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Effects of Functional Group Changes in the *Eco*RI Recognition Site on the Cleavage Reaction Catalyzed by the Endonuclease†

Larry W. McLaughlin,*,‡ Fritz Benseler,‡,§ Erika Graeser,§ Norbert Piel,§ and Stephan Scholtissek§

Department of Chemistry, Boston College, Chestnut Hill, Massachusetts 02167, and Abteilung Chemie, Max-Planck-Institut für experimental Medizin, Göttingen, West Germany

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ABSTRACT: Oligodeoxynucleotides have been prepared that contain changes in the functional group pattern present in the *Eco*RI recognition site. These changes involve "functional group deletions", "functional group reversals", and "displaced functional groups". Steady-state kinetic parameters have been used to characterize the interaction of these modified recognition sites with the *Eco*RI endonuclease. Changes in the functional group pattern have varying effects upon the cleavage reaction. Both the exocyclic amino groups of the two adenine residues and the methyl groups of the thymine residues appear to interact with the endonuclease quite differently. In both cases efficient catalysis was observed when these functional groups were present at the "outer" dA-dT base pair. Selectivity was decreased by over an order of magnitude largely via increases in K_m when these functional groups were deleted. Similar modificatons at the "inner" dA-dT base pair did not alter the kinetic parameters significantly from those observed with the native sequence. Addition of an amimo group to the minor groove at the outer dA-dT base pair resulted in a modified recognition site that interacted with the enzyme, on the basis of observed competitive inhibition kinetics, but was not cleaved.

Sequence specific protein-nucleic acid recognition appears to be a general feature of many biological processes. In order to understand such interactions between macromolecules, it is necessary to examine them at a functional group level. It has been proposed that an important aspect of the recognition process between proteins and nucleic acids involves hydrogen-bond formation between functional groups of the protein amino acid side chains and nucleobase functional groups available in the major and minor grooves of the nucleic acid (Seeman et al., 1976). Complementary patterns of hydrogen-donating and -accepting functional groups would define a particular hydrogen-bonding pattern and assist in determining the affinity of the protein for a given nucleic acid sequence. One approach to decipher a characteristic functional group pattern present in the nucleic acid recognition site involves the sequential deletion or modification of single functional groups from the recognized sequence and the examination of subsequent effects upon protein binding and/or catalysis.

The effects of base analogue substitutions in DNA on restriction endonuclease activity have been reported (Kaplan & Nierlich, 1975; Berkner & Folk, 1977, 1979; Mann & Smith, 1977; Mann et al., 1978; Marchionni & Roufa, 1979; Bodnar et al., 1983). One disadvantage with this approach is that the analogue is introduced at multiple positions, which can complicate subsequent binding and/or catalytic analyses. Oligodeoxynucleotides prepared by chemical and/or enzymatic methods allow the creation of recognition sites with single functional group deletions or modifications. Base analogue substitution in oligodeoxynucleotides has seen some success in the examination of binding and/or catalysis with restriction (Dwyer-Hallquist et al., 1982; Ono et al., 1984; Yolov et al., 1985; Seela & Driller, 1986; Jiricny, et al., 1986; Fliess et al., 1986; Brennan et al., 1986a) and modification (Brennan et al., 1986b) enzymes as well as DNA binding proteins such as the lac repressor (Yansura et al., 1977, 1979; Goeddel et al., 1977, 1978; Fisher & Caruthers, 1979, 1980) and most recently with RNA polymerase-promoter sequence recognition (Dubendorff et al., 1987).

The interaction of restriction endonucleases with their DNA recognition sites [for recent reviews see Jack et al. (1981), Wells et al. (1982), and Modrich (1982)] is an attractive system for studying this phenomenon. Restriction endonucleases recognize a relatively short DNA sequence (commonly four or six base pairs) and do so with a high degree of selectivity. This high sequence selectivity suggests that functional group contacts between the protein and DNA occur at each base pair within the recognition site.

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* Author to whom correspondence should be addressed.
‡ Boston College.
§ Max-Planck-Institut.

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Restriction endonucleases are additionally attractive in that two aspects of protein–nucleic acid interactions can be studied. Functional group requirements can be examined for binding as well as catalysis by the endonuclease. An examination solely of binding between the enzyme and DNA will be of particular interest in cases where catalysis is absent.

The *Eco*RI restriction endonuclease is an obvious choice to use for the study of functional group requirements in DNA recognition sequences. It recognizes the hexameric sequence d(GpApApTpTpC) and catalyzes hydrolysis of the phosphodiester linkage between the dG and dA residues. The enzyme has been highly purified and well characterized (Modrich & Zabel, 1976; Rubin & Modrich, 1978). More recently, the crystal structure of the protein/DNA cocrystal has been reported (Frederick et al., 1984; McClarin et al., 1986). The enzyme is a dimer, and each subunit has a molecular weight of 31 000 (Modrich & Zabel, 1976; Newman et al., 1981; Greene et al., 1981). In the absence of metal ions the protein dimer binds with high affinity to the recognition site (Halford & Johnson, 1980; Jack et al., 1982; Terry et al., 1983). Contacts in the major groove are required for sequence-specific binding as had been suggested by alkylation interference and protection assays (Lu et al., 1981) and then confirmed with the X-ray crystal analysis of the endonuclease/DNA complex (Frederick et al., 1984; McClarin et al., 1986).

The structure of the endonuclease/DNA cocrystal indicates that the nucleic acid undergoes a conformational change upon binding the protein (Frederick et al., 1984). This change results in three "kinks" in the nucleic acid helix, one located at the center of the recognition sequence (neo-1 kink) and two located at both termini of the recognition site (neo-2 kinks). The kinks appear to be necessary so that the protein can fit into the major groove and contact functional groups located on the nucleobases. X-ray crystallographic analysis indicates that binding is facilitated by three base-specific bidentate interactions per monomer (McClarin et al., 1986). Arginine-145 interacts with both N7 positions of the adjacent adenines, and arginine-200 interacts with N7 and O6 of guanine on one of the DNA strands. Glutamic acid 144 interacts with both exocyclic amino groups of the adjacent adenine bases of the complementary strand.

Catalysis by the endonuclease has also been examined in some detail. The enzyme requires Mg^{2+} for hydrolytic activity but not for highly selective binding (Halford & Johnson, 1980; Jack et al., 1982; Terry et al., 1983). The two phosphodiester bonds that hydrolyze in order to produce cleavage of the double-stranded DNA appear to occur sequentially (Modrich & Zabel, 1976; Rubin & Modrich, 1978). In some cases the enzyme can dissociate from the DNA between hydrolytic events (Ruben et al., 1977; Halford et al., 1979). The kinetics of the reaction can additionally be affected by sequences adjacent to the hexameric recognition site (Frederick et al., 1984; Rubin & Modrich, 1978; Halford et al., 1980; Alves et al., 1984). The stereochemistry with respect to phosphorus has been examined by using a modified sequence containing a phosphorothioate diester at the site of hydrolysis (Connolly et al., 1984). The enzyme cleaves preferentially the Rp diastereoisomer.

Although the X-ray crystal structure of the endonuclease/DNA complex is invaluable for identification of binding interactions, it was obtained in the absence of Mg^{2+}, necessary for catalysis. Biochemical solution experiments are still necessary in order to characterize the role of functional group interactions during catalysis. A study of catalysis using a series of modified oligodeoxynucleotides has been reported in a partial analysis of such interactions (Brennan et al., 1986a).

We have prepared a series of 13 modified recognition sequences. These contain three types of functional group modifications. With "deletion modifications" a single functional group has been deleted from the recognition sequence. "Functional group reversals" involve reversing the relative positions of functional groups involved in hydrogen bonding within the recognition sequence. "Functional group displacements" involve moving an exocyclic amino group from the major to the minor groove of the DNA helix. We have avoided using the methylated and brominated derivatives that have been reported previously (Brennan et al., 1986a). We have examined the modified recognition sequences as substrates for the endonuclease and determined steady-state kinetic parameters where applicable. In cases where a modified oligodeoxynucleotide did not function as an enzyme substrate, we have examined them as inhibitors of the reaction using the unmodified sequence to assess binding in the absence of catalytic activity and/or the presence of foreign inhibitory components.

Experimental Procedures

Materials

Sep-Pak cartridges were obtained from Millipore (Milford, MA). [γ-^{32}P]ATP was a product of Amersham Buchler (Amersham, England). DEAE-cellulose[1] circles (DE-81) were obtained from Whatman (Maidstone, England). HPLC was performed on a Du Pont 850 liquid chromatograph equipped with a variable-wavelength detector and a heated column compartment. Thermal melting points of the oligodeoxynucleotides were obtained with a Perkin-Elmer Lambda 3B spectrophotometer equipped with a C570-0701 digital temperature controller and temperature programmer. Solution temperature was measured directly by using an immersible probe and a Telethermometer (Yellow Springs Instrument Co., Yellow Springs, OH). Absorbance and temperature data were collected and stored after analogue to digital conversion (DT-2800, Data Translations, Marlboro, MA) with an IBM-XT personal computer using the ASYST scientific package (MacMillan Software Co., New York). From the absorbance vs temperature graphs first and second derivatives were used to determine T_m values.

Abbreviations. In order to simplify the illustration of modified sequences, the following abbreviations have been used for modified bases: P = purine, 2 = 2-aminopurine, D = 2,6-diaminopurine, I = hypoxanthine, U = uracil, and M = 5-methylcytosine.

Modified Oligodeoxynucleotides. The oligodeoxynucleotides were chemically synthesized by using either phosphite triester (Beaucage & Caruthers, 1981; McBride & Caruthers, 1983; Barone et al., 1984) or phosphate triester (Marugg et al., 1983, 1984) methodology on solid-phase CPG modified supports. The syntheses will be described in detail elsewhere.

Methods

Radioisotopic labeling of the modified oligodeoxynucleotides using T_4 polynucleotide kinase and [γ-^{32}P]ATP was accomplished by the following procedure: To 200 μL of reaction mixture, which contained oligodeoxynucleotide (45 μM), [γ-

[1] Abbreviations: DEAE, diethylaminoethyl; HPLC, high-performance liquid chromatography; BSA, bovine serum albumin; DTT, dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

^{32}P]ATP (2.8 mM, 50 μCi), bovin serum albumin (BSA) (5 μg/mL), 10 mM $MgCl_2$, 10 mM dithiothreitol, and 40 mm Tris-HCl, pH 8.7, was added 24 units of T_4 polynucleotide kinase. The reaction mixture was incubated at 37 °C for 18 h.

Isolation of the labeled fragment proceeded as follows: A Sep-Pak cartridge was prewashed with 5 mL of methanol and 5 mL of distilled water and equilibrated in a solution of 1% methanol/distilled water. The reaction mixture was heated for 2 min at 95 °C to inactivate the enzyme and added directly to the Sep-Pak cartridge. The cartridge was washed with 15–20 mL of 1% methanol/distilled water (excess ATP was eluted in the void volume but a minimum of 10 mL was required to elute the BSA). Subsequently, the phosphorylated oligodeoxynucleotide was eluted with 20 mL of 20% methanol/distilled water. After evaporation to dryness, the product was dissolved in an appropriate amount of distilled water.

Endonuclease Assays. Oligodeoxynucleotide concentrations for the endonuclease assays are given as duplex concentrations, which could be approximated from melting curves obtained by plotting absorbance (260 nm) vs temperature. The enzyme binds to its substrate as a dimer; therefore, we have reported dimer concentrations of endonuclease, and k_{cat} values are reported as phosphodiester bonds hydrolyzed per enzyme dimer. The oligodeoxynucleotides were initially tested as substrates for the endonuclease at 15 °C in 100 μL of reaction mixture containing from 10 to 50 μM oligodeoxynucleotide, 100 mM NaCl, 10 mM $MgSO_4$, 1 mM dithiothreitol, 10 μg/mL bovin serum albumin, and 20 mM Tris-HCl, pH 7.5. The solutions were heated to 65 °C and then cooled slowly to 15 °C. Reactions were initiated by addition of the endonuclease to a concentration of 10–80 nM. Aliquots were withdrawn and analyzed by HPLC using a 4.6 × 250 mm column of ODS-Hypersil developed in 50 mM KH_2PO_4, pH 5.5, and a gradient of 0–70% methanol in 60 min. Substrate oligodeoxynucleotides were completely converted to hydrolyzed products within 2 h. Inactive oligodeoxynucleotides were incubated at 15 °C for a total of 18 h.

Kinetic parameters were obtained by using 5′-^{32}P-labeled oligodeoxynucleotides. Oligodeoxynucleotides had a specific activity of 4000–6000 cpm/pmol (1.8–2.7 Ci/mmol). Duplicate reaction mixtures of 250–500 μL contained from 0.1 to 4.0 μM oligodeoxynucleotide in the buffer described above. Reactions were initiated by the addition of endonuclease to a concentration of 10 or 20 nM. Aliquots of 30 μL were withdrawn at 0-, 2-, 4-, 6-, and 8-min intervals and spotted on DEAE-cellulose circles (2.3 cm in diameter, Whatman DE-81) that had been prewetted with a solution of 0.1 M EDTA. The DE-81 circles were washed for 5 min each in (i) water, (ii) four solutions containing 0.3 M ammonium formate and 0.1 M potassium phosphate, pH 5.4, (iii) ethanol, and (iv) ether. The papers were dried, and the radioactivity was determined by liquid scintillation counting. With this procedure less than 4% of the product oligodeoxynucleotide pCpTpG remained bound to the DEAE-cellulose and more than 96% of the starting fragment d(pCpTpGpApApTpTpCpApG) (or analogue) was retained.

Kinetic parameters (K_m and k_{cat}) were derived from initial velocity data by a computer-assisted linear least-squares fit using the software ASYST.

Inhibition Kinetics. The lack of substrate activity for some oligodeoxynucleotides might be due to the altered functional group characteristics of the recognition site. In this case we wished to determine whether binding occurred between the enzyme and the modified substrate. Inhibition of the hydrolysis of an unmodified recognition sequence in the presence of a modified one would suggest enzyme binding. Alternatively, inhibition could be due to the presence of foreign inhibitors as a result of synthesis and/or isolation of the oligodeoxynucleotide. Thus, inhibition studies were designed such that a parallel experiment on temperature effects could be used to discriminate between these two possibilities (see below).

Kinetic parameters were determined for the dodecamer d(pCpGpCpGpApApTpTpCpGpCpG) essentially as described above. Sequence complementarity between the dodecamer and the modified decamers was absent outside the canonical recognition sequence. We anticipated that this would reduce the possibility of heteroduplex formation involving one strand of dodecamer and one strand of decamer. Cleavage of the dodecamer (0.5 μM) was then assayed in the presence of 0.5–50 μM modified oligodeoxynucleotide using the following procedure: 125 μL of dodecamer (1.0 μM) in the standard assay solution and 125 μL of modified oligodeoxynucleotide (10–100 μM) in the standard assay solution were individually heated to 65 °C and then cooled slowly to 15 °C. The two solutions were then mixed at 15 °C, and the reaction was initiated immediately after mixing by the addition of the endonuclease to a concentration of 40 nM. Aliquots (30 μL) of a control reaction lacking the modified sequence or the reaction mixture containing the modified sequence were spotted on Whatman DE-81 circles and washed as described above.

Only the decamer, d(CpTpGpDpApTpTpCpApG), exhibited any significant inhibition of the hydrolysis of the dodecamer. A K_I was determined at 15 °C in the standard assay solution by monitoring the hydrolysis of varying concentrations of d(pCpGpCpGpApApTpTpCpGpCpG) (0.17, 0.34, 0.50, 0.67 μM) in the presence of 0.15, 0.30, and 0.5 μM d(CpTpGpDpApTpTpCpApG). From duplicate analyses the average of the inverse of the initial velocities was plotted against the inverse of the substrate concentrations and the K_I determined from the apparent K_m observed in each case.

Temperature Experiments. If the inhibition observed above is the result of the presence of a modified *double-stranded* fragment, it should be possible to melt out the observed inhibition by increasing the temperature. Alternatively, inhibition resulting from a foreign contaminant is unlikely to be significantly affected by temperature. Solutions of dodecamer (1.0 μM) and d(CpTpGpDpApTpTpCpApG) (1.0 μM) each in 125 μL of standard assay solution were individually heated to 65 °C and cooled slowly to 15, 25, 30, 35, 40, or 42 °C. Immediately after mixing, the reaction was started by the addition of endonuclease to a concentration of 40 nM. Aliquots from the reaction mixture or a control lacking the modified sequence were spotted on DE-81 circles and washed as described above. Inhibition was measured by monitoring the initial velocity of the reaction mixture as a function of the control reaction.

RESULTS

Substrate Design. The oligodeoxynucleotides containing modified functional group patterns prepared for analysis as substrates fall into three classes: (i) functional group deletions, (ii) functional group reversals, and (iii) functional group displacements. Functional group deletions have been prepared by the single substitution of the appropriate modified base. Thus, deletion of the N2 or O6 of guanine was obtained by the substitution of hypoxanthine, 2-aminopurine, or purine bases, deletion of the N6 of adenine by substitution of purine, and deletion of the methyl group of thymine by substitution of uracil. One of the difficulties with the use of functional group deletions is the resulting loss of hydrogen bonds and

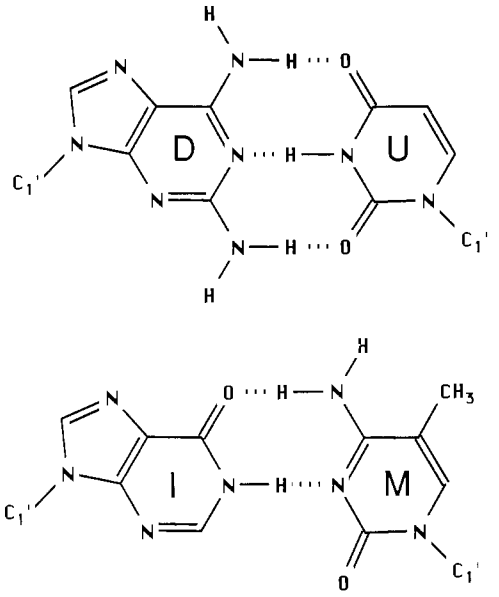

FIGURE 1: Presumed structure of the dD–dU and dI–dM base pairs.

possible alteration of helix character. In order to maintain hydrogen bonding within the DNA helix but alter the functional group characteristics of the recognition site, we have used some modified oligodeoxynucleotides in which the relative positions of certain functional groups have been reversed.

Functional group reversals have been prepared by the substitution of modified base pairs. Replacement of the dG–dC base pair with a dD–dU base pair reverses the relative positions of the exocyclic carbonyl and amino groups located in the major groove (Figure 1). The relative positions of the remaining functional groups remain constant. By analogy with the dG–dC base pair, three hydrogen bonds should exist in the dD–dU base pair. Although the hydrogen of the central hydrogen bond is formally bound to the pyrimidine in the dD–dU base pair and to the purine in the dG–dC base pair, we feel it is unlikely that the endonuclease probes this position of the base pair in the double-stranded form of the nucleic acid. For the same purpose of functional group reversal in the major groove we have used the dI–dM base pair in place of the dA–dT base pair (Figure 1).

The only functional group displacement that has been used in this study occurred when the 2-aminopurine base was substituted for adenine. In this case the exocyclic amino group of adenine has been displaced from the major groove to the minor groove of the dA–dT base pair. Two hydrogen bonds are present in either the dA–dT or d2–dT base pairs.

Reactivity of Native Recognition Sequences. The decamer d(CpTpGpApApTpTpCpApG), containing the unmodified recognition sequence, was hydrolyzed by the endonuclease to produce two products as monitored by HPLC (Figure 2). After the reaction had gone to completion, nucleoside analysis (data not shown) confirmed that hydrolysis had occurred between the dG and dA residues to produce d(CpTpG) and d(pApApTpTpCpCpG).

At a single-strand concentration of 4 μM the 10-mer exhibited a T_m of 40 °C when measured in the enzyme assay solution (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$). On the basis of the melting curve (temperature vs UV absorbance), the decadeoxynucleotide existed almost exclusively in the double-stranded form at 15 °C. Assays were routinely performed at 15 °C, and the Lineweaver–Burk plots obtained for the kinetic study were linear.

After radioisotopic labeling (see Experimental Procedures), kinetic parameters were obtained for both d(pCpTpGpA-

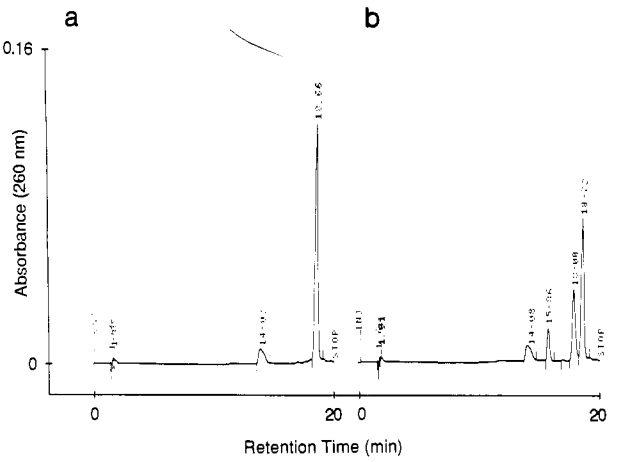

FIGURE 2: HPLC analysis of *Eco*RI-catalyzed hydrolysis of the unmodified decadeoxynucleotide. Analysis at (a) time = 0 min and (b) time = 20 min. Peaks: 14.0 min, BSA; 15.9 min, d(CpTpG); 18.0 min, d(pApApTpTpCpApG); 18.7 min, d(CpTpGpApApT-pTpCpApG). Chromatography conditions as described under Experimental Procedures.

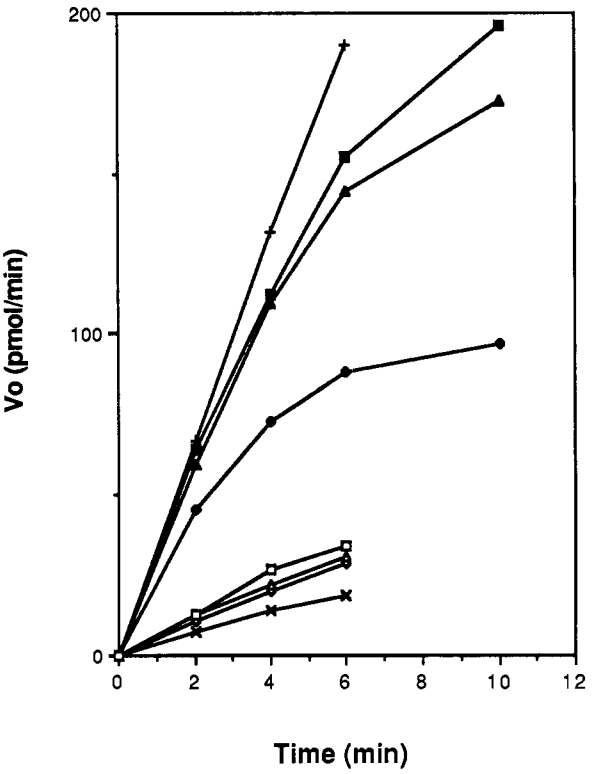

FIGURE 3: Graph of representative initial velocity data at 20 nM enzyme dimer for the unmodified sequence d(pCpTpGpApApT-pTpCpApG) double-stranded concentration of 0.12 (◆), 0.18 (▲), 0.24 (■), and 0.48 (+) μM and for the unmodified sequence d-(pCpGpCpGpApApTpTpCpGpCpG) double-stranded concentration of 0.07 (×), 0.14 (◇), 0.21 (△) and 0.28 (□) μM.

pApTpTpCpApG) and d(pCpGpCpGpApApTpTpCpGpCpG) in the concentration range 0.1–4.0 μM. The rate of phosphodiester bond hydrolysis was linear from 2 to 6 min, and initial velocities could be obtained from this portion of the assays (Figure 3). The enzyme could be saturated with substrate and followed Michaelis–Menten enzyme kinetics. This is in agreement with a recent report (Brennan et al., 1986a). The reaction was additionally first order in enzyme concentration in the range 10–80 nM (data not shown).

Lineweaver–Burk graphs were plotted from the initial velocity data. The decamer exhibited a K_m of 0.12 ± 0.02 μM

Table I: Kinetic Parameters for Modified *Eco*RI Recognition Sites

entry	*Eco*RI substrate	T_m (°C)	K_m[a] (μM)	k_{cat}[a] (min^{-1})	k_{cat}/K_m (rel)
1	*Col*E1 DNA (37 °C)[b]		0.008	4	
2	C T G A A T T C A G	40	0.12 ± 0.01	4.0 ± 0.1	1.0
3	C G C G A A T T C G C G	58	0.13 ± 0.09	0.68 ± 0.15	
4	C T I A A T T C A G	28	0.50 ± 0.07	2.9 ± 0.04	0.17
5	C T 2 A A T T C A G	*c*	*d*	*d*	*d*
6	C T P A A T T C A G	*c*	*d*	*d*	*d*
7	C T D A A T T U A G	36	*d*	*d*	*d*
8	C T G P A T T C A G	29	2.05 ± 0.21	3.9 ± 0.15	0.06
9	C T G A A T U C A G	39	1.66 ± 0.14	2.1 ± 0.09	0.04
10	C T G I A T M C A G	42	*d*	*d*	*d*
11	C T G D A T T C A G	41	*d*	*d*	*d*
12	C T G 2 A T T C A G	30	*d*	*d*	*d*
13	C T G A P T T C A G	28	0.23 ± 0.04	4.0 ± 0.1	0.52
14	C T G A A U T C A G	38	0.20 ± 0.02	2.3 ± 0.1	0.34
15	C T G A I M T C A G	42	*d*	*d*	*d*
16	C T G A 2 T T C A G	30	1.39 ± 0.12	5.2 ± 0.3	0.11

[a] Values are the result of a minimum of two and a maximum of four assays. [b] Modrich and Zabel (1976). [c] Did not exhibit helix to coil transition in the temperature range 10–50 °C and are assumed not to exist as duplexes at 15 °C. [d] No reaction.

and a k_{cat} of 4.0 ± 0.1 phosphodiester bonds hydrolyzed min^{-1} (enzyme dimer)$^{-1}$. The dodecamer exhibited an identical K_m (0.13 ± 0.08 μM) and a somewhat reduced k_{cat} (0.68 ± 0.11 min^{-1}).

Reactivity of Modified Oligodeoxynucleotides. All substrate analogues were initially assayed by HPLC to confirm the site of hydrolysis. Any nonspecific products present in excess of 0.2% of the expected hydrolysis products could be detected with this assay. Seven substrate analogues were inactive by this assay in the presence of the enzyme. In those cases incubation of the reaction mixture continued for a total of 18 h at 15 °C, and less than 0.2% of the starting material had disappeared after that period of time. Six of the analogues functioned as substrates with the endonuclease, and kinetic parameters were determined (Table I). Michaelis–Menten kinetics were observed for all the substrate oligodeoxynucleotides that were hydrolyzed by the enzyme. Graphs of the inverse of the initial velocity vs the inverse of the substrate concentration were linear. The K_m and k_{cat} values for each modified substrate are compiled in Table I. The relative substrate activities of the various analogues have been compared to those of the unmodified sequence by using the relative specificity constant obtained from the relationship k_{cat}/K_m (Table I).

The kinetic data can be summarized as follows. Substitution of purine for adenine or uracil for thymine at the inner dA–dT base pair resulted in a slight increase in the K_m and, in the latter case, a slight decrease in the turnover number. In both cases the specificity constants were reduced only 2- and 3-fold, respectively. On the other hand, substitution of 2-aminopurine at this position resulted in a 9-fold decrease in specificity largely due to an increase in K_m.

Similar substitutions at the outer dA–dT base pair were significantly different. Purine for adenine or uracil for thymine resulted in 17- and 25-fold decreases in the relative k_{cat}/K_m values, respectively, largely via an increase in K_m, but some changes in turnover were observed. Substitution of 2-aminopurine for adenine at this position did not produce a substrate with measurable activity.

The only modification of the dG–dC base pair that had substrate activity was the substitution of hypoxanthine for guanine. In this case the K_m increased 4-fold with a slight decrease in k_{cat}.

Inhibition Studies. Inhibition studies were performed for two reasons: (i) to determine whether the lack of substrate activity observed with some oligodeoxynucleotides was due to foreign inhibitors present in the solution of oligodeoxynucleotide and (ii) to determine whether the endonuclease was in some cases bound to the modified oligodeoxynucleotide but unable to initiate the hydrolytic event.

The dodecamer d(CpGpCpGpApApTpTpCpGpCpG) was used as the endonuclease substrate. At 4 μM single-strand concentration it exhibited a T_m of 58 °C. In the normal endonuclease assay a K_m of 0.13 μM and a k_{cat} of 0.68 min^{-1} were obtained for this dodecamer. Additionally, base complementarity between the dodecamer and modified dodecamers was lacking outside the canonical recognition sequence and, thus, heteroduplex formation would be inhibited.

Only the decamer d(CpTpGpDpApTpTpCpApG) exhibited any inhibition of the hydrolysis of the dodecamer. A Lineweaver–Burk plot indicated that competitive inhibition occurred. (Apparent K_m values of 0.12, 0.38, 0.56, and 1.72 μM were obtained with inhibitor concentrations of 0, 0.15, 0.30, and 0.50 μM, respectively.) Within experimental error identical V_{max} values were obtained with and without inhibitor. A K_I value of 0.038 ± 0.008 μM was obtained from the apparent K_m values. It was necessary to determine whether the observed inhibition resulted from the presence of the *double-stranded* modified sequence or an unknown foreign contaminant. The T_ms of the dodecamer and the 2,6-diaminopurine-containing oligodeoxynucleotide were 58 and 41 °C, respectively. With increasing temperature the modified sequence should undergo helix–coil transition before the unmodified dodecamer. Thus the competitive inhibition observed would be expected to decrease with increasing temperature. Alternatively, inhibition could be expected to be temperature independent for a nonnucleic acid contaminant. We therefore measured initial velocities of the reaction mixture containing d(CpTpGpDpApTpTpCpApG) as a function of a control reaction lacking the modified sequence. The initial velocity of the control increased with temperature as expected (Figure 4, inset). Plotting the initial velocity of the reaction mixture containing the modified sequence as a function of the control reaction indicated a decrease in inhibition most significantly in the range 30–42 °C. At 42 °C the initial velocities of both reaction mixtures were very similar (Figure 4).

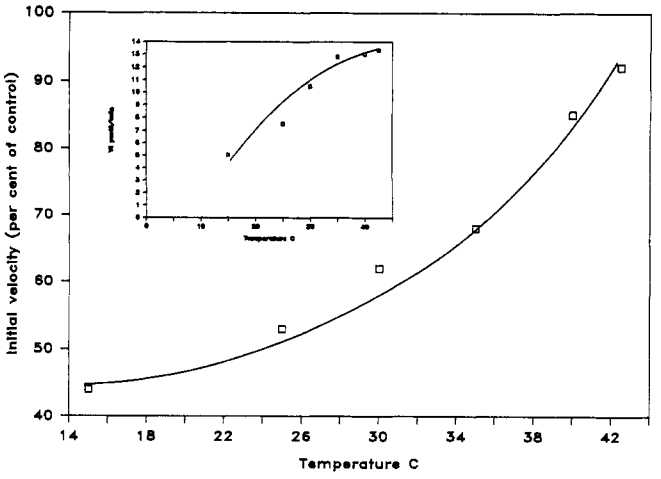

FIGURE 4: Graph of the initial velocity of a reaction mixture containing 0.50 μM d(CpGpCpGpApApTpTpCpGpCpG) and 0.30 μM d(CpTpGpDpApTpTpCpApG) (plotted as a function of a control reaction lacking the modified fragment) vs temperature. Inset: Initial velocity vs temperature of the control reaction.

DISCUSSION

Both the decamer d(CpTpGpApApTpTpCpApG) and the dodecamer d(CpGpCpGpApApTpTpCpGpCpG) were substrates for the endonuclease. They exhibited Michaelis–Menten kinetics as judged from the initial velocities at various enzyme concentrations and from the linear Lineweaver–Burk graphs obtained by plotting the inverse of the substrate concentration against the inverse of the initial rate of hydrolysis. Analysis of the cleavage products indicated that hydrolysis had occurred between the dG and dA residue in both cases. This is in agreement with previous reports involving oligodeoxynucleotide (Greene et al., 1981; Brennan et al., 1986a) and nucleic acid (Hedgepeth et al., 1972) substrates. Previous reports have indicated that a duplex oligodeoxynucleotide is the required substrate. Therefore, we have performed the kinetic analyses at 15 °C where the oligodeoxynucleotides are almost entirely in the duplex form as judged from the T_m data.

Both substrates exhibited identical K_m values within experimental error. These values are much higher than the 0.003–0.03 μM values obtained for natural DNA polymers (Jack et al., 1981), but this is typical for studies involving oligodeoxynucleotide substrates (Greene et al., 1981; Dwyer-Hallquist et al., 1982; Brennan et al., 1986a). These differences appear to be related to the length of the substrate molecule and the stability of the base pairs. The octamer d(TpGpApApTpTpCpA) exhibited a K_m value of 7 μM (Greene et al., 1981). Brennan et al. (1986a) replaced the terminal A–T base pairs with G–C base pairs and reported that the K_m value was reduced to 0.20 μM. We have added terminal G–C base pairs to the octamer described by Greene et al. and observed a K_m value of 0.12 μM. We observed essentially the same K_m value (0.13 μM) for the dodecamer used in the endonuclease/DNA crystal structure. This may reflect differences in endonuclease–DNA binding that require a conformational change in the structure of the DNA (Frederick et al., 1984). However, variations in assay conditions as well as the sequences outside the canonical recognition sequence may account for these differences in Michaelis constants by affecting product release (Rubin & Modrich, 1978).

The k_{cat} value for the 10-mer d(CpTpGpApApTpTpCpApG) of 4.0 min^{-1} is identical with that reported by Greene et al. for the related octamer. The value reported by Brennan et al. is significantly higher but was determined at a higher temperature. The dodecamer d(CpGpCpGpApApTpTpCpGpCpG) exhibited a reduced k_{cat} value (0.68 min^{-1}). The relative difference observed for the decamer and dodecamer appears to be related to the sequence outside the canonical recognition sequence. The observed decrease in k_{cat} with an increase in the G + C content of the sequence adjacent to the recognition site is in agreement with similar observations reported for polymeric DNAs (Rubin & Modrich, 1978; Halford et al., 1980; Alves et al., 1984).

Modified Oligodeoxynucleotides. Modified oligodeoxynucleotides that exhibited cooperative helix–coil transitions in the melting curves obtained from plotting absorbance vs temperature data have been assumed to be duplexes that adopt the B conformation. This has been shown for one of the unmodified sequences by X-ray crystallographic data (Wing et al., 1980).

Approximately half of the modified oligodeoxynucleotides functioned as substrates with the endonuclease (Table I). In some cases the interaction of the endonuclease with the substrate occurred with kinetic values and relative specificity (as measured by k_{cat}/K_m) similar to those of the unmodified sequence. With other modifications the kinetic parameters were significantly different and could be interpreted in some cases as being the result of changes in functional group contacts between enzyme and substrate. Half of the oligodeoxynucleotides did not function as enzyme substrates. Two fragments, d(CpTp2pApApTpCpApG) and d(CpTpPpApApTpTpCpApG), did not form duplex structures to a significant extent at 15 °C. In the other five cases, lack of enzyme activity has been presumed to result from changes in functional group contacts between enzyme and substrate as follows.

Modifications of the dG–dC Base Pair. Deletion of the amino group from the 2-position of guanine results in a 4-fold increase in K_m and a slight decrease in k_{cat}. This results in a 6-fold decrease in the relative specificity constant. This result is of the same relative order as that reported for the hypoxanthine containing octamer (Brennan et al., 1986a). The crystal structure of the enzyme/DNA complex does not indicate any protein contacts in the minor groove of the helix. The observed kinetic effects are likely a result of structural modulation of the helix, possibly affecting the formation of the type II neo-kink upon endonuclease binding (Frederick et al., 1984) rather than functional group contacts.

No cleavage was observed when the position of the exocyclic amino and carbonyl functional groups in the major groove were reversed by substitution of a dD–dU base pair for a dG–dC base pair. Replacing the carbonyl at position 6 of the purine with an amino group would clearly disrupt the bidentate complex reported from the crystal data to occur between Arg-200 and the dG residue of the recognition sequence (McClarin et al., 1986). This sequence did not function as a competitive inhibitor during enzyme-catalyzed hydrolysis of the unmodified dodecamer. The lack of hydrolysis and the inability to function as an inhibitor suggest that this modification prevents the formation of a highly specific enzyme/nucleic acid complex as well as subsequent catalysis under the present conditions.

Modifications of the dA–dT Base Pair. The functional group deletions of the exocyclic amino group from the two adenine residues and of the methyl group from the two thymine residues resulted in dramatically different kinetic parameters. Essentially no change in substrate reactivity was observed when functional group deletions occurred at the "inner" dA–dT base pair. However, the same deletions at the "outer" base pair resulted in more than an order of magnitude decrease in the

relative specificity constant. Protein–nucleic acid binding as analyzed from the crystal data involves the exocyclic amino groups of both adenine residues in a bidentate complex with the carbonyl of Glu-144 (McClarin et al., 1986). The present kinetic data indicate that the exocyclic amino groups of the adenine residues interact quite differently with the endonuclease during catalysis. Catalysis appears to be facilitated largely by contact with the amino group of the outer dA–dT base pair adjacent to the scissile phosphodiester. In the absence of this functional group, the endonuclease can maintain catalytic activity, possibly with contact to the amino group of the inner dA–dT base pair, although much less efficiently.

This observation is confirmed with the data from the two 2-aminopurine substitutions at these positions. Substitution of 2-aminopurine for adenine displaces the amino group from the major groove to the minor groove. Although this substitution may modulate helix structure, when viewed from the major groove, the exocyclic amino group of adenine has been deleted. Substitution of the inner adenine residue by 2-aminopurine produced an endonuclease substrate, although with a specificity constant 17-fold reduced. This result is in agreement with a similar derivative prepared in the octamer series (Brennan et al., 1986a). However, the same modification at the outer base pair resulted in a sequence that was not cleaved by the enzyme. In both cases an amino group has been deleted from the major groove and added to the minor groove. As observed with the purine substitution, the endonuclease was able to discriminate between the two possible positions of the amino group remaining in the major groove and cleaved preferentially the 2-aminopurine-containing oligodeoxynucleotide which maintains the amino group at the outer dA–dT base pair.

Similar discrimination is observed between the methyl groups of the thymine residues. The methyl group of the outer dA–dT base pair is required for efficient catalytic activity by the enzyme, whereas deletion of this group from the inner dA–dT base pair has no significant effect upon catalysis. These results are in agreement with similar demethylated derivatives reported recently (Brennan et al., 1986a) for the octamer series.

Reversing the exocyclic functional groups in the major groove at either dA–dT base pair did not produce an endonuclease substrate. This is somewhat more difficult to interpret. One might expect to observe similar discrimination by the endonuclease with cleavage occurring when the amino group is present on the purine of the outer dA–dT base pair. However, these derivatives introduce a pyrimidine exocyclic amino group. The presence of 5-methylcytosine in either position may add alternate modes of interaction with the enzyme that inhibit binding and/or catalysis. Detailed analysis of these results requires the preparation of the pyrimidine deletion modified sequences (2-pyrimidone-containing oligodeoxynucleotides) in order to assess the importance of the pyrimidine exocyclic functional groups during catalysis.

Inhibition Studies. Inhibition studies indicated that the lack of hydrolysis by the endonuclease was generally a result of the altered DNA fragments and not the result of foreign inhibitory components.

The observation that substitution of 2-aminopurine for adenine at the outer dA–dT base pair produced an inactive substrate, presumably due to the presence of an amino group in the minor groove near the scissle phosphodiester bond, led to the synthesis of d(CpTpGpDpApTpTpCpApG). This modified sequence contains all the functional groups necessary for enzyme binding as determined by X-ray diffraction data (McClarin et al., 1986) and has additionally an exocyclic amino group in the minor groove adjacent to the scissile phosphodiester bond. This modified sequence was not a substrate for the endonuclease and was the only fragment that functioned as a competitive inhibitor of the enzyme.

A similar derivative, d(GpGpApDpTpTpCpC), with 2,6-diaminopurine at the inner dA–dT base pair (Brennan et al., 1986a) is a substrate for the endonuclease, albeit a poor one. It is, however, noteworthy that the enzyme again discriminates in its catalytic function between modifications at the inner and outer dA–dT base pairs. It has been suggested that introduction of the amino group into the minor groove may effect formation of the type I neo-kink (Brennan et al., 1986a). Substitution at the outer dA–dT base pair places the minor groove amino group between the type I and type II neo-kinks. The inhibition studies suggest enzyme binding to d(CpTpGpDpApTpTpCpApG) and presumably formation of the type I and type II neo-kinks. In this case, introduction of the amino group into the minor groove may simply introduce steric crowding about the site of hydrolysis and prevent the catalytic reaction from occurring.

The K_I value of 0.038 μM suggests that the endonuclease interacts with both the native and the 2,6-diaminopurine-containing sequence in a similar manner. This is not unreasonable since all the functional groups necessary for enzyme binding are available. We anticipate that a derivative such as this might be useful for the preparation and study of a long-lived DNA/endonuclease complex in the presence of magnesium.

Conclusions

The recently reported X-ray diffraction data for the endonuclease/DNA complex define the functional group contacts necessary for sequence-specific binding. Kinetic data indicate that the importance of individual functional groups during catalysis may vary significantly. Specifically, only the exocyclic amino group of the outer adenine residue is necessary for efficient catalysis, although both are involved in complex formation (McClarin et al., 1986). The endonuclease discriminates between the inner and outer dA–dT base pairs and, in most cases examined, is more sensitive to functional group alterations at the outer dA–dT base pair, adjacent to the scissle phosphodiester bond.

Addition of an amino group to the minor groove of the DNA fragment at the outer dA–dT base pair results in a oligodeoxynucleotide that appears to bind to the enzyme active site, on the basis of observed competitive inhibition, but is not cleaved by the endonuclease.

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