Chiral Phosphorothioates as Probes of Protein Interactions with Individual DNA Phosphoryl Oxygens: Essential Interactions of *Eco*RI Endonuclease with the Phosphate at pGAATTC†

Michael R. Kurpiewski,‡ Maria Koziolkiewicz,§ Andrzej Wilk,§ Wojciech J. Stec,§ and Linda Jen-Jacobson*,‡

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, and Department of Bioorganic Chemistry, Centre of Molecular and Macromolecular Studies, Polish Academy of Sciences, Lodz, Poland

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ABSTRACT: The contact between *Eco*RI endonuclease and the "primary clamp" phosphate of its recognition site **p**GAATTC is absolutely required for recognition of the canonical and all variant DNA sites. We have probed this contact using oligonucleotides containing single stereospecific (R_P)− or (S_P)− phosphorothioates (Ps). At the GAA**p**TTC position, where the endonuclease interacts with only one phosphoryl oxygen at the central DNA kink, R_P-Ps inhibits and S_P-Ps stimulates binding and cleavage [Lesser *et al.* (1992) *J. Biol. Chem. 267*, 24810−24818]; in contrast, at the **p**GAATTC position both diastereomers inhibit binding. For single-strand substitution, the penalty in binding free energy ($\Delta\Delta G^\circ_{bind}$) is slightly greater for S_P-Ps (+0.9 kcal/mol) than for R_P-Ps (+0.7 kcal/mol). Binding penalties are approximately additive for double-strand substitution (R_P,R_P-Ps or S_P,S_P-Ps). Neither Ps diastereomer in one DNA strand affects the first-order rate constants for cleavage in the unmodified DNA strand, and only S_P-Ps inhibits the cleavage rate constant (3-fold) in the modified DNA strand. Thus, the second-order cleavage rate (including binding and catalysis) is inhibited 14-fold by S_P-Ps and 45-fold by S_P,S_P-Ps. In the canonical complex, the phosphate at **p**GAATTC is completely surrounded by protein and each nonbridging phosphoryl oxygen receives two hydrogen bonds from the endonuclease, such that in either orientation the increased bond length of P−S$^-$ inhibits binding. However, the pro-S_P oxygen interacts with residues that are connected (by proximity or inter-side-chain hydrogen bonding) to side chains with essential roles in catalysis, so cleavage is preferentially inhibited when these side chains are slightly displaced by the S_P-Ps diastereomer.

The contacts between sequence-specific DNA binding proteins and the phosphates of the DNA backbone were at one time considered to make only nonspecific (Coulombic) contributions to binding free energy. More recent evidence from both crystallographic (Aggarwal *et al.*, 1988; Otwinowski *et al.*, 1988; Rosenberg, 1991; Beamer & Pabo, 1992; Ellenberger *et al.*, 1992; Klemm *et al.*, 1994; Kim & Burley, 1994) and biochemical studies (Lesser *et al.*, 1990, 1992) has indicated that the protein−phosphate contacts are often mediated by hydrogen bonds from polypeptide main chain amide groups and/or polar side chains, and make significant contributions to the specificity of the protein−DNA interaction.

We have been using the *Eco*RI endonuclease to dissect the roles of individual protein−phosphate contacts in recognition of the canonical GAATTC site and in discrimination against related DNA sequences. Ethylation interference (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992) and UV photofootprinting (Becker *et al.*, 1988) experiments showed that only six symmetry-related phosphates (three per strand) are crucial to *Eco*RI endonuclease specificity. These key "clamp" contacts **p**N**p**GAA**p**TTC are indispensable to recognition of the canonical base sequence because they anchor and orient the protein recognition helices within the major groove (Rosenberg, 1991; Kim *et al.*, 1996) and stabilize the kinked DNA conformation (Kim *et al.*, 1994; Kumar *et al.*, 1994) in the complex. A set of weaker phosphate contacts, which include those at the scissile G**p**AATTC bonds, contribute nonspecifically to binding free energy.

The "supplementary clamp" contacts at **p**NGAA**p**TTC show characteristic changes in ethylation interference when the endonuclease interacts with any site containing one incorrect base-pair (Lesser *et al.*, 1990) or a methylated adenine. This alteration of the supplementary clamp contacts is associated with "adaptive" structural changes in the protein−DNA complex (Lesser *et al.*, 1990; Jen-Jacobson, 1995) that have been confirmed crystallographically (Wilkosz, 1993) and makes a significant contribution to the energy differential for sequence discrimination (Lesser *et al.*, 1990). By contrast, the "primary clamps" at N**p**GAATTC, completely surrounded by tightly constrained contacts from the endonuclease (Rosenberg, 1991; Kim *et al.*, 1996), are absolutely required for any localized binding at GAATTC and closely related sites; their absence is the signature of nonspecific (catalytically incompetent) complexes (Lesser *et al.*, 1990; Jen-Jacobson, 1995).

Ethylation interference analysis has proven invaluable in identifying these crucial phosphate contacts and their altered roles in variant complexes, but the all-or-nothing effect on protein binding (e.g., 150-fold inhibition at **p**GAATTC;

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* Author to whom correspondence and reprint requests should be addressed. Tel: 412-624-4969. Fax: 412-624-4759. E-mail: LJEN@vms.cis.pitt.edu.

‡ University of Pittsburgh.

§ Polish Academy of Sciences.

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Table 1: Energetics of *Eco*RI Endonuclease Binding to Phosphorothioate Sites

Sequence[a]	K_A [b] M^{-1}	$\Delta\Delta G°_{bind}$ [c] kcal/mol	k_d [d] sec^{-1}	$-\Delta\Delta G°_d{}^{\ddagger}$ [e] kcal/mol
TCGCAGAATTCTGCCGG GCGTCTTAAGACGGCCT	3.3(±0.4)x10^9	0	4.0(±0.7)x10^{-4}	0
R_P-TCGCA**$_{Ps}$**GAATTCTGCCGG GCGT CTTAAGACGGCCT	1.1(±0.1)x10^9	+0.7 ±0.1	1.4(±0.1)x10^{-3}	+0.7 ±0.1
S_P-TCGCA**$_{Ps}$**GAATTCTGCCGG GCGT CTTAAGACGGCCT	7.0(±0.3)x10^8	+0.9 ±0.1	2.2(±0.3)x10^{-3}	+1.0 ±0.1
R_P-TCGCA**$_{Ps}$**GAATTC TGCCGG GCGT CTTAAG**$_{Ps}$**ACGGCCT-R_P	2.9(±0.3)x10^8	+1.4 ±0.1	4.3(±0.7)x10^{-3}	+1.4 ±0.1
S_P-TCGCA**$_{Ps}$**GAATTC TGCCGG GCGT CTTAAG**$_{Ps}$**ACGGCCT-S_P	2.2(±0.3)x10^8	+1.6 ±0.1	7.3(±1.7)x10^{-3}	+1.7 ±0.2

[a] Only one orientation of a site with a single Ps substitution (Figure 1) is shown, but both orientations were tested for each site. [b] Equilibrium association constants K_A are expressed as moles of duplex 17-nt oligomer and endonuclease dimers as the active species. Values of K_A for both orientations were in close agreement; means ± standard deviations of six determinations are shown. The binding buffer was 0.01 M bis-tris-propane, pH 7.5, 0.15 M NaCl, 100 μg/mL bovine serum albumin, and 5 μM dithiothreitol (pH 7.5, 25 °C). [c] The difference in standard binding free energy between the unmodified site and each modified site, calculated as $\Delta\Delta G°_{bind} = -RT \ln (K_A{}^{modified}/K_A{}^{unmodified})$. [d] Dissociation rate constants were obtained at 0.08 M NaCl as described in the legend to Figure 2. Values of k_d for both orientations were in close agreement; means ± standard deviations of four determinations are shown. [e] The difference in free energy of activation for complex dissociation, $\Delta\Delta G°_d{}^{\ddagger} = -RT \ln (k_d{}^{modified}/k_d{}^{unmodified})$. Note that values are tablulated as $-\Delta\Delta G°_d{}^{\ddagger}$.

Becker *et al.*, 1988) does not permit an assessment of how each contact between protein and each individual phosphoryl oxygen contributes to particular aspects of function (e.g., binding, catalysis) in the protein−DNA complex. This kind of precise information will be required to connect high-resolution crystal structures to system-level thermodynamics and kinetics and ultimately to theoretical calculations of interaction energetics.

We have therefore introduced the use of chiral phosphorothioates (Ps)[1] as extremely subtle probes of the stereochemistry of protein contacts with DNA phosphates (Lesser *et al.*, 1992; Koziolkiewicz & Stec, 1992). A prochiral phosphate has P→O bonds of intermediate order and negative charge delocalized across the two nonbridging phosphoryl oxygens, but the predominant resonance form of a phosphorothioate group is O═P−S^- (Frey & Sammons, 1985). The P−S^- single bond is about 0.6 Å longer than the intermediate-order P−O bond (Frey & Sammons, 1985). Thus, a phosphorothioate differs from prochiral phosphate both in bond lengths and in charge localization.

In an earlier paper (Lesser *et al.*, 1992), we showed that a phosphorothioate at the central supplementary clamp phosphate GAA**p**TTC could be used to demonstrate the stereospecificity of the protein−DNA interaction at this position. The *pro-*R_P phosphoryl oxygen (that which, when replaced with sulfur, yields the R_P-Ps) receives a tightly constrained hydrogen bond from the polypeptide main chain amide at Gly^{116}, whereas the *pro-*S_P oxygen points to solvent. We found that single-strand substitution with the R_P-Ps diastereomer, which directs the longer P−S^- bond toward Gly^{116}, inhibited both binding ($\Delta\Delta G°_{bind} > 0$) and the first-order rate constant for cleavage in the modified DNA half-site. By contrast, the S_P-Ps diastereomer in one strand not only stimulated binding ($\Delta\Delta G°_{bind} < 0$) but also stimulated cleavage in the modified DNA half-site.

In this paper we use chiral Ps probes to dissect the functional roles of the contacts to the individual phosphoryl oxygens of the indispensable phosphate at **p**GAATTC. Previous studies (Koziolkiewicz & Stec, 1992) suggested stereoselectivity at this phosphate position, in that oligonucleotides containing R_P-$_{Ps}$GAATTC were cleaved under multiple-turnover conditions, whereas oligonucleotides containing S_P-$_{Ps}$GAATTC were not cleaved at all. We show here that single-strand substitution with either diastereomer inhibits *Eco*RI endonuclease binding ($\Delta\Delta G°_{bind} = +0.7$ to $+0.9$ kcal/mol), although the penalty in binding free energy $\Delta\Delta G°_{bind}$ is slightly greater for the S_P-Ps diastereomer. Neither diastereomer inhibits the cleavage rate constant for the unmodified DNA half-site, implying that there is no major structural change in the complex. However, the first-order cleavage-rate constant for the modified DNA half-site is more pronouncedly inhibited by S_P-Ps (about 3-fold) than by R_P-Ps (about 25%). Thus, the combined inhibition of binding and catalysis is 14-fold for single-strand S_P-Ps substitution and 45-fold for double-strand S_P,S_P-Ps substitution. Detailed consideration of the structure of the complex indicates that the inhibition of binding reflects an unfavorable effect of P−S^- on the interactions with multiple protein elements that normally contact both nonbridging phosphoryl oxygens of the phosphate at **p**GAATTC and that replacement of the *pro-*S_P oxygen with sulfur preferentially inhibits the first-order cleavage rate constant by affecting the positions of residues that participate in catalysis.

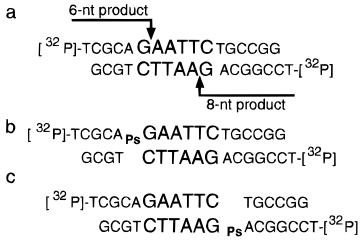

FIGURE 1: Design of oligonucleotide substrates. The upper sequence (a) shows the canonical duplex 17-nt oligomer. *Eco*RI endonuclease cleavage in the upper strand produces an end-labeled 6-nt product; cleavage in the lower strand produces an 8-nt product (see Figure 4). In all experiments, each site with one Ps substitution was tested in both orientations as shown in (b) and (c).

MATERIALS AND METHODS

Oligonucleotide Substrates. *Eco*RI endonuclease was prepared as described previously (Jen-Jacobson *et al.*, 1983). All experiments used synthetic duplex 17-nt oligonucleotides (Figure 1) carrying the GAATTC site off center so that cleavage in each of the DNA strands gives rise to a

[1] Abbreviations: Ps, phosphorothioate; nt, nucleotide(s); bis-tris, [bis-(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

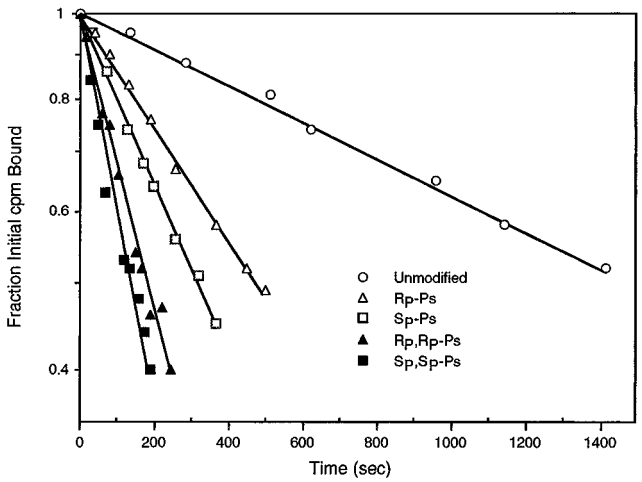

FIGURE 2: Kinetics of dissociation of endonuclease−DNA complexes. Complexes between *Eco*RI endonuclease (8 nM) and each of the radiolabeled oligonucleotides (10 nM) were formed under solution conditions described in Table 1, except these data were obtained at 0.08 M NaCl. Curves represent individual experiments. The means of multiple experiments like these are reported in Table 1.

distinguishable product. The sequences are the same as those used in our earlier work (Lesser *et al.*, 1990, 1992). Stereospecific phosphorothioate substitutions were placed in these oligonucleotides at A_{Ps}GAATTC by synthesis and block coupling of separately purified R_P-A_{Ps}G and S_P-A_{Ps}G dinucleotides by the methods previously described (Lesser *et al.*, 1992). The completed R_P- and S_P-oligonucleotides were purified, and their stereochemical purity (99.5% for S_P-Ps and 95% for R_P-Ps) was demonstrated according to Lesser *et al.* (1992). These Ps oligonucleotides were annealed with unmodified complementary strands to produce heteroduplexes or with Ps-modified complementary strands to produce homoduplexes. End labeling and repurification of duplexes were performed as described (Lesser *et al.*, 1992).

Other Methods. Equilibrium binding (Lesser *et al.*, 1990, 1992), dissociation kinetics (Lesser *et al.*, 1992; Jen-Jacobson *et al.*, 1986), and cleavage kinetics (Lesser *et al.*, 1990, 1992) were measured, and ethylation-interference footprinting (Lesser *et al.*, 1990, 1992) was performed as described, with minor changes in protein and/or DNA concentrations as given below in the legends to the figures and tables.

RESULTS

Effects of Phosphorothioates on Endonuclease−DNA Binding. The introduction of a single Ps into one strand of a duplex oligonucleotide inhibits equilibrium binding. As shown in Table 1, the penalty in standard binding free energy $\Delta\Delta G^\circ_{bind}$, relative to the parent unmodified DNA, is +0.7 kcal/mol for R_P-Ps and slightly larger (+0.9 kcal/mol) for S_P-Ps. In both cases, the penalty was the same regardless of which strand of the duplex was modified, that is, for either orientation shown in Figure 1. These penalties are significantly larger than those observed previously ($\Delta\Delta G^\circ_{bind}$ = +0.3 kcal/mol) for the unfavorable R_P-Ps at GAA$_{\mathbf{Ps}}$TTC (Lesser *et al.*, 1992).

Given the standard deviations in the measurements of the equilibrium association constant K_A (Table 1), it is hard to be confident of the small difference in $\Delta\Delta G^\circ_{bind}$ between the single-strand R_P-Ps and S_P-Ps substitutions. However, the difference can be confirmed by measuring the kinetics of dissociation of preformed protein−DNA complexes (Figure 2); the S_P-Ps complex clearly dissociated faster than did the R_P-Ps complex.

It is noteworthy that the changes in energy of activation for complex dissociation, calculated from the dissociation rate constants k_d as $\Delta\Delta G^{\circ\ddagger}_d = -RT \ln (k_d^{modified}/k_d^{unmodified})$, give binding penalties very similar to the $\Delta\Delta G^\circ_{bind}$ values calculated from equilibrium binding (Table 1). That is, $\Delta\Delta G^\circ_{bind} \approx -\Delta\Delta G^{\circ\ddagger}_d$. (The signs are opposite because we are comparing $\Delta\Delta G^\circ_{bind}$ for the association reaction to $\Delta\Delta G^{\circ\ddagger}_d$ for the dissociation reaction.) This is expected if the modification affects neither the free energy level of the hypothetical noncovalent "transition state" for complex dissociation nor the frequency factor for decomposition of

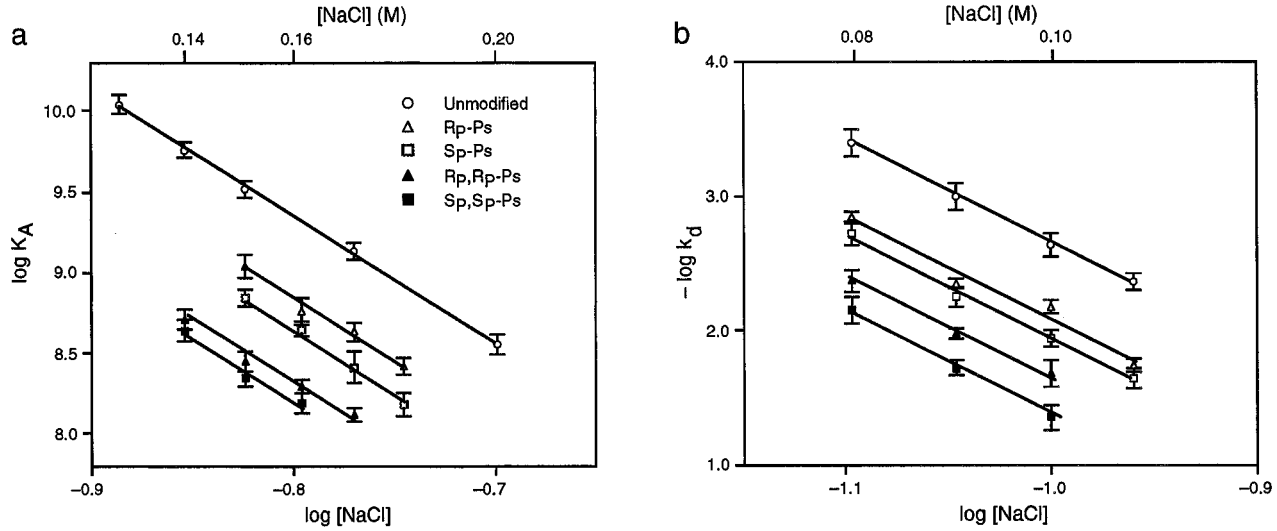

FIGURE 3: Dependence on NaCl concentration of the *Eco*RI endonuclease interaction with canonical and (R_P)- and (S_P)-phosphorothioate-modified substrates. For sites containing single-strand Ps substitution, both orientations (Figure 1b,c) were tested and showed no significant differences. Points show combined means ± the standard deviations of three independent determinations for each orientation (six total). For sites containing double-strand Ps substitution, points show means ± standard deviations of three to four independent determinations. (a) The logarithm of the equilibrium binding constant (K_A^{obs}) is plotted versus the logarithm of the NaCl concentration. (b) The negative logarithm of the dissociation rate constant (k_d) is plotted versus the logarithm of the NaCl concentration. Symbols are the same in both panels.

this transition state to the free protein and DNA (Jen-Jacobson, 1995).

When both strands were modified in R_P,R_P- or S_P,S_P-Ps homoduplexes, the penalties in $\Delta\Delta G°_{bind}$ and $\Delta\Delta G°^{‡}_{d}$ were close to the sums of the penalties for the constituent single-strand modifications. Both equilibrium and dissociation kinetic data indicated that the energetic effects of two R_P-Ps substitutions were additive, within experimental error. For the S_P,S_P-Ps homoduplex, the $\Delta\Delta G°^{‡}_{d}$ and $\Delta\Delta G°_{bind}$ values were slightly less than the sum of the penalties for single S_P-Ps substitutions, but the standard deviations were such that we cannot determine with confidence whether the penalties for S_P-Ps substitutions are additive or slightly subadditive.

The approximately additive behavior for **p**$_S$GAATTC substitutions should be contrasted with that of Ps substitutions at GAA**p**$_S$TTC (Lesser *et al.*, 1992), where double Ps substitutions in homoduplexes and in R_P,S_P-Ps heteroduplexes always gave values of $\Delta\Delta G°_{bind}$ that were less favorable (i.e., more positive or less negative) than predicted from the sum of the $\Delta\Delta G°_{bind}$ values for the constituent single-strand substitutions.

Measurements of the dependence of K_A and k_d on cation concentration (Figure 3) show that the observed binding *differences* between complexes with Ps-oligonucleotides pertain at all cation concentrations. Furthermore, the slopes of the salt dependence curves for equilibrium binding ($\partial \log K_A/\partial \log [NaCl] = -7.5 \pm 0.5$) and for dissociation kinetics ($\partial \log k_d/\partial \log [NaCl] = 7.1 \pm 0.4$) were the same for all Ps derivatives and for the unmodified parent oligonucleotide. Changes in these slopes have been observed in other modified DNA sites (Lesser *et al.*, 1990; Mossing & Record, 1985; Jen-Jacobson *et al*., 1991) as indicators of structural "adaptations" in the protein−DNA complexes, but in the present cases there is no indication of even minor adaptation. We have confirmed this inference by ethylation interference footprinting (data not shown): the phosphate footprints made by *Eco*RI endonuclease on the various Ps-substituted oligonucleotides are qualitatively and quantitatively indistinguishable from those on the parent unmodified sequence.

Cleavage of Phosphorothioate-Substituted DNA. There were three key elements in our experimental strategy to measure the effects of Ps substitution on catalysis: First, we designed oligonucleotide substrates (Figure 1) that located the GAATTC recognition site eccentrically (but in constant flanking sequence) so we could distinguish between cleavages in each of the DNA strands. Second, we made stereospecific Ps substitutions in only one strand of these duplexes, such that each substrate had one modified and one unmodified DNA strand. Third, we measured first-order cleavage rate constants (for both orientations of the eccentric site) after addition of Mg^{2+} to preformed endonuclease−DNA complexes, at high concentrations of endonuclease and DNA and with endonuclease in excess, so that all DNA was enzyme bound. This procedure yields true first-order rate constants for the strand cleavage steps, independent of any differences in binding affinity or product release. [We (Jen-Jacobson *et al.*, 1996; our unpublished results) have shown that the affinity of *Eco*RI endonuclease−DNA complexes for Mg^{2+} generally does not change, even when there are profound structural adaptations in the complex.] Under these conditions, the formation of the distinguishable 6-nt and 8-nt

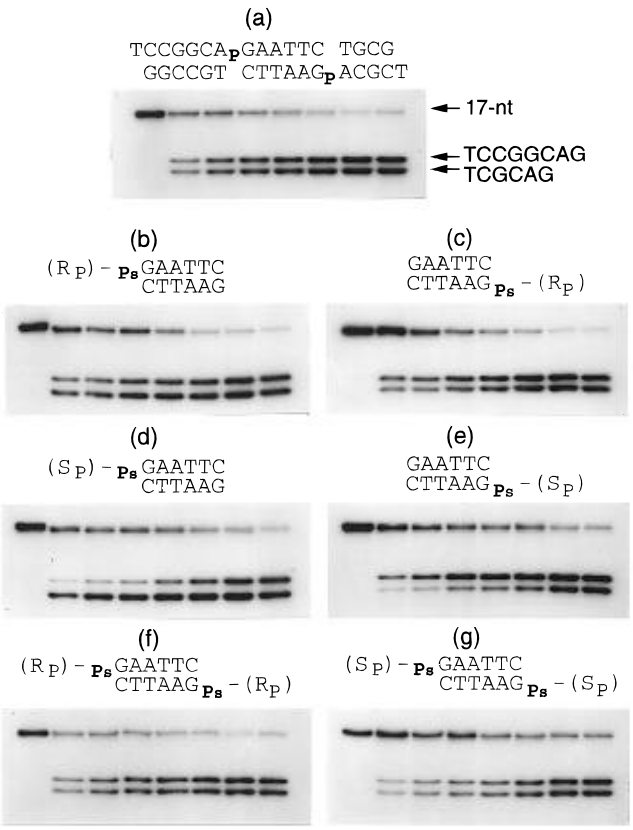

FIGURE 4: Single-turnover cleavage kinetics of canonical and (R_P)- and (S_P)-phosphorothioate-modified substrates. A representative time course for each orientation of a site containing Ps in one strand is shown. Reaction times in seconds (left to right) for each panel were as follows: (a) 0, 0.9, 1.9, 2.6, 3, 4, 5, 6; (b) 0, 1.1, 1.8, 2.3, 3, 5, 6, 8; (c) 0, 1.1, 1.6, 2.1, 3.0, 5.5, 8, 10; (d) 0, 1.2, 2.0, 2.8, 5.0, 8.0,10.0, 15.0; (e) 0, 1.1, 2.0, 2.9, 5.0, 7.0, 10.0, 15.0; (f) 0, 0.9, 2.0, 3.0, 4.0, 5.0, 8.0, 15.0; (g) 0, 1.3, 2.2, 3.0, 4.0, 5.0, 8.0, 10.0. Only a fraction of the complete time course (14 points) used for the calculation of cleavage rate constants (Table 2) is shown. These experiments were performed at 12 mM Mg^{2+} (Lesser *et al.*, 1993); some earlier data (Lesser *et al.*, 1990, 1992) were obtained at 5 mM Mg^{2+}.

products resulting from cleavage in each DNA strand is a parallel-sequential process (Lesser *et al.*, 1990).

With the unmodified duplex as substrate, the two DNA strands are cleaved at equal rates and the cleavage rate constants for the nicked intermediates are approximately the same as for the intact duplex (Lesser *et al.*, 1990), such that the 6-nt and 8-nt products appear at equal rates (Figure 4). Single-strand substitution of either R_P-Ps or S_P-Ps had no effect (Table 2) on the first-order rate constants for cleavage in the unmodified DNA strand (k_1). We have previously pointed out (Lesser *et al.*, 1990) that k_1, representing a cleavage event at a point remote from the locus of modification, reports on global rather than local changes in the protein−DNA interface. Discernible structural adaptations in the complex are invariably associated with changes in k_1 (Lesser *et al.*, 1990, 1992; Jen-Jacobson *et al.*, 1991; Jen-Jacobson, 1995), and conversely, unaltered values of k_1 are observed only in complexes that have not undergone global structural adjustments (Lesser *et al.*, 1990, 1993). Thus, the unaltered k_1 values for Ps substitutions at **p**GAATTC imply that the complexes remain essentially isomorphous with the canonical complex, supporting the inferences from the unaltered salt dependence and ethylation interference.

Table 2: First-Order and Relative Second-Order Cleavage Rate Constants[a]

Sequence [b]	k_1 [c] sec^{-1}	$\Delta\Delta G^{\circ\ddagger}_1$ kcal/mol [d]	k_2 [c] sec^{-1}	$\Delta\Delta G^{\circ\ddagger}_2$ kcal/mol	Relative [e] $k_1 \times K_A$ [Fold inhibition] [f]	Relative $k_2 \times K_A$ [Fold inhibition]
TCGCAGAATTCTGCCGG GCGTCTTAAGACGGCCT	0.9 ± 0.1	0	0.9 ± 0.1	0	1	1
R_P-TCGCA**ps**GAATTCTGCCGG GCGT CTTAAGACGGCCT	0.9 ± 0.1	0 ± 0.1	0.7 ± 0.1	+ 0.1 ± 0.1	0.33 ± 0.07 [3]	0.26 ± 0.06 [4]
S_P-TCGCA**ps**GAATTCTGCCGG GCGT CTTAAGACGGCCT	0.9 ± 0.2	0 ± 0.1	0.3 ± 0.1	+0.7 ± 0.1	0.21 ± 0.06 [5]	0.07 ± 0.03 [14]
R_P-TCGCA**ps**GAATTC TGCCGG GCGT CTTAAG**ps**ACGGCCT-R_P	1.1 ± 0.2	− 0.1 ± 0.1	0.9 ± 0.2	0 ± 0.1	0.11 ± 0.03 [9]	0.09 ± 0.03 [11]
S_P-TCGCA**ps**GAATTC TGCCGG GCGT CTTAAG**ps**ACGGCCT-S_P	0.3 ± 0.1	+0.7 ± 0.2	0.3 ± 0.1	+0.7 ± 0.2	0.022 ± 0.009 [45]	0.022 ± 0.009 [45]

[a] First-order rate constants were measured from densitometric data on gels similar to those in Figure 4, calculated from a full parallel-sequential model as described by Lesser *et al*. (1990). [b] Only one orientation of a site with a single Ps substitution (Figure 1) is shown. Both orientations were tested for each site containing a single Ps substitution. [c] For each site with Ps substitution in one strand, k_1 denotes the rate constant for cleavage in the unmodified DNA strand and k_2 denotes that for cleavage in the modified strand. For sites with Ps in both strands, k_1 was arbitrarily assigned to the top strand (Figure 1). Values for the single-substituted sites were independent of orientation (Figure 1); means ± standard deviations of both orientations (total of six determinations) are shown. Values for the double-substituted sites are means ± standard deviations of three determinations. [d] The difference in energy of activation $\Delta G^{\ddagger}$, relative to the unmodified site $= -RT \ln [k_{cleave}(\text{mod})/k_{cleave}(\text{unmod})]$, where the subscripts 1 and 2 refer to the rate constants k_1 and k_2, respectively. [e] For each site, the apparent second-order rate constant $k_{cleave}K_A$ relative to that for the unmodified site. [f] Fold inhibition, in brackets, is the reciprocal of the relative $k_{cleave}K_A$.

Substitution of R_P-Ps in one DNA strand had at most a very slight (~25%) inhibitory effect on the first-order cleavage rate constant (k_2) for the modified strand, but S_P-Ps substitution inhibited k_2 by about 3-fold (Table 2). This change in k_2 (but not k_1) is reflected visibly as an unequal rate of appearance of the 6-nt and 8-nt products (Figure 4). This was not a consequence of the eccentric location of the GAATTC site in the 17-nt substrate, since the strand containing an S_P-Ps was always cleaved slower, regardless of whether the "top" or the "bottom" DNA strand was modified (Figure 4). In S_P,S_P-Ps homoduplexes, both k_1 and k_2 were inhibited by about 3-fold (Table 2), resulting in slower but equal rates of appearance of both 6-nt and 8-nt products (Figure 4).

In earlier experiments (Koziolkiewicz & Stec, 1992) under multiple-turnover conditions (i.e., DNA in large molar excess to endonuclease), it was observed that the R_P,R_P-Ps derivative of **p**GAATTC was cleaved, but the S_P,S_P-Ps derivative was not cleaved at all. Although those experiments correctly identified the stereospecificity of Ps action at this position, we can now see that the effect and its stereospecificity have a far more subtle basis than was previously supposed. Single-strand S_P-Ps substitution at this position has only slightly greater inhibitory effects on both binding and cleavage than does R_P-Ps, but the apparent difference between the diastereomers is amplified by double-strand substitution and under multiple-turnover conditions, where the apparent second-order rate constant for the overall reaction is determined by the product of the binding and cleavage rate constants (i.e., $k_{cleave}K_A$). (The parameter $k_{cleave}K_A$ is analogous to the familiar k_{cat}/K_M used in steady-state kinetics, where k_{cat} and K_M are the catalytic and Michaelis−Menten constants.) For these Ps substitutions, our data (Table 2) show that the second-order rate constant for cleavage in the modified half-site is inhibited 14-fold for S_P-Ps and 45-fold for S_P,S_P-Ps, compared to only 11-fold for R_P,R_P-Ps. Thus, under multiple-turnover conditions, the 4-fold kinetic advantage of R_P,R_P-Ps over S_P,S_P-Ps apparently resulted (Koziolkiewicz & Stec, 1992) in the classification of these substrates as "cleaved" and "not cleaved", respectively. This is an excellent illustration of how multiple-turnover experiments, or the use of steady-state parameters K_M and k_{cat} determined under such conditions, may yield less useful information than a careful experimental allocation of the effects as between binding and individual kinetic constants for the bond-breaking steps.

DISCUSSION

Protein Contacts at ***p****GAATTC.* The structure of the cocrystalline complex between *Eco*RI endonuclease and DNA (Rosenberg, 1991; Kim *et al.*, 1996) shows that the nonbridging oxygens of the primary clamp phosphate at **p**GAATTC lie within hydrogen-bonding distance (2.8−3.4 Å) of four groups on the protein (Figure 5A).

The *pro*-R_P oxygen (pointing right in Figure 5A) is directed toward the DNA major groove and receives contacts from the guanidino group of Arg^{203} and from Lys^{148} ϵ-NH_3^+. The *pro*-S_P oxygen (pointing left in Figure 5A) receives hydrogen bonds from the side chain amide of Asn^{149} and the main chain amide of Lys^{89}. As a result of these positions and interactions, the primary clamp phosphate at **p**GAATTC is almost completely buried in surrounding protein elements (Figure 5B).

To understand the structural basis for the differential effects of Ps diastereomers on binding and cleavage, it is necessary to understand how the *pro*-R_P and *pro*-S_P phosphoryl oxygens differ in their participation in an elaborate network of residue connectivities and hydrogen bonds between side chains, which serves to buttress the protein−phosphate contacts and to provide "communication" with protein elements involved in sequence recognition and catalysis:

(a) Arg^{203} also hydrogen bonds to the upstream phosphate at **p**AGAATTC and to a water molecule that mediates recognition of O^6 and N^7 of guanine (not shown in Figure 5).

(b) Lys^{148} ϵ-NH_3^+ is hydrogen bonded to the side chain carbonyls of Asn^{149} and Glu^{144}. In turn, Glu^{144} is connected by side chain hydrogen bonds (not shown) to the guanidino

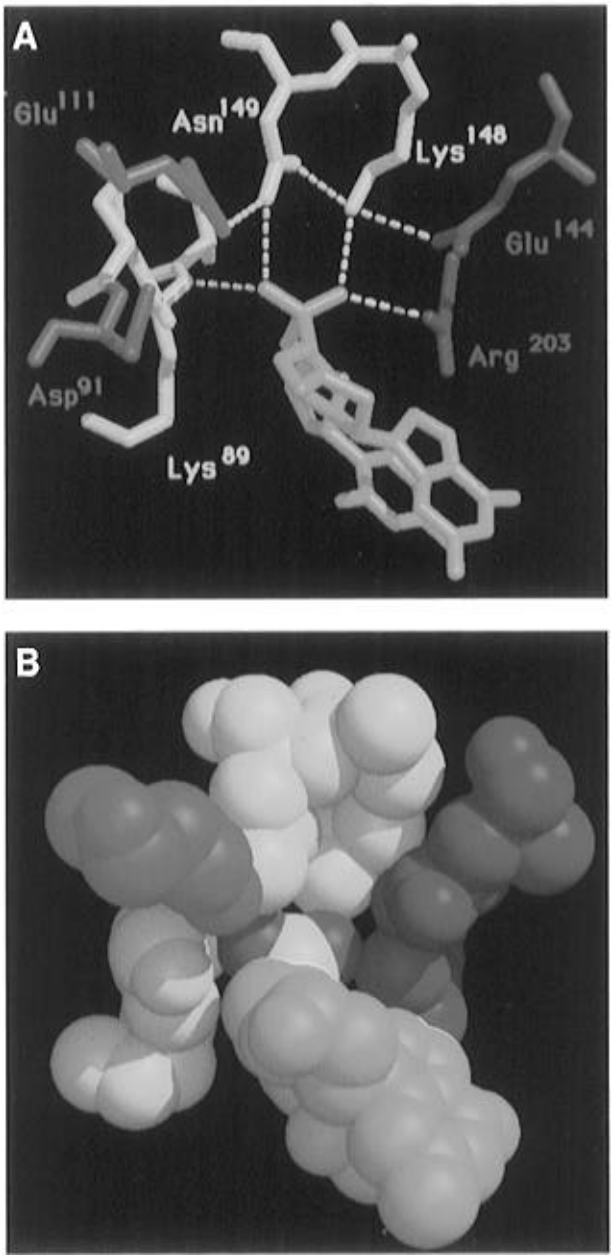

FIGURE 5: Interactions of *Eco*RI endonuclease with the phosphate at **p**GAATTC. Models were drawn with MIDAS Plus software using the atomic coordinates of the *Eco*RI endonuclease−TCGCGAATTCGCG complex (Rosenberg, 1991; Kim *et al.*, 1996; Brookhaven Protein Data Bank accession number 1rie). (a, top) The region surrounding the phosphate **p**GAATTC (CpG in light blue). The *pro*-S_P phosphoryl oxygen points to the left and the *pro*-R_P oxygen to the right. The active site residues Glu^{111} (green) and Asp^{91} (magenta) are at the left. Hydrogen bonds are shown as dashed lines. Note that the oligonucleotides used in our experiments (Figure 1) have an A in place of a C in this region. (b, bottom) Space-filling model of the same view shown in (A). Colors are as in (A) except that the phosphate at C**p**GAATTC has been recolored to show the phosphoryl oxygens (red) and the phosphorus atom (yellow). Asp^{91} is omitted for clarity.

group of Arg^{145}, which also interacts with the N^7 of the inner adenine and with the scissile phosphate at G**p**AATTC.

(c) The side chain amide of Asn^{149} is hydrogen bonded to a side chain carbonyl of Glu^{111}, which participates in the catalytic mechanism (see below).

(d) Lys^{148} and Asn^{149} lie at the C-terminal end of the "α-4 helix", which also includes the recognition residue Arg^{145}.

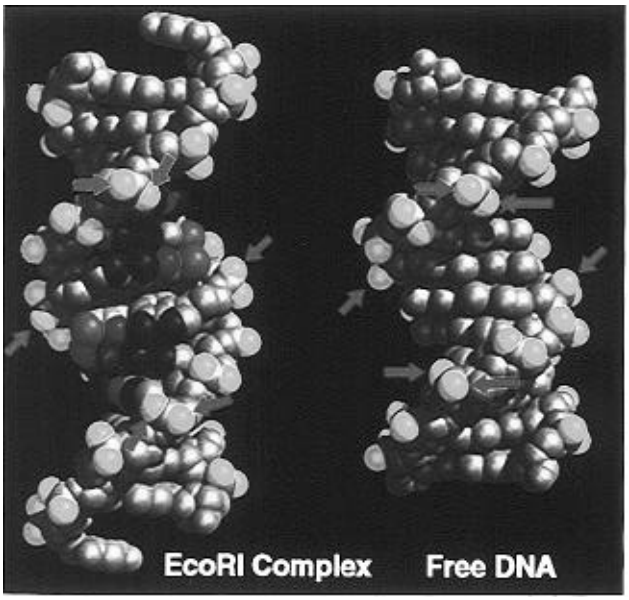

FIGURE 6: Comparison of the DNA conformations in the *Eco*RI endonuclease−TCGCGAATTCGCG complex (left; Rosenberg, 1991; Kim *et al.*, 1996) and the free duplex oligodeoxynucleotide CGCGAATTCGCG (right; Dickerson & Drew, 1981; Drew *et al.*, 1981; PDB accession number 1bna). The two models are aligned at the upper phosphate **p**GAATTC. Major groove groups on the bases recognized by *Eco*RI endonuclease are shown in red (guanine O^6 and thymine O^4), blue (adenine N^6 and N^7), and green (thymine CH_3). The green arrows indicate phosphoryl oxygens (yellow) contacted by *Eco*RI endonuclease at **p**GAATTC (top and bottom) and at GAA**p**TTC (left and right). The flanking supplementary clamp contacts at **p**NGAATTC are not indicated.

(e) Lys^{89} lies in the β-bridge (Rosenberg, 1991; Kim *et al.*, 1996) which follows the course of the DNA backbone at the 5′-side of the GAATTC site. At one end, this element provides hydrogen bonds from Ser^{86} OH and Ser^{87} NH to the phosphate at **p**AGAATTC. At the other end, the β-bridge provides Asp^{91}, which participates in the catalytic mechanism (see below).

The overall effect of this construction, therefore, is that the primary clamp phosphates at **p**GAATTC are intimately coupled with the entire recognition interface. It is hardly surprising that introduction of a bulky ethyl group on either phosphoryl oxygen strongly inhibits endonuclease binding to the canonical GAATTC site (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992) and to closely related sites (Lesser *et al.*, 1990; Jen-Jacobson *et al.*, 1991). Every cleavage-competent complex with any DNA site, even those with transition-state interaction free energy penalties up to +13 kcal/mol, must include the contacts at **p**GAATTC on both DNA strands (Lesser *et al.*, 1990; Jen-Jacobson *et al.*, 1991; Jen-Jacobson *et al.*, 1996). As we have shown, even the subtle phosphorothioate modification produces pronounced effects on binding and catalysis.

The phosphate contacts at **p**GAATTC probably have a crucial role in the characteristic "kinked" distortion (Rosenberg, 1991; Kim *et al.*, 1994, 1996; Kumar *et al.*, 1994) of the DNA when complexed with *Eco*RI endonuclease (Figure 6). The distortion involves a net unwinding of the DNA (by 30°) and opening of the major groove (by about 3 Å) so that recognition elements of the protein can be inserted to make hydrogen bonds and van der Waals contacts to functional groups on the bases. Base recognition would be impossible without this distortion.

In the free DNA (Figure 6), the phosphates at **p**GAATTC in the two DNA strands were rotated with respect to each other around the helical screw. As *Eco*RI endonuclease binds, however, it unwinds the DNA to bring these phosphates into alignment with each other along the major groove face on which lie the groups on the bases that are recognized. We believe that these primary clamp phosphates are the points at which the enzyme, in effect, "grasps" the DNA to stabilize the unwound conformation (or to exert an unwinding force to "strain" the DNA; Kumar *et al.*, 1994). Additional points of grasp are provided by the supplementary clamp contacts at **p**AGAA**p**TTC. The central contacts at GAA**p**TTC (Figure 6) lie at the points of kinking of the DNA backbone (Rosenberg, 1991; Kim *et al.*, 1994, 1996) and may be crucial to stabilizing this feature of the conformation. Thus, the recognition of the GAATTC bases and the phosphate clamps should be regarded as an integral, cooperative process.

Phosphorothioate Effects on Endonuclease Binding. Because both phosphoryl oxygens at **p**GAATTC are hydrogen bonded to protein in a highly precise network that is sensitive to bond lengths and angles and charge distribution, both R_P-Ps and S_P-Ps inhibit endonuclease binding.

The R_P-Ps substitution directs P−S$^-$ toward Lys^{148} and Arg^{203}, with a bond length increased by about 0.6 Å (Frey & Sammons, 1985) compared with prochiral phosphate. It is possible, but not certain, that the replacement of a partial negative charge with a full negative charge would make the interaction with these positively charged side chains slightly more favorable. On the other hand, although both the Lys^{148} and Arg^{203} side chains are relatively flexible, their participation in a network with other protein−DNA and intra-protein interactions (see above) suggests that even relatively minor movements to accommodate P−S$^-$ might have unfavorable energetic consequences.

The R_P-Ps also directs P═O, with a bond length about 0.06 Å shorter than in prochiral phosphate (Frey & Sammons, 1985), toward Asn^{149} and the polypeptide chain at Lys^{89}. We believe that this replacement of the *pro*-S_P P→O with P═O would have little structural or energetic consequence.

The S_P-Ps substitution directs P−S$^-$ toward Asn^{149} and the polypeptide main chain amide at Lys^{89} (to the left in Figure 5A). The original distance from the *pro*-S_P oxygen to Lys^{89} NH was about 2.8 Å, and it seems probable that the added 0.6 Å length of the P−S$^-$ bond can be accommodated only by some adjustments of position and unfavorable energetic consequences. The stereospecific inhibition of catalysis by the S_P-Ps derivative implies that there are also some adjustments of the β-bridge and/or Asn^{149} (see below). This may account for the observation (Table 1) that S_P-Ps inhibits binding slightly more than does R_P-Ps, but there is no basis on which to account for the absolute magnitudes of the changes in binding free energy.

It is also noteworthy that the $\Delta\Delta G°_{bind}$ values for Ps substitutions are additive (within experimental error) in the R_P,R_P homoduplex and additive or slightly subadditive in the S_P,S_P homoduplex (Table 1). Energetic additivity is generally only possible when the perturbation to the interface has purely local consequences and does not cause significant changes in the overall structure of the complex (Jen-Jacobson, 1995); structural adaptation, however minor, is invariably associated with nonadditivity of $\Delta\Delta G°_{bind}$ values (Carter *et al.*, 1984; Wells, 1990; Lesser *et al.*, 1992; Jen-Jacobson, 1995). The inference that there is little or no adaptation in these Ps-modified complexes is further supported by the unaltered ethylation interference footprints, the unaltered salt dependence of binding (Figure 3), and the fact that neither R_P-Ps nor S_P-Ps affects the cleavage rate constant for the unmodified DNA strand.

Stereospecific Effect of S_P-Ps on Cleavage Rate Constants. In previous studies (Koziolkiewicz & Stec, 1992) it was simply reported that S_P,S_P-Ps was not cleaved by *Eco*RI endonuclease, but we have now dissected out and quantitated the effects of Ps diastereomers on binding and on the individual rate constants for cleavage in each of the DNA half-sites. None of the single-strand substitutions at **p**GAATTC affect k_1 for cleavage in the unmodified DNA strand, consistent with the absence of structural adaptation in these complexes. The stereospecific effect of S_P-Ps on the rate constant k_2 for cleavage in the modified DNA half-site (3-fold inhibition; Table 2 and Figure 4) may be understood from the interactions between *Eco*RI endonuclease and the *pro*-S_P phosphoryl oxygen (Figure 5A). As noted above, we believe that a P−S$^-$ directed toward the β-bridge and Asn^{149} would only be accommodated by some minor displacement of these elements. This, in turn, would affect the positions of Asp^{91} and Glu^{111}, albeit to an unknown extent. Both of these side chains have essential roles in catalysis.

The side chain carboxyls of Asp^{91} and Glu^{111} chelate the essential Mg^{2+} cofactor in the active site (Rosenberg, 1991; Kim *et al.*, 1996). Mutation of Glu^{111} abolishes catalytic activity with little effect on binding (King *et al*., 1989). There is no direct genetic evidence that Asp^{91} is involved in catalysis, but Asp residues are found in analogous positions in the active site Asp^{94} of *Bam*HI endonuclease (Winkler, 1992; Newman et al., 1994a,b) and Asp^{74} of *Eco*RV endonuclease (Winkler, 1992; Newman et al., 1994a,b; 1995; Kostrewa & Winkler, 1995), and mutation of the analogous Asp^{94} of *Bam*HI endonuclease abolishes catalytic activity (Xu & Schildkraut, 1991; Dorner & Schildkraut, 1994).

We therefore propose that the 3-fold inhibition of k_2 (Table 2) by a single S_P-Ps in the immediate vicinity is a consequence of minor displacement of Asp^{91} and/or Glu^{111}. The inference is further supported by the absence of inhibitory effect when R_P-Ps directs the nondisruptive P═O toward these protein elements. The fact that double-strand S_P,S_P-Ps substitution produces the same degree of inhibition, but in both DNA strands (Table 2), indicates that the transition-state effects are independent in the two half-sites, consistent with this interpretation of a strictly local effect.

It is also noteworthy that for the R_P,R_P-Ps derivative, where there is a substantial penalty in binding free energy ($\Delta\Delta G°_{bind} = +1.6$ kcal/mol, Table 1), the values of the rate constants k_1 and k_2 are unchanged; that is, in terms of free energies, $\Delta\Delta G^‡ = 0$ (Table 2). Since the free energy of activation $\Delta G^‡$ is the energy difference between the unactivated enzyme−DNA complex and the transition-state complex, the fact that $\Delta\Delta G°_{bind} > 0$ and $\Delta\Delta G^‡ = 0$ necessarily implies that the protein−DNA interactions affected by the R_P-Ps substitution(s) make precisely the same energetic contributions in the unactivated endonuclease-DNA complex and in the transition-state complex. That is, an energetic penalty paid at the level of binding need not be paid again in the transition state. We had previously observed similar behavior for Ps substitutions at GAA$_{\mathbf{p}}$TTC (Lesser *et al.*, 1992) and for multiple substitutions of purine for adenine (Lesser *et*

al., 1993). Taken together, these cases imply that the transition-state complex must bear a very close structural resemblance to the unactivated endonuclease−DNA complex seen in the crystal structure (Rosenberg, 1991; Kim *et al.*, 1996). In particular, the distorted DNA conformation must be very similar in the unactivated and transition-state complexes. We have therefore termed the unactivated endonuclease−DNA complex a "pre-transition-state" complex (Lesser *et al.*, 1993), in which both protein and DNA already depart significantly from their ground-state conformations and are poised to enter the transition state.

Methods for Evaluating Protein−Phosphate Contacts. Given the subtlety with which phosphorothioates can be used to probe the significance of individual protein−phosphate contacts to the functions of a protein−DNA complex, it seems worthwhile to make a comparison with the information available from ethylation interference experiments. As a practical matter, ethylation interference footprinting (Siebenlist & Gilbert, 1980; Lu *et al*., 1981; Ebright, 1986) allows a rapid survey of possible protein interactions with many DNA phosphates in and around a recognition site. Our work with *Eco*RI endonuclease (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992) indicates that ethylation of a given phosphate interferes strongly with binding when that phosphate is directly contacted by polar side chains or by polypeptide amides but not when only water-mediated contacts are made. The method has also proven extremely useful for detecting a variety of structural adaptations in modified complexes (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992; Jen-Jacobson *et al*., 1991; Jen-Jacobson *et al*., 1996).

We caution that it is not simple to interpret ethylation effects on protein−DNA binding in terms of detailed molecular structure. The ethylation reaction (using ethylnitrosourea; Siebenlist & Gilbert, 1980) is not stereoselective and thus produces a mixture of (R_P)- and (S_P)-ethylphosphotriesters at each DNA phosphate. If only one diastereomer inhibits protein−DNA binding, the interference effect will be no more than 2-fold. That is, strong interference (much greater than 2-fold) can only be observed if both diastereomers inhibit binding. Such strong interference was used to identify the important contacts made by *Eco*RI endonuclease at **p**N**p**GAA**p**TTC (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992; Jen-Jacobson *et al*., 1991).

For the phosphate at **p**GAATTC, both the structure (Figure 5) and our results with Ps substitutions make it reasonable that ethylation of either phosphoryl oxygen inhibits binding. However, for the phosphate at GAA**p**TTC both the structure (Rosenberg, 1991; Kim *et al.*, 1996) and results with Ps substitutions (Lesser *et al.*, 1992) show that only the *pro*-R_P oxygen interacts with protein, yet the strong ethylation interference indicates that ethylation of either oxygen inhibits binding. Why does ethylation of the *pro*-S_P oxygen interfere with binding?

Examination of the structure indicates that an ethyl group added to the *pro*-S_P oxygen at GAA**p**TTC would come into steric conflict with the adjacent deoxyribose when the DNA attempts to assume the characteristic kinked conformation in the complex with *Eco*RI endonuclease. In other words, an ethyl group directed away from Gly^{116} inhibits binding not because of steric conflict with the protein but because it has an unfavorable effect on the ability of the DNA to attain a particular distorted conformation. By contrast, the minimal structural perturbations introduced by a phosphorothioate are extremely unlikely to affect DNA conformation or distortability.

Our results suggest that the stereoselectivity of Ps effects can form an empirical basis for determining whether one or both phosphoryl oxygens at a given position are contacted by protein. At phosphates where the protein makes a tight hydrogen-bonding interaction to only one of the phosphoryl oxygens, one Ps diastereomer will improve binding (relative to the unmodified DNA) and the other will be inhibitory, as we observed for the supplementary clamps at GAA**p**TTC (Lesser *et al.*, 1992). At phosphates where the protein forms direct interactions to both phosphoryl oxygens, both Ps diastereomers will be inhibitory but one Ps diastereomer may nonetheless be favored, as we showed here for the primary clamp phosphate contacts at **p**GAATTC. Finally, at phosphates that receive only water-mediated contacts, or that form only salt links to flexible Lys or Arg side chains, there is little or no ethylation interference with binding (Becker *et al.*, 1988; Lesser *et al.*, 1990, 1992) and multiple-turnover cleavage experiments (Koziolkiewicz & Stec, 1992) indicate little or no effect of Ps substitution.

For enzymes like restriction endonucleases that interact with specific DNA sites, the exquisite sensitivity of the transition state to the most subtle perturbations may produce a stereoselective effect of Ps diastereomers (located at positions other than the scissile phosphodiester bond) on cleavage rate constants, as we observed here. This stereoselectivity, when interpreted in conjunction with a crystal structure, may yield extremely detailed information about how networked protein−phosphate interactions serve to "buttress" the precise positions of catalytic site residues.

REFERENCES

Aggarwal, A. K., Rodgers, D. W., Drottar, M., Ptashne, M., & Harrison, S. C. (1988) *Science 242*, 899−907.

Beamer, L. J., & Pabo, C. O. (1992) *J. Mol. Biol. 227*, 177−196.

Becker, M. M., Lesser, D., Kurpiewski, M., Baranger, A., & Jen-Jacobson, L. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6247−6251.

Carter, P. J., Winter, G., Wilkinson, A. J., & Fersht, A. R. (1990) *Cell 38*, 835−840.

Dickerson, R. E., & Drew, H. R. (1981) *J. Mol. Biol. 149*, 761−786.

Dorner, L. F., & Schildkraut, I. (1994) *Nucleic Acids Res. 22*, 1068−1074.

Drew, H. R., Wing, R. M., Takano, T., Broka, C., Tanaka, S., Itakura, K., & Dickerson, R. E. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2179−2183.

Ebright, R. (1986) in *Protein Structure Design and Folding* (Oxender, D. L., Ed.) pp 207−219, Alan R. Liss, Inc., New York, NY.

Ellenberger, T. E., Brandl, C. J., Struhl, K., & Harrison, S. C. (1992) *Cell 71*, 1223−1237.

Frey, P. A., & Sammons, R. D. (1985) *Science 228*, 541−545.

Jen-Jacobson, L. (1995) *Methods Enzymol. 259*, 305−344.

Jen-Jacobson, L., Kurpiewski, M., Lesser, D., Grable, J., Boyer, H. W., Rosenberg, J. M., & Greene, P. J. (1983) *J. Biol. Chem. 258*, 14638−14646.

Jen-Jacobson, L., Lesser, D., & Kurpiewski, M. (1986) *Cell 45*, 619−629.

Jen-Jacobson, L., Lesser, D. R., & Kurpiewski, M. R. (1991) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Vol. 5, pp 141−170, Springer-Verlag, Berlin.

Jen-Jacobson, L., Engler, L. E., Lesser, D. R., Kurpiewski, M. R., Yee, C., & McVerry, B. (1996) *EMBO J. 15*, 2870−2882.

Kim, J. L., & Burley, S. K. (1994) *Nat. Struct. Biol. 1*, 638−657.

Kim, Y., Choi, J., Grable, J. C., Chandrasekhar, K., Greene, P., Hager, P., & Rosenberg, J. M. (1994) in *Structural Biology: The State of the Art* (Sarma, R. H., & Sarma, M. H., Eds.) pp 225−246, Adenine Press, New York.

Kim, Y., Choi, J., Grable, J. C., Chandrasekhar, K., Wilkosz, P., Greene, P., Hager, P., & Rosenberg, J. M. (1996) *Proteins* (in press).

King, K., Benkovic, S. J., Modrich, P. (1989) *J. Biol. Chem. 264*, 11807−11815.

Klemm, J. D., Rould, M. A., Aurora, R., Herr, W., & Pabo, C. O. (1994) *Cell 77*, 21−32.

Kostrewa, D., & Winkler, F. K. (1995) *Biochemistry 34*, 683−696.

Koziolkiewicz, M., & Stec. W. J. (1992) *Biochemistry 31*, 9460−9466.

Kumar, S., Duan, Y., Kollman, P. A., & Rosenberg, J. M. (1994) *J. Biomol. Struct. Dyn. 12*, p 487−525.

Lesser, D. R., Kurpiewski, M. R., & Jen-Jacobson, L. (1990) *Science 250*, 776−786.

Lesser, D. R., Grajkowski, A., Kurpiewski, M. R., Koziolkiewicz, M., Stec, W. J., & Jen-Jacobson, L. (1992) *J. Biol. Chem. 267*, 24810−24818.

Lesser, D. R., Kurpiewski, M. R., Waters, T., Connolly, B. A., & Jen-Jacobson, L. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7548−7552.

Lu, A. L., Jack., W. E., Modrich, P. (1981) *J. Biol. Chem. 256*, 13200−13206.

Mossing, M. C. & Record, M. T. (1985) *J. Mol. Biol. 186*, 295−305.

Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., & Aggarwal, A. K. (1994a) *Nature 368*, 660−664.

Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., & Aggarwal, A. K. (1994b) *Structure 2*, 439−452.

Newman, M., Strzelecka, T., Dorner, L. F., Schildkraut, I., & Aggarwal, A. K. (1995) *Science 269*, 656−663.

Otwinowski, Z., Schevitz, R. W., Zhang, R.-G., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., & Sigler, P. B. (1988) *Nature 336*, 321−329.

Rosenberg, J. M. (1991) *Curr. Opin. Struct. Biol. 1*, 104−113.

Siebenlist, U., & Gilbert, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 122−126.

Wells, J. A. (1990) *Biochemistry 29*, 8509−8517.

Wilkosz, P. (1993) Ph.D. Thesis, University of Pittsburgh, Pittsburgh, PA.

Winkler, F. K. (1992) *Curr. Opin. Struct. Biol. 2*, 93−99.

Xu, S.-y., & Schildkraut, I. (1991) *J. Biol. Chem. 266*, 4425−4429.

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