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Importance of phosphate contacts for sequence recognition by *Eco*RI restriction enzyme☆

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Abstract

We have studied the importance of charge and hydrogen-bonding potential of the phosphodiester backbone for binding and cleavage by *Eco*RI restriction endonuclease. We used 12-mer oligodeoxynucleotide substrates with single substitutions of phosphates by chiral methylphosphonates at each position of the recognition sequence –pGpApApTpTpCp–. Binding was moderately reduced between 4- and 400-fold more or less equally for the R_P and S_P-analogues mainly caused by missing charge interaction. The range of cleavage effects was much wider. Four substrates were not cleaved at all. At both flanking positions and in the purine half of the sequence up to the central position, cleavage was more impaired than binding and differences between R_P and S_P diastereomeres were more pronounced. These effects are easily interpreted by direct phosphate contacts seen in the crystal structure. For the effects of substitutions in the pyrimidine half of the recognition sequence, more indirect effects have to be discussed.

Sequence specific interaction of restriction endonucleases with their recognition sequences in general is overdetermined to ensure the high accuracy of these enzymes [1,2]. In the case of *Eco*RI, 16 hydrogen bonds and eight hydrophobic interactions contact directly the six base pairs of the recognition sequence to which 16 hydrogen bonds to the phosphodiester backbone are added [3]. While many efforts were made to study the base contacts, the role of phosphate contacts was attributed for long time to a more unspecific clamping of enzyme to DNA to allow base contacts to form. Ethylation interference data demonstrated that a more steric hindrance of a close proximity of enzyme and DNA could be established in the purine half of the recognition sequence [4–6]. This was verified and enlarged to cleavage data by Koziolkiewicz and Stec [7] using O-ethyl-phosphotriester analogues of oligodeoxynucleotides. In the same paper also, phosphorothioate analogues were used and the diastereomers were studied separately. Some of the latter substrates were also used to establish the importance of contacts to the phosphates in front and in the middle of the recognition sequence for kinking the DNA [8,9]. The phosphorothioate substitution localizes the negative charge on the sulphur moiety, which is larger than oxygen, and the P–S^- bond is 0.6 Å longer than the P–O bond (Fig. 1). Therefore, direct contacts have to be slightly reorganized but charge interactions through space are mainly undisturbed. The ethoxy substitution eliminates the charge. Therefore, it interferes with charge interactions. Furthermore, a bulky substituent is added which may sterically interfere with the protein even if a real contact is not made. We have synthesized methylphosphonate (P-Me) stereospecifically substituted substrates that eliminate the charge at the position of substitution and present only one oxygen moiety for hydrogen-bonding interactions. Therefore, direct contacts to the oxygen moieties are probed and charge interactions are disturbed. However, the methyl group is even less bulky than sulphur and no steric interference is to be expected. Our results complement those with other substrates and give a detailed view upon the importance of charge and hydrogen-bonding interactions between *Eco*RI restriction endonuclease and the entire phosphodiester backbone of its recognition sequence.

☆ *Abbreviations:* P-Me, methylphosphonate, short names of the oligodeoxynucleotides are defined in Table 1.

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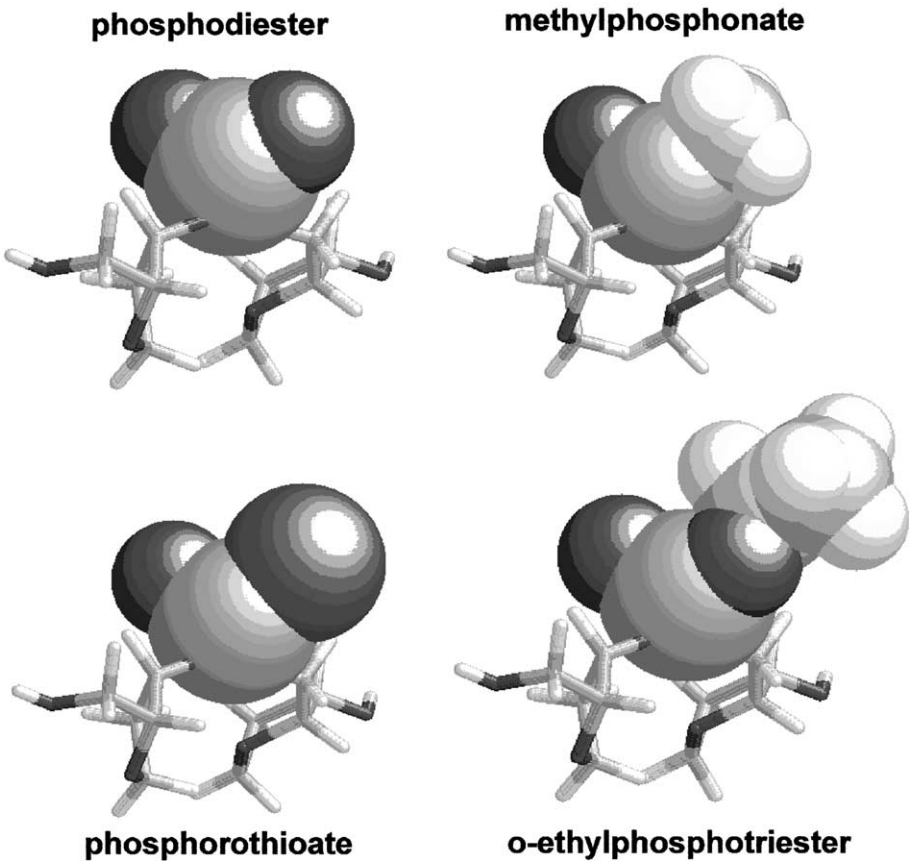

Fig. 1. Comparison of the R_P-P-Me structure with the canonical phosphodiester as well as S_P-phosphorothioate and S_P-*O*-ethyl-phosphotriester. Note that the nomenclature of the stereochemical orientation of the P-Me substitution is opposite to that for the phosphorothioate substitutions because of the chemical nature of the methyl group. The view is from 5′ to 3′. Only the molecules on the left-hand side are negatively charged.

Materials and methods

Enzyme preparation. The *Eco*RI endonuclease, which carries a C-terminal His6-affinity tag, was prepared using Ni-NTA-agarose (Qiagen) and phosphocellulose [10].

Synthesis of oligodeoxynucleotides. Synthesis of dodecamers carrying P-Me substitution was carried out in two steps. The first step involved the synthesis of dinucleosides carrying P-Me linkage and their inclusion into the dodecamer sequence was accomplished in the second step. The synthetic strategy is analogous to already published procedure in an earlier communication by Srivastava et al. [11]. The isomeric purity at the P-Me linkage was ascertained by ^{31}P-NMR spectra wherein only singlet was observed for each diastereomer in the range of 31–33 ppm. Chemical shift values of the P-signal for R_P and S_P diastereomers were clearly separated. Loschner and Engels [12], on the basis of 2D-NMR and ^{31}P-NMR spectra, have observed that R_P isomer exhibits phosphorous chemical shift at higher field than the S_P isomer. Therefore, we have assigned R_P and S_P configurations to the diastereomers on the basis of ^{31}P chemical shift values for each isomer and are in conformity with the reported observation [12].

Characterization of oligodeoxynucleotides. The dodecamers were characterized for the presence of P-Me linkage as well as for the proper molecular composition. Presence of P-Me linkage was established by the ammonia hydrolysis studies of the P-Me dodecamers whereas the molecular characterization was confirmed by electrospray ionization mass spectroscopy (ESI-MS) wherein the molecular ion peaks at appropriate mass range were obtained for all phosphate containing and modified dodecamers.

Radioactive labelling of oligodeoxynucleotides. Oligodeoxynucleotide (10–50 pmol) was incubated with 20 μCi [α-^{32}P]ddATP (Amersham) and 2.5 U terminal deoxynucleotidyl transferase in 20 μl of 200 mM K-cacodylate, pH 7.2, 1 mM $CoCl_2$, 0.1 mM DTT, and 0.01% v/v Triton at 37 °C for 5–6 h. The labelled oligodeoxynucleotide was purified with the Qiaquick Nucleotide Removal Kit according to supplier's (Qiagen) protocol.

Binding of oligodeoxynucleotides. Binding of oligodeoxynucleotide substrates was determined in electrophoretic mobility shift assays. Fifty nM labelled control oligodeoxynucleotides (CTRLS) were incubated with varying enzyme concentrations (10–1000 nM) in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 25 mM EDTA for 30 min at 22 °C and then applied to a 20 × 20 cm polyacrylamide gel (5.8% acrylamide, 0.2% *N*, *N*′-methylenebisacrylamide, 100 mM Tris/borate, pH 8.3, and 2.3 mM EDTA) at 5 V/cm for 3–4 h. P-Me substrates were measured in competition to CTRL using 50 nM CTRL, 100 nM *Eco*RI, and varying concentrations of P-Me oligodeoxynucleotides (0.1–200 μM). Gels were dried, quantified in a Fuji X Bas 1000 PhosphorImager and Mac Bas 2.0 (Fuji), and the binding constants were calculated as described [13].

Cleavage of oligodeoxynucleotides. One μM labelled oligodeoxynucleotide was cleaved by 100 nM *Eco*RI endonuclease in 20 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 10 mM $MgCl_2$ at 22 °C. Aliquots were withdrawn after several time points according to the cleavability of the substrate and the reaction was stopped by adding a fourth of the volume of 80 mM EDTA, 4 mM SDS, and 1% bromophenol blue. The samples were applied to 18% denaturing PAGE. The initial cleavage velocities were used to calculate k_{app} (min^{-1}).

Results

The introduction of methyl group in place of the hydroxy at the phosphorous creates chirality having R_P or S_P configuration. The two diastereomers differ in their biochemical and biophysical properties. Hence, it is all the more important to obtain stereochemically pure oligomers. The chimeric dodecamers reported in this study contain stereoregular P-Me at the pre-selected position within and outside the *Eco*RI recognition sequence. All the dodecamers were obtained in good quantities and homogeneity as judged by the reversed phase HPLC.

The presence of P-Me linkage was confirmed by heating the dodecamer with concentrated ammonia. This treatment is known to specifically hydrolyse P-Me without affecting the phosphodiester linkage. Aliquots from the reaction mixture of each dodecamer were subjected to RP-HPLC wherein peaks corresponding to the hydrolytic products due to cleavage at the P-Me linkage were observed. As expected there was no hydrolysis in the case of control dodecamer under similar condition. Thus, confirming the presence of P-Me linkage in the case of chimeric dodecamers. Further in the mass spectra molecular ion peaks were observed at 3645.44 and 3643.40 for the control and modified dodecamers, respectively. The difference in molecular mass is because of the methyl substitution in place of the hydroxy group. Since the dodecamers are palindromic, it was expected that they would form duplex in the solution. Accordingly, a peak at 7289 for the control dodecamer and a peak at 7286 for the modified dodecamers were observed. Thus, confirming the duplex structure.

Binding of *Eco*RI to the oligodeoxynucleotide substrates was measured by the electrophoretic mobility shift assay. Labelled CTRL was used directly and in competition to all P-Me substrates studied. The binding constant of the canonical oligodeoxynucleotide without any substitutions was rather low compared with other oligodeoxynucleotide substrates (Table 1, Fig. 2A, [6,14–17]). This may in part be attributed to its length of only 12 nucleotides. The method may also contribute, as it is known that slight variations of the buffer conditions may cause rather strong variations in binding strength [18]. Our main interest was the characterization of the influence of P-Me substitutions in comparison to the unmodified sequence. The competition measurements are ideally suited for that purpose and directly show the difference to the unmodified oligodeoxynucleotide, even if its binding constant may be underestimated (Fig. 2B). P-Me substitutions in the two halves of the recognition sequence cause different effects. In the purine half, they only moderately reduce binding by factors of 4–90 while in the pyrimidine half the reduction is roughly fivefold higher (Table 1). The effect of the orientation of the methyl group at an individual position is rather low with the exception of the substitution of the phosphate to be cleaved where the R_P-analogue is accepted more than 20-fold better than the S_P-analogue.

Because the oligodeoxynucleotides are self-complementary, the cleavage in the two single strands is not distinguishable (Fig. 3). Therefore, in the denaturing gel, we detect only the first cut within one single strand. The enzyme may cleave the second strand simultaneously but it is more likely that the double strand falls apart and the intact single strand anneals with another one to

Table 1
Binding and cleavage of P-Me oligodeoxynucleotides by *Eco*RI restriction endonuclease

Oligodeoxynucleotide		K_{ass}[a] (M^{-1})	$K_{ass}^{CTRL}/K_{ass}^{P\text{-}Me}$	k_{app}[b] (min^{-1})	$k_{app}^{CTRL}/k_{app}^{P\text{-}Me}$
Name	Sequence				
CTRL	GACGAATTCGTC	6.1×10^6		30.4	
CG1-R	GAC$_{P\text{-}Me}$GAATTCGTC	7.8×10^5	8	0.06	507
CG1-S	GAC$_{P\text{-}Me}$GAATTCGTC	6.3×10^5	10	0.1	304
GA-R	GACG$_{P\text{-}Me}$AATTCGTC	1.5×10^6	4	n.d.	$\propto$
GA-S	GACG$_{P\text{-}Me}$AATTCGTC	7.6×10^4	80	n.d.	$\propto$
AA-R	GACGA$_{P\text{-}ME}$ATTCGTC	1.3×10^6	5	1.1	27
AA-S	GACGA$_{P\text{-}Me}$ATTCGTC	8.1×10^5	8	0.1	304
AT-R	GACGAA$_{P\text{-}Me}$TTCGTC	6.0×10^4	102	0.44	69
AT-S	GACGAA$_{P\text{-}Me}$TTCGTC	2.0×10^4	305	n.d.	$\propto$
TT-R	GACGAAT$_{P\text{-}Me}$TCGTC	1.4×10^4	436	1.3	23
TT-S	GACGAAT$_{P\text{-}Me}$TCGTC	4.1×10^4	149	1.1	28
TC-S	GACGAATT$_{P\text{-}Me}$CGTC	2.3×10^5	27	6.5	5
CG2-R	GACGAATTC$_{P\text{-}Me}$GTC	1.8×10^4	339	0.0044	6909
CG2-S	GACGAATTC$_{P\text{-}Me}$GTC	1.0×10^5	61	n.d.	$\propto$

n.d. = Not detectable.
[a] Measured by competition to CTRL in electrophoretic mobility shift assays. Binding constants are accurate within a factor of 3.
[b] Cleavage rates are accurate within ±10%.

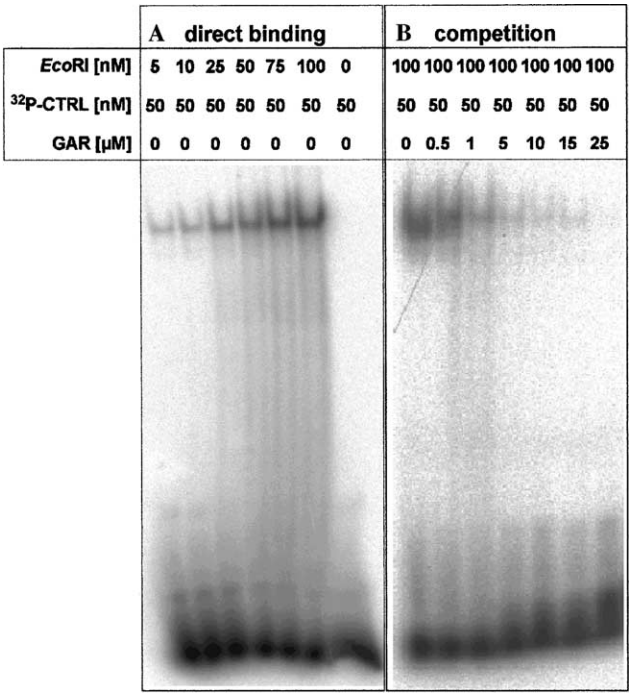

Fig. 2. Determination of binding of *Eco*RI endonuclease to P-Me substrates by electrophoretic mobility shift experiments. (A) Direct binding of radioactive labelled CTRL oligodeoxynucleotide. (B) Competition of CTRL binding by unlabelled GA-R_P-methylphosphonate substrate. Binding constants are listed in Table 1.

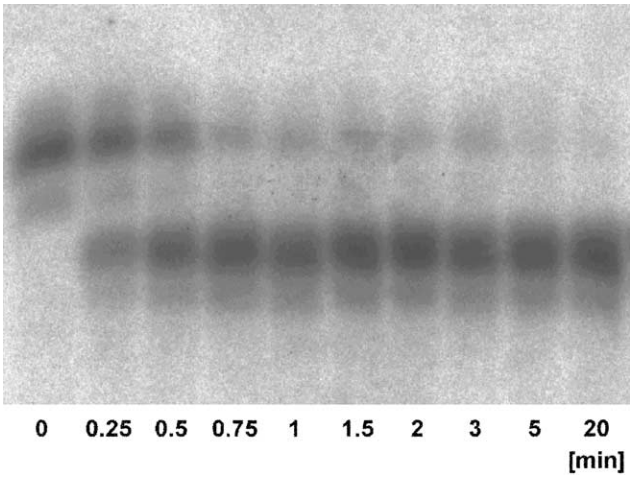

Fig. 3. Cleavage of 1 μM CTRL substrate by 0.05 μM *Eco*RI endonuclease. Cleavage rates are listed in Table 1.

form a new double stranded substrate [19]. In each case, the instability of the short oligodeoxynucleotide products forces the dissociation of enzyme product complex that will therefore not be the rate-limiting step of the reaction. The cleavage of the unmodified substrate is within the range of cleavage rates measured for other oligodeoxynucleotide substrates [6,14–16,19]. This suggests complete saturation of the enzyme substrate complex and an underestimation of binding strength in the electrophoretic mobility shift experiments.

The range of cleavage rates measured for the modified oligodeoxynucleotides is much wider than the range of binding constants which reaches from only fivefold less for TC-S to no cleavage within hours for GA-R, GA-S, AT-S, and CG2-S (Table 1). Comparing cleavage and binding shows no obvious correlation. For example, TT-R has the strongest reduction of binding strength but cleaves rather fast while CG2-S binds even better than TT-R but a cleavage is not detectable.

Discussion

The P-Me moiety is ideally suited to determine the importance of the negative charge at a phosphate residue for the interaction with a protein. Furthermore, the methyl group is only modestly larger than oxygen but it refuses to accept a hydrogen bond from an amino acid residue (Fig. 1). Therefore, in length and orientation fixed hydrogen-bonding interactions as well as often less localized charge attraction are disturbed.

We want to discuss in detail the measured effects of these substitutions on binding and cleavage by the *Eco*RI endonuclease for each position in the light of the contacts, which are seen in the cocrystal structure (Fig. 4, [20]). We use the stereochemical nomenclature applicable for methylphosphonates, which deviates from that for O-ethyl-phosphotriester and phosphorothioate substitutions (see Fig. 1 and its legend for the positioning of R_P and S_P substitutions).

The remarkable retardation in the catalytic activity of *Eco*RI with CG1-R and CG1-S may be attributed to the extensive hydrogen-bonding network extending almost equally over *pro*-R_P and *pro*-S_P phosphoryl oxygen atoms. The *pro*-R_p phosphoryl oxygen is hydrogen bonded to the amide NH of Lys89 as well as slightly less optimal to Asn149 and substitution of this oxygen with methyl group prevents the formation of these specific contacts. Similarly, the *pro*-S_P oxygen is oriented to form a hydrogen bond with Lys148 and two localized water molecules. The former contact is impaired twice when a methyl group replaces this oxygen because of missing charge and hydrogen bond acceptor moiety. The missing charge may also influence Arg203 nearby which is engaged in a base contact and only slightly too far away for a strong hydrogen bond. The large reduction of cleavage activity accompanied by an only modest impairment of binding suggests that interactions with the primary clamp phosphates at pGAATTC are intimately interwoven with the entire recognition process and the modification at these phosphates which removes negative charge interferes with its coupling to catalysis. Accordingly, O-ethyl-phosphotriester substrates interfered even more with cleavage [7] which indicates an

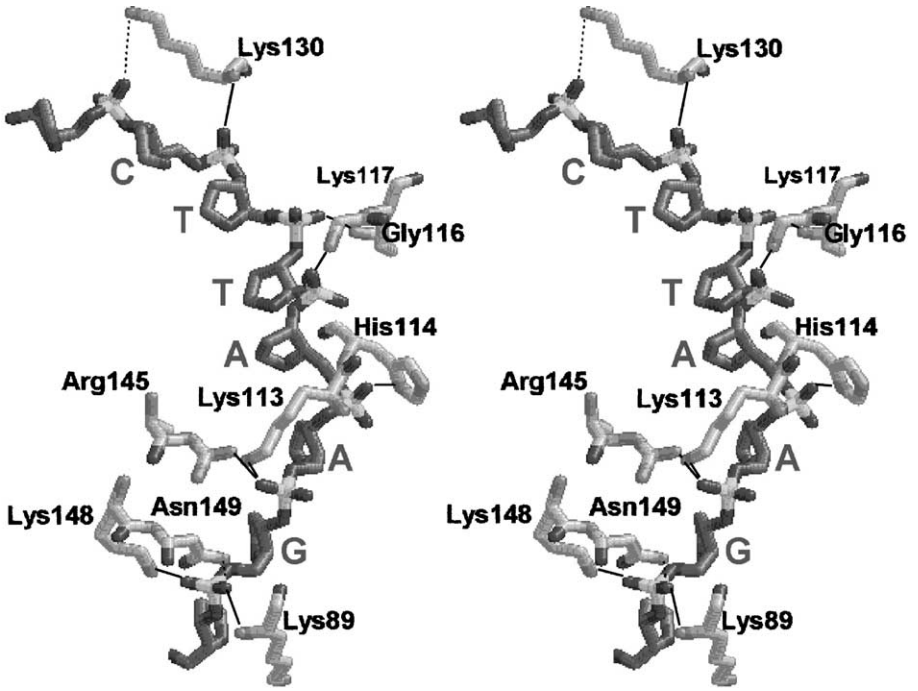

Fig. 4. Stereo view of the phosphate contacts to one phosphodiester backbone, which runs from 5′ to 3′ from bottom to top (generated from 1eri.pdb).

additional steric hindrance while phosphorothioate substitutions showed only moderate effects on cleavage and binding [9]. Therefore, the negative charge at this position plays a major role in recognition.

The P-Me substitution at the scissile phosphate resulted in completely resistant analogues (GA-R and GA-S). Several mechanisms of phosphodiester hydrolysis are discussed. In all of them, Mg^{2+} binds to the scissile phosphate and partially neutralizes the negative charge spread over both non-bridging oxygen atoms. Both R_P and S_P methyl groups at this phosphate eliminate negative charge and thereby may prevent divalent metal ion from binding. Such an effect should not be seen in binding measurements, which have been performed already in the absence of Mg^{2+} to prevent cleavage. Accordingly, GA-R is the best-bound P-Me substrate. GA-S is bound much worse because the *pro*-S_P oxygen accepts two hydrogen bonds, one from Lys113 which is essential for cleavage by reducing the additional negative charge of the transition state [10] and a second one from Arg145 which is additionally engaged in contacting the outer adenine base and Glu144 of the other subunit. Therefore, binding of S_P P-Me isomer is substantially decreased. These results strongly support the importance of phosphate charge interaction with the amino acid residues at the enzyme active site predicted by the crystal structure.

AA-R is bound slightly better and cleaved 10 times faster than AA-S because the *pro*-S_P oxygen is in hydrogen-bonding distance to His114 and a localized water molecule while the *pro*-R_P oxygen has no binding partner in the cocrystal structure. The rather good binding of AA-S may be attributed to a possible substitution of the hydrogen bond by a hydrophobic interaction between the methyl group and the imidazole ring after rotating the imidazole ring of histidine by 180°. This compensates the loss of binding consequent to removal of the negative charge on the phosphorous. This would even preserve the positioning of His114 by Glu170. A corresponding contact of an amino acid side chain with a P-Me was found for the *Eco*RV mutant T94V [21]. But the loss of negative charge for both substrates may be propagated to Lys113 and the rest of active centre directly next to His114, which may be responsible for the large drop in cleavage rate, by 30- and 300-fold. Phosphorothioate as well as O-ethyl-phosphotriester substitutions also showed the importance of the negative charge at the position of the *pro*-S_P oxygen.

According to one proposed mechanism of phosphodiester hydrolysis by *Eco*RI, the *pro*-S_P phosphoryl oxygen of the phosphate 3′ to the scissile phosphate acts as a base to protonate the hydrolytic water molecule [22]. This would imply a reduction in cleavage rate as observed in the present study namely AA-S isomer that is cleaved substantially lower than the all phosphate containing dodecamer. But if the *pro*-S_P phosphoryl oxygen plays an essential role in catalysis, one would expect an even larger reduction in cleavage rate like the one found for H-phosphonates at this position [23]. Either the postulated reorientation of His114 also compensates

mechanistically for the loss of the water activating oxygen or the phosphate has a more indirect role in the catalytic mechanism as discussed in the recent review of [2].

Diastereomers of the P-Me substitution at the central phosphate GAApTTC also behave differently. Enzyme accepted only the R_P isomer (AT-R) whereas the other isomer (AT-S) was completely resistant to cleavage. The polypeptide backbone near Gly116 is closely positioned to this central phosphate. The *pro*-S_P phosphoryl oxygen, projecting towards major groove, is in a hydrogen-bonding position with the amide NH of Gly116. Its substitution by a methyl group not only prevents hydrogen bond formation it may also push Gly116 slightly away from the DNA. Because Gly116 is in the same β-strand as the amino acid residues of the active centre (Glu111 and Lys113), this strongly reduces the catalytic action. Similarly, the corresponding phosphorothioate substrate is cleaved more slowly and its diastereomer analogue slightly better than the unmodified substrate [9]. No amino acid contact to *pro*-R_P oxygen but two localized water molecules are detectable in the cocrystal structure. Lys117 stands somewhat between this and the next phosphate but the loss of the negative charge is evident for AT-R as well. Only 4.2 and 3.5 Å away are the α- and β-methylene groups of Gln115 that couples base recognition by the extended chain motif to catalysis [16]. Although a hydrophobic contact is possible without larger conformational changes because the β-methylene group already contacts the methyl group of the inner thymine residue, which is also postulated for the S_P-P-Me [24], an even slight repositioning of this residue should interfere with binding as well as cleavage. A similar hydrophobic interaction may be the cause of cleavage reduction of the O-ethyl phosphotriester substituted oligodeoxynucleotides [9] but it is indistinguishable from steric interference. Although from the studies with several phosphate substituted substrates it may seem that the charge at the central position is of major importance, a much stronger influence may be exerted by the exact positioning of β3 with the catalytic centre via Gln115 or Gly116.

Methyl substitution for either *pro*-R_P or *pro*-S_P oxygen at the phosphate GAATpTC does not prevent hydrolysis of DNA. DNA cleavage occurs at almost equal rates for the two diastereomers and is moderately reduced compared to the unmodified substrate. Therefore, it may be said that charge interaction at this phosphate is not crucial for enzyme activity. This is rather surprising because Lys117 is in hydrogen-bonding distance to the *pro*-R_P oxygen and stays in contact at least part of the time during molecular dynamics simulation [25]. Accordingly, TT-S is bound better than TT-R. However, this difference of a factor of 2 is very low against the 100-fold drop in binding strength compared with the unmodified substrate. It seems therefore that the hydrogen bond is not as important as charge, which is recognized through space, and not by a localized interaction and that this ionic interaction is important for strong binding but is not propagated to the active centre. In accordance with that, charged phosphorothioate substitutions show no effect [7]. The situation changes with O-ethyl-phosphotriester substitutions because the TT-R analogue was cleaved readily while the TT-S analogue was cleaved only poorly [7]. The experimental design where cleavage was measured after a fixed time point may have hidden a slight reduction in cleavage rate of the TT-R analogue. The larger reduction of the TT-S analogue may indicate a slight steric interference with the first base contacting residue of the extended chain motif Met137. Altogether, GAATpTC is more important for binding than for cleavage.

Similarly, TC-S analogue also exhibited considerable amount of hydrolysis. The extent of cleavage was highest in TC-S among the other P-Me substrates. This is rather surprising because only the *pro*-S_P oxygen is in hydrogen-bonding distance to the amide NH of Lys130 and a localized water molecule. Since we were unable to synthesize the other isomer TC-R, nothing can be said about it directly. However, it is also clear from the studies with other modifications that the contact seen in the cocrystal structure is not important for the enzyme. Moreover a network of hydration shell is extended in the minor groove of DNA in the region of TTC sequence. These phosphates may be important for mediating with protein through the hydration layer.

Replacement of the GAATTCp phosphate with P-Me results in a totally resistant analogue (CG2-S) and the other analogue (CG2-R) with extremely low activity. Although Lys130 is nearby and long enough for a hydrogen bond, no direct contact to this phosphate is seen in the cocrystal structure. A simple ionic interaction cannot explain this strong reduction in cleavage especially because mutants at this position are rather active [26]. One might argue that Lys130 and Arg131 and their positive charges are important for a first approach to the DNA because they are located at the tip of the inner arm, which is laid around the DNA to bring the extended chain (137–142) deep into the major groove. In accordance with that, phosphorothioate substitutions at this position lead to undisturbed cleavage while O-ethyl-phosphotriester substitutions inhibit slightly the enzymatic action. But the P-Me substitutions inhibit binding rather strongly and cleavage even more. The present study provides direct evidence on the importance of flanking phosphate residues for initial approach by the enzyme. It may well be that another phenomenon comes into play. The K130A, K130E, and R131E mutants cannot bind DNA in the absence of divalent cations [26] and flanking sequences drastically influence their cleavage activity [27]. The effect of divalent cations was postulated to result from binding to the Asp–Xaa–Asp

Ca^{2+}-binding motif built by Asp133 and Asp135. But the triple mutant K130E/D133A/D135A still needs divalent cations for sequence specific binding (O. Rosati, unpublished observation). Therefore, subtle differences in the positioning of the inner arm may render alternative more stable enzyme conformations, which can be influenced by surrounding sequences or lead to inactivity. Such an inactive conformation was detected for the wild-type enzyme in stopped flow experiments as long as it was not preincubated with Mg^{2+} or DNA [19]. It is also possible that a hydrophobic interaction between the methyl group and the lysine side chain whose α-methylene group is 4.1 Å away stabilizes an alternative conformation. The considerably faster cleavage of O-ethyl-phosphotriester substituted substrates would then reflect their larger volume, leading to a different positioning of Lys130.

It becomes more and more obvious that the *Eco*RI restriction endonuclease behaves like a fine-tuned molecular machine, which only exerts its catalytic action after several conformational changes triggered by a precise interaction with its DNA substrate. These conformational changes are not localized but comprise even those parts of the enzyme that are far away from the DNA. For example, the N-terminal arms, which start close together on the rear part of the enzyme [28], are necessary for catalytic activity [29]. Therefore, part of the effects, which result from disturbing direct interactions to the DNA, will result even from subtle influences on enzyme conformation, which are not explainable from the inspection of the direct surrounding.

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