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A new affinity reagent for the site-specific, covalent attachment of DNA to active-site nucleophiles: application to the *Eco*RI and *Rsr*I restriction and modification enzymes

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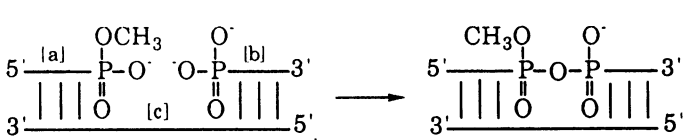
ABSTRACT

A modified oligodeoxyribonucleotide duplex containing an unnatural internucleotide trisubstituted 3′ to 5′ pyrophosphate bond in one strand [5′(oligo$_1$)3′-P(OCH$_3$) P-5′(oligo$_2$) 3′] reacts with nucleophiles in aqueous media by acting as a phosphorylating affinity reagent. When interacted with a protein, a portion of the oligonucleotide [—P-5′(oligo$_2$)3′] becomes attached to an amino acid nucleophilic group through a phosphate of the O-methyl-modified pyrophosphate linkage. We demonstrate the affinity labeling of nucleophilic groups at the active sites of the *Eco*RI and *Rsr*I restriction and modification enzymes with an oligodeoxyribonucleotide duplex containing a modified scissile bond in the *Eco*RI recognition site. With the *Eco*RI and *Rsr*I endonucleases in molar excess approximately 1% of the oligonucleotide becomes attached to the protein, and with the companion methyltransferases the yield approaches 40% for the *Eco*RI enzyme and 30% for the *Rsr*I methyltransferase. Crosslinking proceeds only upon formation of a sequence-specific enzyme-DNA complex, and generates a covalent bond between the 3′-phosphate of the modified pyrophosphate in the substrate and a nucleophilic group at the active site of the enzyme. The reaction results in the elimination of an oligodeoxyribonucleotide remnant that contains the 3′-O-methylphosphate [5′(oligo$_1$)3′-P(OCH$_3$)] derived from the modified phosphate of the pyrophosphate linkage. Hydrolysis properties of the covalent protein-DNA adducts indicate that phosphoamide (P-N) bonds are formed with the *Eco*RI endonuclease and methyltransferase.

INTRODUCTION

Covalently crosslinking a substrate to an enzyme is an established means of identifying the binding sites or regions of a protein that are in intimate contact with its ligand. In the case of the DNA restriction and modification enzymes, however, only a few such experiments have been reported (1,2). One of the main impediments to crosslinking DNA to proteins that recognize specific sequences has been the limited number of chemically active groups that can be introduced into a DNA duplex without significantly influencing either its base-pairing capability or its capacity to be bound specifically by the protein. Compared to other regions of the duplex where potentially reactive groups might be introduced, the modification of internucleotide phosphodiester bonds might produce lesser distortions in the DNA structure or disruptions of specific contacts and thus be desirable targets for the introduction of a crosslinking function.

In the present work we prepared a novel affinity reagent for the covalent modification of nucleophiles residing in the binding sites of proteins or enzymes that interact with DNA. We modified a double-strand oligodeoxyribonucleotide by incorporating a trisubstituted pyrophosphate internucleotide bond in place of the natural phosphodiester bond at a single, defined position in the nucleotide chain. The trisubstituted pyrophosphate internucleotide bond is formed by the chemical ligation (3) of an oligodeoxyribonucleotide with an O-methyl substituted 3′-phosphate [a] to another oligonucleotide with a 5′-phosphate [b] using a third, complementary strand [c] as a template to appose the two phosphate groups:



A water-soluble carbodiimide effects the phosphoanhydride bond formation (4).

Single-strand oligodeoxyribonucleotides containing trisubstituted pyrophosphate internucleotide bonds are sufficiently stable in aqueous solutions at pH values from 6 to 8, in the

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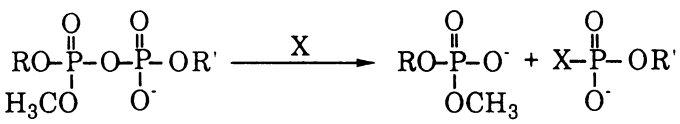
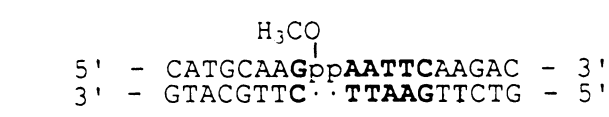
absence of strong nucleophiles, to avoid spontaneous degradation. The reaction of nucleophiles with such pyrophosphate analogues leads to attack on the bond, according to the following scheme (4):



where R and R′ represent the oligodeoxyribonucleotide chains surrounding the bond and X the nucleophile.

The trisubstituted pyrophosphate may be regarded as a phosphorylating reagent, acting on the nucleophile and covalently modifying it with a segment of oligodeoxyribonucleotide from the original duplex. The inability of restriction enzymes to hydrolyze pyrophosphate bonds, as demonstrated previously with the *Eco*RII endonuclease (5) and in this work is a desired characteristic of these substrates. The introduction of a pyrophosphate internucleotide bond into DNA appears not to have a significant influence on the structure and thermal stability of double-strand DNA (6) and thus should not interfere with binding of the enzyme (Fig.1). This combination of properties suggests that double-strand oligodeoxyribonucleotides containing a trisubstituted pyrophosphate bond might react according to the scheme outlined above with nucleophilic groups in the active sites of DNA restriction and modification enzymes upon formation of sequence-specific enzyme-DNA complexes.

In this work we examine the interaction of a duplex octadecadeoxyribonucleotide with the *Eco*RI and *Rsr*I restriction endonucleases and their cognate methyltransferases. The duplex (I) contains the recognition sequence, d(GAATTC), of these four enzymes, with one strand containing a methoxy-substituted pyrophosphate bond as the connection between the G and A residues that is the position attacked by the two endonucleases:



where the two dots represents a phosphodiester bond in the strand opposite the substituted pyrophosphate.

A methyl group was chosen as the alkyl substitutent because of its relatively small size and, therefore, presumably lesser steric effect on enzyme-DNA complex formation. Computer-displayed models of the duplex (I) with the natural phosphodiester internucleotide bond and two pyrophosphoryl-modified analogues are presented in Figure 1.

MATERIALS AND METHODS

Oligonucleotides

Synthesis of d(pAATTCAAGAC) [*b*]. All oligonucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer (Biotechnology Center, University of Illinois). The decadeoxyribonucleotide d(pAATTCAAGAC) [b] was formed with a 5′-phosphorylating agent purchased from Clontech. A small portion of this decanucleotide was prepared with a 5′-hydroxyl to allow for subsequent 5′-^{32}P labelling by reaction with [γ-^{32}P]ATP (DuPont) and T4 polynucleotide kinase using standard procedures. The labeled decanucleotide was purified on a NENSORB 20 Nucleic Acids Purification Cartridge (DuPont) and was used for the preparation of duplex (I) to incorporate a radioactive phosphorus into the pyrophosphate bond. Unlabeled d(pAATTCAAGAC) was combined with the 5′-^{32}P-labelled decadeoxyribonucleotide to give the desired specific radioactivities.

Oligonucleotides were purified by ion-exchange chromatography on a Beckman Model 420 HPLC system. The chromatography employed an FPLC Mono Q column (Pharmacia) that was eluted at 1 ml/min. The buffers were 0.18 M NaCl, 0.01 M NaOH (buffer A) and 0.9 M NaCl, 0.01 M NaOH (buffer B). The components were separated by elution with a gradient of 0 to 50% B over 72 minutes. After purification, the oligonucleotides were desalted by gel-filtration on NAP-5 or NAP-10 columns (Pharmacia) using water as an eluent.

Synthesis of d(CATGCAAGp). Initial experiments used a sample prepared by Dr. T.S.Oretskaya of Moscow State University using H-phosphonate chemistry and 2-[2-(4-methoxytrityloxy) ethylsulfonyl]ethyl-H-phosphonate for the 3′phosphorylation (7). Alternatively, d(CATGCAAGp) was obtained by the phosphoramidite method on an Applied Biosystems 380A DNA synthesizer. The first condensation was with a 5′-phosphorylation agent (5′ Phosphate−ON) (Clontech, Palo Alto, CA), using a CP6-glass support bearing any deoxyribonucleoside as a target for the attachment of the phosphate that would ultimately become the 3′-P of the product. Subsequently, the normal oligonucleotide synthesis protocol was performed. This procedure, when completed by the ammonolysis deblocking step, leads to an oligonucleotide with a 3′-phosphate. Samples prepared either way behaved identically after purification.

Synthesis of d(CATGCAAGpOCH$_3$) [*a*]. Alkylation of the 3′-phosphate of d(CATGCAAGp) was essentially as described in (8), and was carried out in a volume of 50−100 μl at 4°C overnight. The reaction contained 0.05 to 0.1 μmol of oligonucleotide, 0.4 M 2-[N-morpholino]ethanesulfonic acid (MES) buffer (pH 4.5), 1.6 M $MgCl_2$, 6 M methanol and 5 to 8 mg (26.1 to 41.7 μmol) N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC). After partial purification of the mixture by gel-filtration on an NAP-5 column, d(CATGCAAGpOCH$_3$) was isolated by HPLC on a 0.4×15 cm Adsorbosphere C-8 column (Alltech), and desalted by multiple evaporations to dryness from 50% ethanol at 50°C. The conversion of the 3′-phosphate to methoxyester was 80−90%, and the yield after HPLC-purification was 50%.

Synthesis of duplex (I). Equimolar amounts of d(CATGCAAGpOCH$_3$) [a], [5′-^{32}P]d(pAATTCAAGAC) [b] and d(GTCTTGAATTCTTGCATG) [c] (prepared by standard phosphoramidate chemistry) (~ 0.5 nmol of each) in 10 μl of 0.05 M MES-HCl (pH 6.0), 0.02 M $MgCl_2$ were treated with 0.2 M EDC for 5−6 days at 4°C in the dark (2,4). Periodically, 0.2 μl aliquots were removed and analyzed by electrophoresis on 20% PAG containing 7 M urea followed by autoradiography to locate the isotope. The yield of modified octadecanucleotide, {d[CATGGCAAGp(OCH$_3$)^{32}pAATTCAAGAC]} where p(OCH$_3$)^{32}p represents the labeled, substituted 3′ to 5′ pyrophosphate internucleotide bond, was 25−30%. The oligonucleotide fraction was separated from the other components of the reaction mixture by gel-filtration on a NAP-5 column using water as the eluent.

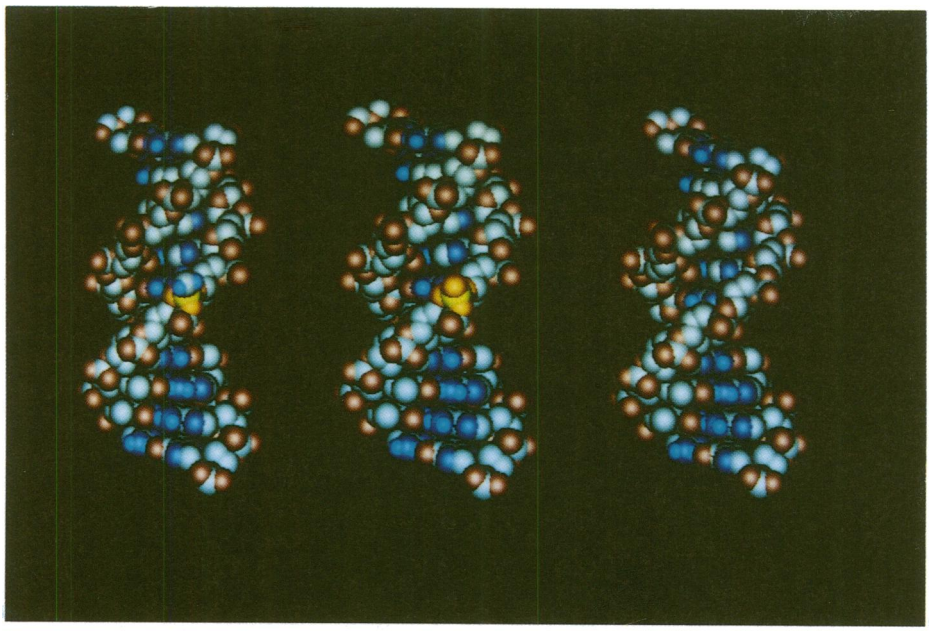


Fig. 1. Graphic representations of eleven-base pair segments centered about the site of modification in duplexes I, II, and III. Duplex octadecadeoxyribonucleotides were modeled containing a methoxy-substituted pyrophosphate (I), a pyrophosphate (II), or a phosphodiester (III) linkage at the scissile bond in the sequence d(GAATTC) of the top strand as given in the text. These structures were generated by calculating the coordinates of a B-form unmodified duplex (III) using QUANTA (Polygen), and manually disconnecting the phosphorus of the scissile phosphodiester bond from the 3'-hydroxyl of the G residue. An added O-methylphosphate (I) or unsubstituted phosphate (II) was then introduced into the structure at the discontinuity by appropriately connecting it to the oligonucleotide fragments so that van der Waals constraints were not violated and ideal bond lengths and angles were maintained throughout. The added phosphorus and oxygen atoms are indicated in yellow and orange, respectively. These models are displayed on an IBM Professional Graphics Display using the program SPHERE of the pdViewer package by A. R. Crofts and H. H. Robinson of the University of Illinois.

Synthesis of duplex (II). The octadecanucleotide d(CATGCAAGp-^{32}pAATTCAAGAC), with an unsubstituted internucleotide 3' to 5' pyrophosphate bond (indicated as p^{32}p) was obtained similarly by EDC-induced condensation of d(CATGCAAGp) with [5'-^{32}P]d(pAATTCAAGAC) in a double-strand complex with the template d(GTCTTGAATTCTTGCATG). The yield was 85–90%. Single-strand d(CATGCAAGp^{32}pAATTCAA-GAC) was purified by electrophoresis on 20% PAG containing 7 M urea followed by elution from the gel by the crush-and-soak method.

Enzymes

*Eco*RI endonuclease (R·*Eco*RI) and methyltransferase (M·*Eco*RI) were gifts of Dr. P. Modrich. *Rsr*I endonuclease (R·*Rsr*I) and methyltransferase (M·*Rsr*I), purified according to procedures described in (9) and (10), were kindly provided by Drs. C. Aiken and W. Kaszubska. T4 polynucleotide kinase was purchased from BRL Life Technologies, Inc.

Endonuclease assay

Cleavage by R·*Eco*RI or R·*Rsr*I was assayed in a manner similar to that described (11). Aliquots of duplexes II and III were dried in microfuge tubes *in vacuo*, redissolved in 10 μl of *Eco*RI buffer (20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA) or *Rsr*I buffer (50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 1 mM DTT), heated to 70–80°C for 5 min, and allowed to cool to room temperature for 10–15 min. After dissolution of the oligonucleotides by shaking and collection of the solution by centrifugation, the tubes were placed in a 20°C water bath for 3–5 min. R·*Eco*RI and R·*Rsr*I were diluted into reaction buffer immediately prior to use, and reactions were initiated by adding an equal volume (10 μl) of diluted enzyme to the reaction mixture. The final concentration of substrate was 100 to 500 nM. Aliquots (2 μl) were withdrawn at 2 min intervals and spotted onto DEAE-cellulose TLC sheets (Whatman, Inc). The plates were subjected to homochromatography using homomix 6 (12). The radioactivity in the substrate and product spots was located by autoradiography, the labelled areas excised, and their activity quantified by Cerenkov counting using a Beckman LS 1801 liquid-scintillation counter. Turnover numbers (k_{cat}) were determined as described in (13).

Crosslinking oligonucleotide to enzyme

The oligonucleotide fraction obtained after chemical ligation (about 0.5 μM in total oligonucleotide) was incubated with enzyme (1 μM) in the appropriate buffer at 37°C for 20–24 hours. The buffers for the crosslinking reaction were: for R·*Eco*RI—20 mM Tris-HCl (or methylimidazole-HCl) (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT; for M·*Eco*RI—50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 5 mM DTT, 2 μM sinefungin; for R·*Rsr*I—50 mM Tris-HCl (or methylimidazole–HCl) (pH 8.5), 1 mM EDTA, 1 mM DTT; for M·*Rsr*I—50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 5 mM DTT, 2 μM sinefungin. The progress of the crosslinking reaction

was monitored by electrophoresis on PAG containing sodium dodecylsulfate (SDS) (14). For this purpose an equal volume (20 μl) of 2× sample buffer (200 mM Tris-HCl (pH 6.8), 3% SDS, 40% glycerol, 3% β-mercaptoethanol and 0.01% bromphenol blue) was added, the sample heated for 5 min at 95°C, and loaded on a 20% SDS-PAG. After electrophoresis, the gel was exposed at −70°C for 4−18 hours on Kodak XAR-5 X-ray film to locate the label. The yield of chemical crosslinking was determined by liquid scintillation counting of excised gel bands containing the crosslinked enzyme and the remaining oligonucleotides.

Analysis of the covalent bond between oligonucleotide and enzyme

The radioactive, crosslinked R.*Eco*RI or M·*Eco*RI products were purified by gel-filtration on a NAP-5 column and concentrated by evaporation *in vacuo* in a Speed Vac centrifuge. To the resulting solution, 0.5 M HCl, 8 M NH_2OH-HCl (pH 5) or 0.5 M NaOH were added to final concentrations of 0.1 M, 4 M and 0.1 M, respectively. The reaction mixtures were incubated for 20 min, 2 hours and 30 min respectively at 37°C, loaded on a 20% SDS-PAG, and the proportion of the radioactivity remaining attached to the enzyme determined after electrophoresis.

RESULTS AND DISCUSSION

Preparation of the affinity reagent

Duplex I, with a methyloxy-substituted pyrophosphate internucleotide bond at the scissile bond in one strand of the recognition site of the *Eco*RI restriction endonuclease, was prepared by chemical ligation (3,4) as described above. The yield was 25−30% after 5−6 days of incubation (Fig. 2). Attempts to purify the modified octadecanucleotide (the top strand of duplex I) by SDS-PAG electrophoresis were unsuccessful because of partial hydrolysis of the substituted pyrophosphate linkage during the crush-and-soak elution from the gel. Therefore, entire oligonucleotide chemical ligation mixtures were used for crosslinking experiments without purification beyond the removal of low molecular weight components by gel-filtration chromatography. In these mixtures only 25−30% of the mixture was the activated substrate and thus potentially reactive with the enzymes. The remainder of the mixture consisted of unligated double-strand complexes and may have been bound by the enzymes. Binding of the inert substrates could compete with the reactive oligonucleotide and thereby reduce the cross-linking yields. It is likely that a more rapid purification procedure or one carried out at low temperatures would allow purification of the reagent. Electroelution from the electrophoresis gel or ion-exchange chromatography followed by rapid gel-filtration could yield a purer product.

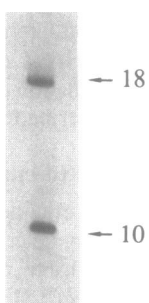


Fig. 2. Electrophoretic analysis of the chemical ligation mixture used to form duplex I. [5′-^{32}P]d(pAATTCAAGAC) and d(CATGCAAGp(OCH_3)) were incubated with a template strand as described in Materials and Methods with EDC. The product d(CATGCAAG(OCH_3)p[^{32}P]pAATTCAAGAC) (indicated by 18) and the labelled, unreacted precursor (indicated by 10) were detected by autoradiography.

Interaction of *Eco*RI and *Rsr*I restriction endonucleases with duplex containing unmodified pyrophosphate

In order to determine whether *Eco*RI and *Rsr*I restriction endonucleases would bind the pyrophosphate-modified double-strand oligonucleotide, we studied the ability of the enzymes to cleave an analogue duplex. Instead of the actual crosslinking reagent, (I), we used its unsubstituted pyrophosphate analogue,

```
5' - CATGCAAGppAATTCAAGAC - 3'
3' - GTACGTTC··TTAAGTTCTG - 5'      (II)
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Modeling indicates that duplex II is structurally similar to duplex I (Fig. 1). However, the pyrophosphate linkage is not as chemically reactive, and consequently, the unsubstituted internucleotide pyrophosphate bonds do not serve as activated phosphorylating agents (15). As a control we used the same duplex lacking phosphodiester bond alterations,

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5' - CATGCAAGAATTCAAGAC - 3'
3' - GTACGTTCTTAAGTTCTG - 5'        (III)
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We found that the top strand of duplex II is cleaved by neither restriction endonuclease (data not shown). However both R·*Eco*RI and R·*Rsr*I cut the bottom strand of duplex II (Table 1). To quantify potential disruptive influences due to the introduction of the pyrophosphate internucleotide bond, we compared the kinetics of bottom strand cleavage for duplexes II and III and found no significant differences.

These data show that both endonucleases bind and cut the unmodified GAATTC site in duplex II, but fail to cut the pyrophosphate-containing site. That the unmodified strand is cut suggests that the enzymes might also recognize methylsubstituted pyrophosphate-containing GAATTC sites since a methyl group is relatively small. Although a duplex containing the modified pyrophosphate was not assayed for catalytic competence, the sequence-specific linking of duplex I to the enzymes (see below) substantiates this expectation. Furthermore, previous ethylation-interference studies with *Eco*RI endonuclease indicate that ethylation of the scissile phosphodiester bond is relatively

Table 1. Effect of pyrophosphate linkage on the hydrolysis of the opposite strand.

	Substrate[a]	Endonuclease *Eco*RI Turnover Number[b]	Endonuclease *Rsr*I Turnover Number[b]
(II)	5' - CATGCAAGppAATTCAAGAC - 3'[c] 3' - GTACGTTC··TTAAGTTCTG^{32}p - 5'	3.4±0.5	2.9±0.4
(III)	5' - CATGCAAGAATTCAAGAC - 3' 3' - GTACGTTCTTAAGTTCTG^{32}p - 5'	2.6±0.4	2.5±0.3

[a]The bottom strand was labelled with [γ-^{32}P]ATP and T4 polynucleotide kinase.
[b]Turnover numbers are k_{cat} values and were determined as described in Materials and Methods.
[c]The recognition sequence is indicated in boldface and the dots indicate the phosphodiester bond in the strand opposite the pyrophosphate linkage.

nondisruptive of specific complex formation (16, 17). A still larger, aromatic derivative of the *Taq*I scissile phosphodiester bond has recently been shown to crosslink to the cognate ENase (18).

Crosslinking of duplex (I) with *Eco*RI and *Rsr*I restriction and modification enzymes

A typical regime for crosslinking the substrate to an enzyme incubated 1 μM enzyme and 0.5 μM oligonucleotide duplex (containing about 25% activated substrate) for 18–24 hours. In order to detect the covalent complex, the reaction mixture was subjected to SDS-PAG electrophoresis. Control experiments with 5′-^{32}P-labeled unmodified duplex III confirmed that this procedure completely separates noncovalently bound substrate and hydrolyzed products from the enzymes which are retarded in their migration as a result of the modification. The protein was visualized by staining the gels with silver and the labelled oligonucleotides and products of crosslinking were located by autoradiography. M·*Eco*RI crosslinked more efficiently than R·*Eco*RI (Fig. 3). Under these electrophoresis conditions the two labeled proteins migrated similarly in spite of their differences in M_r. When subjected to longer times of electrophoresis the labeled oligonucleotide-endonuclease complex migrated more rapidly than the affinity-labeled methyltransferase. This result argues against the possibility that the labeled product formed with the endonuclease is in fact due to a contamination of the nuclease preparation with the methyltransferase (19).

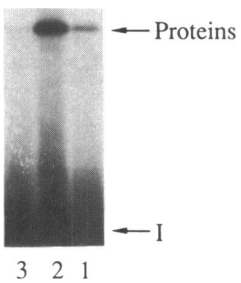

Fig. 3. Electrophoretic analysis of the crosslinking of duplex I to *Eco*RI endonuclease and methyltransferase. Duplex I with ^{32}P in the 3′-phosphate of the modified pyrophosphate as shown in the legend to Fig. 2, was incubated with the endonuclease (lane 1), the methyltransferase (lane 2), or in the absence of enzyme and the reaction mixtures processed as described in Materials and Methods. The positions of migration of the duplex (I) and the oligonucleotide-modified proteins (Proteins) are indicated.

We found that all four enzymes crosslinked to duplex I. In order to optimize the reactions, we varied pH values in the range from 7.0 to 9.0, buffer compositions, temperatures, and reaction times. We list the optimal conditions in Table 2. In all cases the best yields of substrate attachment were at pH values approximately 0.5 unit higher than the pH optimum for catalysis by the respective enzymes. For R·*Eco*RI and R·*Rsr*I a buffer containing 1-methylimidazole instead of Tris gave 1.5-fold higher yields of product. For M·*Eco*RI and M·*Rsr*I the best yields were obtained in Tris buffer. There were no significant temperature dependences of yield over the range of 20°to 37°C. The optimal reaction time was approximately 20 to 24 hours, after which no further radioactivity associated with the protein fraction. The addition of Mg^{2+} to the crosslinking reactions with endonuclease did not change the yields of product.

To show specific binding, duplex I was incubated under the conditions shown in Table 2 with R·*Eco*RI that had been preheated for 10 min at 95°C. No radioactivity was found associated with protein. Under conditions where the formation of the enzyme-substrate complex is unlikely, i.e., in buffers containing 0.5 M NaCl or 0.1% SDS, crosslinking was likewise not observed. We also failed to see nonspecific crosslinking of duplex I to high concentrations of bovine serum albumin. Neither did we detect any radioactivity associated with any of the enzymes upon incubation with labelled, unmodified duplex III or with labeled duplex II, which contains an unsubstituted pyrophosphate internucleotide bond at the same position. In these control experiments unmodified duplex III with either an internal ^{32}P-

Table 2. Crosslinking of duplex I to of the *Eco*RI and *Rsr*I restriction and modification enzymes.

Enzyme	Reaction Components[a]	pH	Attached Label (%)	Attached Activated Substrate[b] (%)
R·*Eco*RI	20 mM Methylimidazole 100 mM NaCl 1 mM EDTA 1 mM DTT	8.0	0.2–0.28	0.8–1.0
M·*Eco*RI	50 mM Tris 5 mM EDTA 5 mM DTT 2 μM Sinefungin	8.5	10–12	35–40
R·*Rsr*I	50 mM Methylimidazole 1 mM EDTA 1 mM DTT	8.5	0.2–0.25	0.8–1.0
M·*Rsr*I	50 mM Tris 5 mM EDTA 5 mM DTT 2 μM Sinefungin	8.5	6–9	25–30

[a]Typically, enzyme was present at 1 μM, total oligodeoxyribonucleotide duplex at 0.5 μM, and the reaction mixtures were incubated at 25–37°C for 18–24 h.
[b]The attached activated substrate values are corrected to account for the presence of labelled, unligated oligonucleotide precursors in the reaction mixtures (see text).

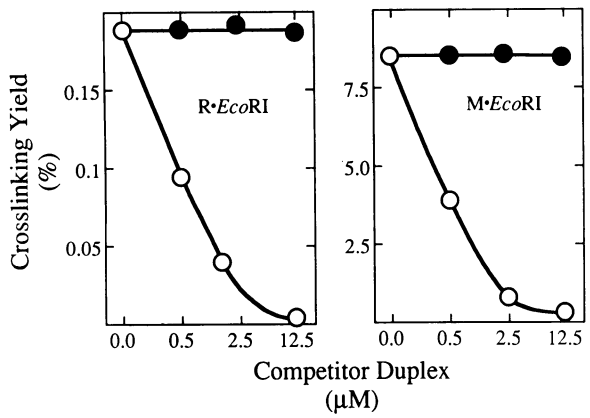

Fig. 4. Crosslinking duplex I to *Eco*RI endonuclease and methyltransferase in the presence of an unlabeled duplex containing an *Eco*RI site (III) (○) or a duplex lacking the recognition sequence (IV) (●). Duplex I labeled with ^{32}P in the 3′-phosphate of the modified pyrophosphate (0.5 μM) was incubated under the conditions given in Table 2 with increasing amounts of unlabeled DNA described in Results. The yields were determined by electrophoretic analysis as described in Materials and Methods and plotted as a function of the amount of added unlabeled DNA.

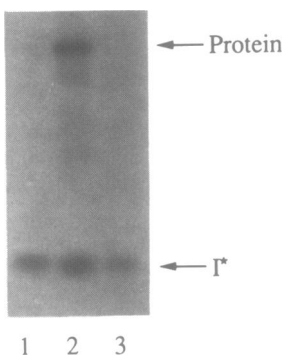

Fig. 5. Stability of isolated *Eco*RI methyltransferase-[^{32}P] duplex I. The isolated covalent complex (Protein) between the methyltransferase and duplex I (lane 2) labelled as in the legend to Fig. 2 was treated with 0.1 M HCl (lane 1) or 4 M NH_2OH at pH 5 (lane 3) as described in Materials and Methods. The released oligonucleotide fragment is indicated by I*. Radiolabel was detected by autoradiography.

label located in the top strand between G and A at the scissile bond, or with a 5′-end ^{32}P-label was used. Similarly, the pyrophosphate-modified duplex (II) was ^{32}P-labeled at the internal phosphate (CATGCAAGp^{32}pAATTCAAGAC) or at the 5′-phosphate of the top strand. No radioactivity associated with protein when any of these substrates was used. Collectively, the results show that duplex I must be bound by the enzymes and that only the methoxy-substituted pyrophosphate acts as an affinity reagent. That enzyme-bound radioactivity was not detected using 5′-^{32}P-labeled duplex (I) as a substrate shows that the phosphate to the 3′ side of the trisubstituted pyrophosphate bond (i.e., the phosphate lacking the methoxy substituent) reacts with the nucleophiles on the enzymes. These findings indicate that the decanucleotide fragment of I, [5′-^{32}P]pAATTCAAGAC, probably becomes covalently attached to the enzyme through its 5′-phosphate.

Sequence specificity of the crosslinking of *Eco*RI restriction-modification enzymes to duplex I

In order to demonstrate that the attachment of substrate to enzyme was specific for the DNA recognition sequence, crosslinking was undertaken in the presence of increasing amounts of unlabeled, unmodified duplex III. Increasing the concentration of the competitor duplex lead to inhibition of the crosslinking reaction (Fig. 4). A 25-fold excess of competitor almost completely blocked the attachment of labeled substrate to enzyme. At the same time the presence at the same concentrations of the oligodeoxyribonucleotide duplex

5′-AAGCCTGCTTATTTATACTAACTTGAGC-3′ (IV)
3′- TCGGACGTATAAATATGATTGAACTCGC-5′

that lacks an *Eco*RI recognition site had no significant effect on the reaction. These results indicate that the crosslinkings are sequence specific (Fig. 4).

Nature of the covalent bond between the *Eco*RI restriction and modification enzymes and the oligodeoxyribonucleotide

To determine the nature of the covalent bonds linking the oligonucleotide to the *Eco*RI enzymes, crosslinked enzymes were isolated and subjected to several tests. The intermolecular covalent linkage to M·*Eco*RI was stable in 0.1 NaOH solution (data not shown), but was hydrolyzed by treatment with 0.1 M HCl or 4 M NH_2OH at pH 5 (Fig. 5). Identical results were obtained with the crosslinked R·*Eco*RI product. The acid and acidic-hydroxylamine lability and alkaline stability are diagnostic for phosphoamide bonds (20). The acid lability of the linkage excludes mixed anhydride linkages to Glu or Asp. Ester linkages to Ser, Thr, or Tyr are unlikely because acidic NH_2OH fails to efficiently cleave these bonds. Thus, these results suggest that phosphorus to nitrogen bonds link the enzymes to the substrate in these two complexes. His, Lys, and Arg are likely targets for the observed crosslinkings.

CONCLUSIONS

Chemical ligation, which was developed as an alternative method of assembling oligonucleotides for chemical syntheses, is also a means of constructing modified internucleotide bonds in DNA duplexes. We have developed a reproducible synthesis for a novel type of crosslinking reagent that can be used to covalently attach oligonucleotide substrates to nucleophilic groups in the binding sites of enzymes or proteins that bind DNA. This reagent, in the form of an octadecadeoxyribonucleotide duplex containing a single methyloxysubstituted 3′ to 5′ pyrophosphate internucleotide bond, reacted with the recognition sites of the *Eco*RI and *Rsr*I restriction and modification enzymes to form covalently-linked complexes. The covalent attachment of enzyme to modified oligonucleotide duplex likely occurred specifically at the sites of the enzymes that are involved in DNA sequence recognition. Initial characterizations indicate that P-N bonds link the *Eco*RI endonuclease and methyltransferase to the oligonucleotide.

The differences in reactivity among the four enzymes of the *Eco*RI and *Rsr*I restriction-modification systems (Table 2) probably reflect the differences in the local environments of the substituted pyrophosphate bond with respect to potential nucleophiles in the active sites. Presumably both the identity and availability of nucleophilic groups as well as the affinity of the

enzyme for the substrate would influence the yield of crosslinked product. Of the four enzymes we examined structural information is available for only R·*Eco*RI (21). The structure suggests that Lys 113, His 114 and Arg 145 are near the scissile bond and thus one or more of these amino acids might bear the nucleophiles leading to the formation of the phosphoamide linkage we observed.

Because the activated, substituted pyrophosphate linkage can be synthesized at any location in a sequence, these reagents should be useful for identifying the regions of proteins and enzymes that are near particular phosphodiester bonds.

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