco*RV restriction endonuclease. (A) Time = 0 (no cleavage). (B) Time = 195 min (reaction complete). The identities of the substrates and products are shown. Prolonging the reaction time did not lead to any further degradation of the products, showing the absence of nonspecific nucleases.

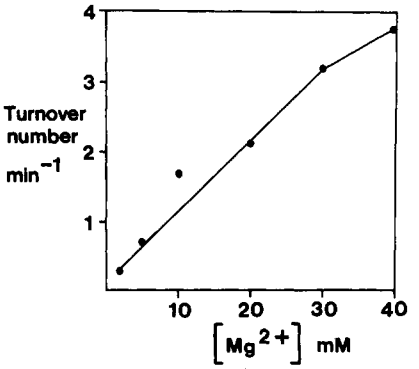

FIGURE 3: Effect of $[Mg^{2+}]$ on the rate of cleavage of R_p-d(GACGATsATCGTC) by the *Eco*RV restriction endonuclease. In all cases the ionic strengths of the reaction buffers were kept constant by appropriate adjustments to the [NaCl].

This was found in this case with the fast eluting peak corresponding to the R_p isomer and the slow S_p. This was shown using nucleases of known stereospecificities for phosphorothioates. Snake venom phosphodiesterase cleaves phosphorothioates having the R_p configuration but does not digest those of S_p (Burgers & Eckstein, 1978). With nuclease P1 these stereospecificities are reversed (Potter et al., 1983). Thus, digesting the resolved diastereomers with both nucleases and analysis of the deoxynucleoside products clearly established their absolute configurations at phosphorus as shown in Table I.

When R_p- and S_p-d(GACGATsATCGTC) were incubated with the *Eco*RV endonuclease, only the R_p isomer was found to be a substrate (Figure 2). Analysis of the products formed gave d(GACGAT) and d-ps(ATCGTC), confirming hydrolysis at the correct position (Figure 2). Prolonged hydrolysis with large quantities of *Eco*RV led to no measurable cleavage of the S_p isomer. An identical situation has been seen with the *Eco*R1 endonuclease where R_p but not S_p phosphorothioates are cut (Connolly et al., 1984a,b). In a recent study, the effect of phosphorothioates (of R_p configuration) in plasmid DNA on *Eco*RV cleavage has been investigated (Olsen et al., 1990). These plasmids contained a single phosphorothioate group at the *Eco*RV cleavage site (GATsATC), but only one of the DNA strands in the duplex was phosphorothioate substituted. Here the unmodified strand was nicked by the endonuclease while the phosphorothioate strand was refractory. These experiments are not strictly comparable to ours as our dodecamer has both strands substituted with phosphorothioate. It is not known if doubly substituted plasmid DNA can be cleaved, perhaps by using larger quantities of endonuclease.

The rate of cleavage of R_p-d(GACGATsATCGTC) by the *Eco*RV endonuclease was very dependent on Mg^{2+} concentration as shown in Figure 3. Hydrolysis increased as the Mg^{2+} levels were varied from 2 to 40 mM (the ionic strength of the assay solutions was kept the same by an appropriate decrease in NaCl levels). Previously we have observed a similar, but not identical, effect with dp(GACGATATCGTC), the parent dodecamer containing an additional 5′-phosphate group (Newman et al., 1990b). Here rates increased up to 20 mM $MgCl_2$ but remained relatively constant between 20 and 40 mM $MgCl_2$. Very recently we have shown that d(GACGATATCGTC), the true parent, has a very similar Mg^{2+} dependence on rate as the phosphorothioate (Waters and Connolly, unpublished observations). The origins of these requirements for high concentrations of Mg^{2+} remain obscure. Nevertheless, with plasmid substrates it has been noted that cognate sequence cleavage exhibits a low K_d for Mg^{2+}, whereas cutting at nearly cognate sequences shows a high K_d (Taylor & Halford, 1989). It has been suggested that this is important in the DNA ligase catalyzed repair of noncognate sequences incorrectly cut by the endonuclease (Taylor et al., 1990). With noncognate sequences the low affinity for Mg^{2+} means that only one subunit of the dimeric endonuclease contains Mg^{2+} and as a consequence only one of the DNA strands is cut. This nicked intermediate is a substrate for cellular DNA ligase. Short oligodeoxynucleotides, which may lack some of the endonuclease contacts to phosphate groups flanking the GATATC site, may behave similarly to noncognate plasmid sequences and so need high Mg^{2+} levels. Under identical Mg^{2+} concentrations (10 mM $MgCl_2$) we have observed that R_p-d(GACGATsATCGTC) is cut at 9% of the rate of d(GACGATATCGTC).

The evaluation of the stereochemical outcome of the *Eco*RV endonuclease using R_p-d(GACGATsATCGTC) is by a standard method illustrated in Figure 4. Hydrolysis in $H_2{}^{18}O$

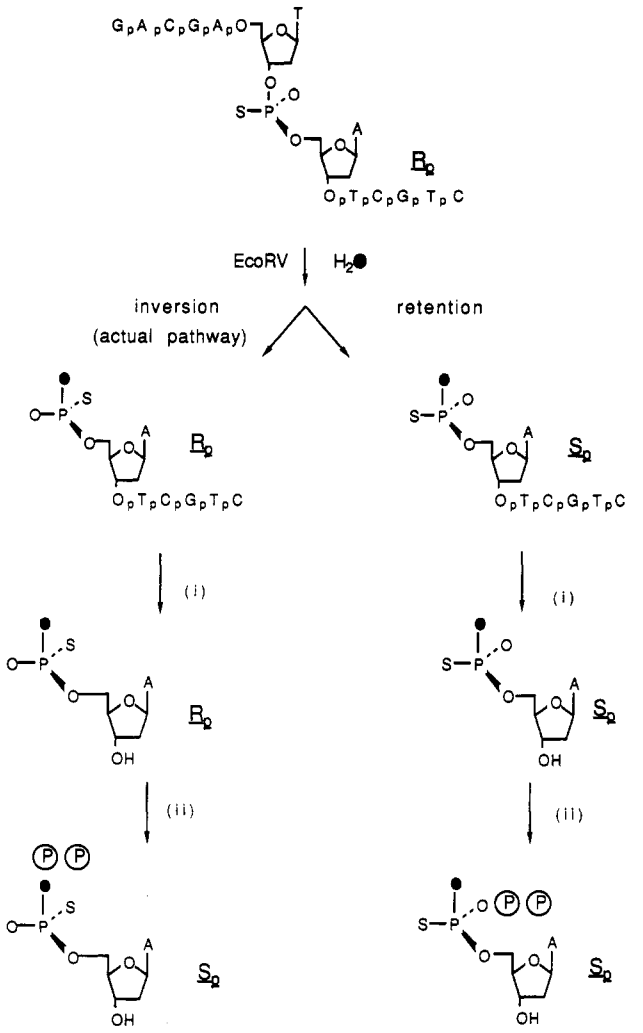

FIGURE 4: Possible stereochemical outcomes due to the *Eco*RV-catalyzed hydrolysis of R_p-d(GACGATsATCGTC) in $H_2{}^{18}O$. The consequences of this reaction occurring with both inversion and retention of configuration at phosphorus are shown. However, inversion is the actual pathway found. ● = ^{18}O. (i) Nuclease P1 and alkaline phosphatase. (ii) Adenylate kinase, pyruvate kinase, ATP, and phosphoenolpyruvate.

yields [^{18}O]dps(ATCGTC) of one of the two configurations shown. There is no need to separate this oligodeoxynucleotide from the coproduct d(GACGAT). Rather, when HPLC had shown that the *Eco*RV reaction was complete, the $H_2{}^{18}O$ was removed by freeze-drying and the two products dissolved in H_2O for further digestion with nuclease P1. This nuclease cleaves all of the remaining intact phosphodiester bonds, giving [^{18}O]dAMPS and dCMP, dGMP, TMP, and dAMP. Addition of alkaline phosphatase removes the 5′-phosphates but not the 5′-phosphorothioate, giving [^{18}O]dAMPS and dC, dG, T, and dA. Due to its negative charges the [^{18}O]dAMPS is easily purified from the deoxynucleosides by ion-exchange chromatography on DEAE columns. It should be noted that the complete hydrolysis observed with *Eco*RV, the nuclease P1 digestion being performed in H_2O rather than $H_2{}^{18}O$, and the inability of nuclease P1 to cut R_p-phosphorothioates all ensure that the ^{18}O incorporated into [^{18}O]dAMPS is solely due to the *Eco*RV endonuclease action. The high levels of Mg^{2+} (40 mM) and *Eco*RV endonuclease (43 μM) ensured that the hydrolysis proceeded rapidly and was complete in about 3 h. The two [^{18}O]dAMPS species shown in Figure 4 can be distinguished by stereospecific phosphorylation with adenylate kinase and pyruvate kinase, which give only S_p-[^{18}O]dATPαS (Brody & Frey, 1981). As this figure shows,

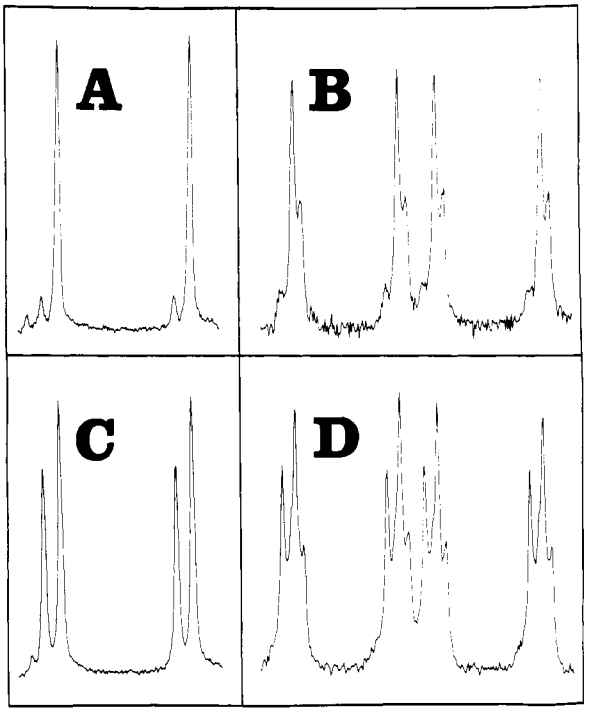

FIGURE 5: ^{31}P NMR spectra of the [^{18}O]dATPαS produced as the final product of the *Eco*RV-catalyzed hydrolysis of R_p-d(GACGATsATCGTC) (see Figure 4 for the complete reaction scheme). (A) α-Phosphorus region of [^{18}O]dATPαS. (B) β-Phosphorus region of [^{18}O]dATPαS. (C) α-Phosphorus region of [^{18}O]dATPαS to which has been added about 0.5 equiv of dATPαS. (d) β-Phosphorus region of [^{18}O]dATPαS to which has been added about 0.5 equiv of dATPαS. The α-phosphorus resonances occur at about 44 ppm and the β-phosphorus resonances at about −21.5 ppm. Part C shows that the upfield shift at the α-phosphorus due to the presence of ^{18}O is 3.4 Hz. Part D shows the upfield shift at the β-phosphorus due to this isotope is 2.4 Hz.

inversion of configuration by the endonuclease results in S_p-[α,β-^{18}O]dATPαS (i.e., the ^{18}O is in a bridging position between the α- and β-phosphorus atoms). Conversely, retention gives S_p-[α-^{18}O]dATPαS with the isotope attached to the α-phosphorus alone. It is possible to distinguish between these using the ^{18}O-induced upfield shift seen in ^{31}P NMR spectroscopy (Cohn & Hu, 1978; Lowe & Sproat, 1978; Lutz et al., 1978). This effect causes ^{18}O-containing phosphates to resonate at higher field than ^{16}O-containing species. Furthermore, the magnitude of the upfield shift increases with P–O bond order. Figure 5 shows the α (Figure 5A) and β (Figure 5B) regions of the ^{31}P NMR spectrum of the S_p-[^{18}O]dATPαS produced. The small peaks to the left are S_p-dATPαS (all oxygen-16), which arise as the $H_2{}^{18}O$ used only contained 91% ^{18}O (the remainder being mainly ^{16}O). The ^{18}O-induced upfield shift is best viewed after the addition of S_p-dATPαS. Figure 5C shows an upfield shift at the α-phosphorus of 3.4 Hz. These spectra were recorded at 145.78 MHz, and so this chemical shift is almost identical to those recorded previously at 81 MHz for S_p-[α,β-^{18}O]dATPαS (Connolly et al., 1984a) and agrees well with very many recorded spectra of S_p-[α,β-^{18}O]ATPαS [see review by Frey (1989)]. This suggests that the S_p-[^{18}O]dATPαS we have produced has the oxygen atom bridging the α- and β-phosphorus atoms, and confirmation of this comes from Figure 5D. Here a chemical shift of 2.4 Hz is seen for the β-phosphorus atom, corresponding (when allowance is made for the difference in field strength at which the spectra were recorded) to those observed previously for S_p-[α,β-^{18}O]ATPαS and S_p-[α,β-^{18}O]dATPαS (Connolly et al., 1984a,c). The alternative ^{18}O-labeled S_p-[α-^{18}O]dATPαS would be expected

to show a 5.2-Hz shift for the α-phosphorothioate and no shift for the β-phosphate (Connolly et al., 1984c; Jarvest & Lowe, 1979).

These results show that the *Eco*RV-catalyzed hydrolysis proceeds with inversion of configuration at phosphorus. The simplest interpretation is that the reaction proceeds by direct nucleophilic attack of H_2O at the scissile phosphate, with a trigonal bipyramidal transition state, and without the intermediacy of a covalent enzyme–substrate species. Studies with very many enzymes have shown that when a substrate is directly converted to product via a single displacment reaction, inversion of configuration at phosphorus is observed. For reactions involving a covalent intermediate two such displacements, each proceeding with inversion and so resulting in net retention of configuration, are required (see references in the introduction). An identical result was seen with the *Eco*R1 restriction endonuclease, suggesting that both proteins, which have a similar array of three amino acids at their active sites, use the same hydrolytic mechanism. However, despite having a crystal structure for the *Eco*RV endonuclease and knowing the stereochemical outcome, it is still difficult to formulate a full mechanism, probably because the crystal structure was determined in the absence of Mg^{2+}. Site-directed mutagenesis has shown that Glu45, Asp74, Asp90, and Lys92 are all essential for *Eco*RV activity (Selent et al., 1992). On the basis of these results and the crystal structure it has been proposed that Asp74 and Asp90 coordinate Mg^{2+}. It was further proposed that this cation bound to the scissile phosphate, leading to its polarization and increasing its susceptibility to nucleophilic attack. Lys92 was inferred to have an involvement in the stabilization of the negative charges on the phosphate in the transition state. However, it was not possible to decide if Asp74, Asp90, or the more remote Glu45 acted as a general base to activate the water molecule, so allowing nucleophilic attack by a hydroxide ion. Furthermore, it was not clear whether the attacking water molecule was also bound to the catalytic Mg^{2+} during catalysis or remained unliganded to the metal. Precedents for features of this mechanism have been observed with several other nucleases. Staphylococcal nuclease is Ca^{2+} activated, and three acidic side chains are liganded to the metal. One of these has been suggested to function as a general base by activating a Ca^{2+}-bound water molecule. A positively charged residue provides transition-state stabilization (Cotton et al., 1979; Serpersu et al., 1987, 1989; Hibler, 1987; Weber et al., 1991). The 3′,5′-exonuclease activity of *E. coli* DNA polymerase 1 depends on two metal ions (possibly Zn^{2+} and Mg^{2+}). Four acidic amino acids are involved in both their binding and water activation. Here transition-state stabilization is thought to be mediated via one of the metal ions (Beese & Steitz, 1991; Derbyshire et al., 1991). *E. coli* RNase H requires divalent cations and contains three essential acidic residues (Kanaya et al., 1990). It is thought to have a similar mechanism to the 3′,5′-exonuclease (Yang et al., 1991). Bovine pancreatic DNase 1 contains a Mg^{2+} bound to Glu39 and also the scissile phosphate. Two histidine residues provide general acid–base catalysis serving both to activate H_2O and to protonate the leaving group. An Arg residue may be involved in transition-state stabilization (Suck & Oefner, 1986; Suck et al., 1988; Lahm & Suck, 1991). Despite the importance of Glu45, Asp74, Asp90, and Lys92 suggested by mutagenesis and the precedents given above, close examination of the crystal structure reveals that none of these residues (or indeed any other amino acids) are correctly positioned for general base catalysis and H_2O activation in the *Eco*RV DNA complex (Winkler, 1992; Winkler et al., 1992). Slight conformational changes on Mg^{2+} binding would not be expected to alter this. Either the changes on Mg^{2+} complexation are larger than expected, leading to the correct positioning of an active site base, or the *Eco*RV hydrolytic mechanism has as yet unsuspected additional features. Clearly, full elucidation of the mechanism requires further experimentation.

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