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Oligodeoxynucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine as affinity labels for the Eco RV restriction endonuclease and modification methylase

Theo T.Nikiforov and Bernard A.Connolly*[1]
Department of Biochemistry, University of Southampton, Bassett Crescent East, Southampton, SO9 3TU and [1]Department of Biochemistry and Genetics, The University, Newcastle-upon-Tyne, NE2 4HH, UK

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ABSTRACT

4-Thiothymidine and 6-thiodeoxyguanosine were incorporated into synthetic dodecamers containing the recognition site d(GATATC) of the enzymes Eco RV endonuclease and Eco RV methyltransferase. Upon irradiation with long wavelength UV light (340–360 nm), these oligodeoxynucleotides were photochemically crosslinked to both enzymes. The yields were up to 35% with the methyltransferase, but lower (up to 6%) with the endonuclease. Oligodeoxynucleotides containing 4-thiothymidine generally gave higher yields of crosslinking than those containing 6-thiodeoxyguanosine. Although both specific (i.e. those containing the d(GATATC) sequence) and non-specific (lacking this sequence) photoreactive oligodeoxynucleotides gave rise to crosslinked products, the use of a non-reactive, competitive substrate oligodeoxynucleotide suppressed the crosslinking, indicating that the reaction takes place at the enzymes' active sites. Oligodeoxynucleotides containing 4-thiocyanatothymidine or 6-thiocyanatodeoxyguanosine were also prepared by treatment of the title oligomers with CNBr and KCN. The dodecamers containing 4-thiocyanatothymidine were found to covalently modify both enzymes under study, with levels of crosslinking reaching up to 42% with the endonuclease and up to 12% with the methyltransferase. No crosslinking was observed with oligodeoxynucleotides containing 6-thiocyanatodeoxyguanosine.

INTRODUCTION

Photochemical crosslinking has been widely used in the study of DNA- and RNA-binding proteins [1, 2]. Underivatized nucleic acids can undergo photochemical reactions and thus their photocrosslinking with nucleic acid-binding proteins has been realized by irradiation with short wavelength UV light (254 nm). However, in most cases the yields of the desired crosslinked proteins are low. Therefore other, more photosensitive base analogues, which often absorb at longer wavelengths, have been introduced into nucleic acids. 5-Bromouracil is such a widely used photoreactive base analogue. Its use leads to significantly increased yields of photocrosslinked products upon irradiation at about 310 nm [3–6]. An advantage of this compound is its availability as the corresponding phosphoramidite of 5-bromo-2-deoxyuridine, which can be easily introduced at any desired position of synthetic oligodeoxynucleotides using standard phosphoramidite chemistry. The successful use of other photoreactive nucleotides incorporated into DNA has also been described. These include 5-azido-2-deoxyuridine (incorporated enzymatically via its 5-triphosphate); [7, 8]; 8-azido-2-deoxyadenosine (incorporated enzymatically) [8]; an azido derivative of 2-deoxyuridine (incorporated enzymatically) [9]; and an oligodeoxynucleotide containing a 5-terminal azido function and prepared by a chemical approach [10].

The photoreactivity of 4-thiopyrimidines has long been recognized (see [11] for a review), and their uses in a number of biological photoaffinity labelling experiments described [12–14]. Very recently short mRNA molecules containing several 4-thiouridine bases have been prepared enzymatically and used in photocrosslinking to ribosomes [15]. Many tRNAs contain 4-thiouridine, which can be used as an intrinsic photoaffinity probe to study tRNA tertiary structure [16]. Less data is available on 6-thioguanosine and its deoxy analog, but some reports suggest that it too is photoreactive and therefore potentially useful for photoaffinity labelling [17]. Until recently, the introduction of these modified residues at predetermined positions of synthetic nucleic acid oligomers has not been possible.

We have recently developed highly efficient methods for the introduction of both 4-thiothymidine [18–20] and 6-thiodeoxyguanosine [20, 21] residues into synthetic oligodeoxynucleotides using phosphoramidite chemistry. This has enabled us to study the possible use of oligodeoxynucleotides containing these modified bases in photoaffinity labelling of two

* To whom correspondence should be addressed

DNA-modifying enzymes, the Eco RV endonuclease and the Eco RV methyltransferase. The results presented in this paper show that oligodeoxynucleotides containing these residues can indeed be used as photoaffinity labels for these DNA-binding enzymes, and therefore could find wide application in biochemistry and molecular biology.

The chemical crosslinking of nucleic acid-binding proteins (without the need for photochemical activation) to their substrates has also been reported and usually involves the attachment of a chemically reactive group to the 3- or 5-end of an otherwise unmodified substrate molecule. A good example is the affinity labelling of RNA polymerase with oligonucleotides containing reactive phosphorylating agents at their 5-termini [22]. Because the chemically reactive centre is located outside of the actual region of the substrate molecule, which is recognized and bound to the enzyme, crosslinking might in this case occur to amino acid residues that are not necessarily part of the binding/catalytic apparatus. To circumvent this potential problem, the preparation and use in crosslinking experiments of a chemically reactive deoxyguanosine derivative has been described [23].

It has previously been observed that 4-thiopyrimidines can be converted to thiocyanato derivatives on treatment with CNBr/KCN and that these compounds are very sensitive to attack by nucleophiles [24, 25]. We have now found that treatment of the oligodeoxynucleotides containing 4-thiothymidine with CNBr/KCN yields molecules containing 4-thiocyanatothymidine. These can be chemically crosslinked (in the dark) to both DNA-binding enzymes under study. Since the reactive 4-thiocyanatothymidine residue is part of the enzymes' recognition sequence, the reacting amino acid residues should be part of the enzymes' binding sites. We have found that the reactivity of these chemical affinity labels complements that of the photochemically reactive, 4-thiothymidine-containing substrate analogues, thus further broadening their usefulness.

MATERIALS AND METHODS

EcoRV endonuclease and Eco RV methyltransferase were overexpressed using recombinant plasmids in *E. coli* and isolated as previously described [26, 27]. Protein concentrations were determined using a BioRad protein determination kit, with BSA as a standard. All oligodeoxynucleotides were synthesized on an Applied Biosystems 380A DNA synthesizer using standard phosphoramidite chemistry. Oligodeoxynucleotides containing 4-thiothymidine and 6-thiodeoxyguanosine were prepared as previously reported [19–21], and purified by reverse-phase HPLC.

5′-[^{32}P]-Phosphorylated oligodeoxynucleotides were prepared using [γ-^{32}P]-ATP and polynucleotide kinase. S-Adenosyl-L-methionine (AdoMet) was from Boehringer Mannheim, sinefungin was purchased from Calbiochem.

Preparation of oligodeoxynucleotides containing 4-thiocyanatothymidine and 6-thiocyanatodeoxyguanosine residues

An oligodeoxynucleotide containing either 4-thiothymidine or 6-thiodeoxyguanosine (approximately 1 OD) was dissolved in 150 μl of 7 M urea, 50 mM HEPES, pH 7.5. Then, 30 μl of a 1 M KCN solution in the same buffer and 50 μl of a freshly prepared 0.15 M CNBr solution, also in the same buffer were added and the resulting mixture kept on ice for 30 min. The course of the reaction was followed by HPLC and more CNBr was added if necessary. When the starting material disappeared completely, the mixture was applied directly to a NAP-5 gel filtration column (Pharmacia), equilibrated with 5 mM MES, pH 6.5, 10 mM NaCl, and the product eluted with the same buffer. Fractions containing the product were pooled and concentrated in vacuum. The products were then stored frozen at −20°C. Products containing 6-thiocyanatodeoxyguanosine were found to be quite stable, showing little or no decomposition after a three week period of storage, including several freeze-thaw cycles, but the oligodeoxynucleotides containing 4-thiocyanatothymidine decomposed relatively rapidly and so were used within one week of their preparation. Deoxynucleoside composition analysis of oligodeoxynucleotides was carried out as previously described, using a mixture of snake venom phosphodiesterase and alkaline phosphatase (both obtained from Boehringer Mannheim) [18, 20, 21].

Photocrosslinking of oligodeoxynucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine to the Eco RV endonuclease and Eco RV methyltransferase

Photoaffinity labelling was generally carried out in 60–100 μl reaction mixtures, which were placed on a piece of Parafilm and irradiated with the long wavelength UV light of a hand-held, dual-wavelength Mineralight UVGL-25 lamp, at a distance of 1.5–2 cm, at room temperature. To completely remove all light <310 nm, a Pyrex plate was placed between the samples and the light source. Enzymes were used in a concentration of 3–10 μM. The concentration of the photoreactive oligodeoxynucleotides ranged from 5 to 30 μM, and they were added in 2–3 aliquots to the reaction mixture, followed by 10–12 min of irradiation after the addition of each aliquot. Before starting the irradiation, the reaction mixtures were left at room temperature for 15 min to allow complex formation. For the Eco RV methyltransferase crosslinking, the reaction buffer comprised 50 mM HEPES, pH 7.5, 100 mM NaCl, and 1 mM sinefungin or S-adenosyl-L-methionine. With the Eco RV endonuclease, a buffer containing 50 mM HEPES, pH 7.5, 100 mM NaCl, and either 10 mM $MgCl_2$ or 1 mM EDTA was used. Analysis of the extent of crosslinking was performed by the separation of free and crosslinked enzyme using denaturing SDS polyacrylamide gel electrophoresis according to Laemmli [28]. Proteins were visualized by silver staining using a kit obtained from Stratagene. When radioactively labelled oligodeoxynucleotides were used, the gels were dried and exposed to X-ray films.

Table 1. Yields of crosslinked products obtained by photochemical and chemical affinity labelling of Eco RV endonuclease and Eco RV methyltransferase

Oligodeoxynucleotide	Enzyme		
	Methyltransferase	Endonuclease ($+Mg^{2+}$/$-Mg^{2+}$)	
d(GAC[^{6S}G]ATATCGTC)	8%	6%	0%
d(GACTAC[^{6S}G]TAGTC)	5%	traces	0%
d(GACGA[^{4S}T]ATCGTC)	18%	traces	traces
d(GACGATA[^{4S}T]CGTC)	35%	6%	traces
d(GACGA[^{4S}T]A[^{4S}T]CGTC)	10%	traces	0%
d(GAC[^{4S}T]ACGTAGTC)	traces	traces	0%
d(GAC[^{6SCN}G]ATATCGTC)	0%	0%	n.d.
d(GACGA[^{4SCN}T]ATCGTC)	8%	12%	n.d.
d(GACGATA[^{4SCN}T]CGTC)	12%	42%	n.d.
d(GAC[^{4SCN}T]ACGTAGTC)	5%	30%	n.d.

Affinity labelling with oligodeoxynucleotides containing 4-thiocyanatothymidine or 6-thiocyanatodeoxyguanosine

The chemical crosslinking was performed using identical concentrations of enzymes and oligodeoxynucleotides as above and the same buffer conditions, but at 37°C and without UV irradiation. Here, all experiments with the endonuclease were carried out in the presence of 10 mM $MgCl_2$. The reactive oligodeoxynucleotides were added in 2–3 aliquots, allowing 1–2 h of incubation before addition of the next aliquot. Analysis by electrophoresis was performed as above.

RESULTS

The recognition sequence of both Eco RV enzymes is d(GATATC). We have previously shown that the self-complementary dodecamer d(GACGATATCGTC) is a substrate for both proteins [29, 30]. Using methods recently developed in our laboratory, we have introduced 4-thiothymidine and 6-thiodeoxyguanosine residues into the Eco RV recognition hexamer at the centre of this dodecamer. A doubly modified dodecamer, d(GACGA[^{4S}T]A[^{4S}T]CGTC), was also prepared. As controls, d(GAC[^{4S}T]ACGTAGTC) and d(GACTAC[^{6S}G]TAGTC) were synthesized. These are self-complementary dodecamers which contain the thiobases but lack the EcoRV recognition hexamer. All these oligodeoxynucleotides are listed in Table 1. Irradiation of a mixture of the Eco RV methyltransferase and d(GACGA[^{4S}T]ATCGTC) at 340–360 nm, as described in the experimental section, gave rise to a new protein band on a denaturing SDS gel (Fig. 1). The apparent molecular weight of this new band is approximately 36000, which is consistent with the attachment of one of the DNA strands to the enzyme (molecular weight of the Eco RV methyltransferase is 32000, the molecular weight of one of the strands of the dodecamer is about 3600). With d(GACGATA[^{4S}T]CGTC) this new band was again formed, but a second, weaker new protein band, corresponding to the attachment of both substrate DNA strands to the enzyme was additionally seen (Fig. 1). Scanning of these silver stained gels showed that the level of photocrosslinking to the methyltransferase with d(GACGA[^{4S}T]ATCGTC) and d(GACGATA[^{4S}T]CGTC) was 18% and 35%, respectively. With the doubly modified dodecamer d(GACGA[^{4S}T]A[^{4S}T]CGTC) levels of 10% photocrosslinking to the methyltransferase were observed (Fig. 1). This lower level of crosslinking as compared to singly modified oligomers may be due to poorer binding of the oligodeoxynucleotide containing two thiothymidine residues to the methyltransferase. However, when this enzyme was incubated and irradiated with d(GAC[^{4S}T]ACGTAGTC), a dodecamer that contains 4-thiothymidine but lacks the Eco RV recognition site, very little photocrosslinking was seen (Fig. 1). These results are summarized in Table 1.

To prove that the two new bands observed when d(GACGATA[^{4S}T]CGTC) was irradiated with the methyltransferase were indeed products of covalent protein oligodeoxynucleotide attachment the experiment was repeated using 5-[^{32}P]-d(pGACGATA[^{4S}T]CGTC). After separation by denaturing gel electrophoresis, the gel was dried and autoradiographed. Fig. 2 shows both the silver stained gel and the autoradiogram, where two radioactive protein bands can be seen that correspond in position to the two new bands visible on the silver stained gel. Control experiments showed that, 1) the Eco RV methyltransferase retains full enzyme activity when irradiated at 340–360 nm under the conditions used for crosslinking but in the absence of 4-thiothymidine-containing oligodeoxynucleotides; 2) irradiation of the methyltransferase with d(GACGATATCGTC), the parent oligodeoxynucleotide lacking 4-thiothymidine, does not lead to the formation of any new protein bands as assessed by silver staining of denaturing gels; 3) incubation of the methyltransferase with 4-thiothymidine-containing oligodeoxynucleotides without UV irradiation does also not lead to any new protein bands as assessed both by staining and autoradiography of denaturing gels. The latter finding confirms that 4-thiothymidine-containing oligodeoxynucleotides are true photoaffinity labels. When d(GACGATA[^{4S}T]CGTC) was irradiated with the methyltransferase in the presence of a competitive unmodified twenty-mer, d(CACCCAAGATATCTTGGGTG), the degree of photocrosslinking was reduced. Furthermore, as the concentration of this twenty-mer was increased, the level of photocrosslinking

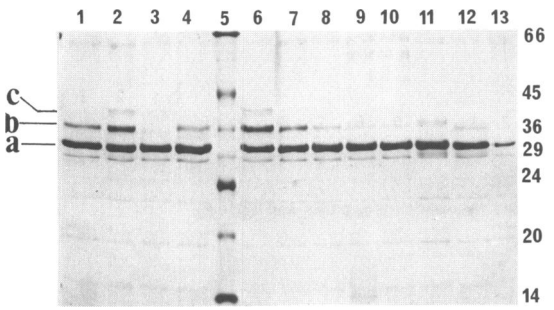

Figure 1. Photoaffinity labelling of Eco RV methyltransferase with oligodeoxynucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine. Analysis by SDS-PAGE, visualization by silver staining. Lane 1, reaction with d(GACGA[^{4S}T]ATCGTC); lane 2, reaction with d(GACGATA[^{4S}T]CGTC); lane 3, reaction with d(GAC[^{4S}T]ACGTAGTC); lane 4, reaction with d(GACGA[^{4S}T]A[^{4S}T]CGTC); lane 5, molecular weight marker (SDS 7, Sigma), molecular weights in thousands are given on the right; lane 6, reaction with d(GACGATA[^{4S}T]CGTC); lanes 7–10, reaction with d(GACGATA[^{4S}T]CGTC) as in lane 6, but in the presence of increasing concentrations of the non-photoreactive, competitive substrate oligodeoxynucleotide d(CACCCAAGATATCTTGGGTG); lane 11, reaction with d(GAC[^{6S}G]ATATCGTC); lane 12, reaction with d(GACTAC[^{6S}G]TAGTC); lane 13, unmodified Eco RV methyltransferase. a, unmodified Eco RV methyltransferase; b, methyltransferase covalently attached to one strand of the dodecamer; c, methyltransferase covalently attached to both strands of the dodecamer.

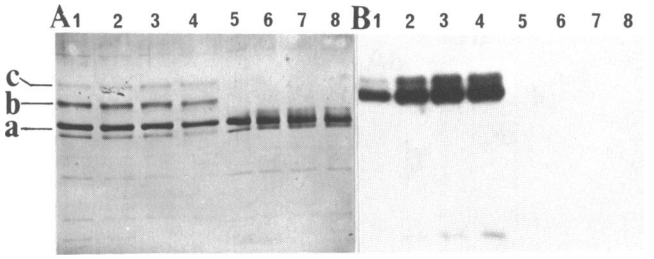

Figure 2. Photoaffinity labelling of Eco RV methyltransferase with 5-[^{32}P]-d(pGACGATA[^{4S}T]CGTC). Panel A, silver-stained SDS-PAGE; panel B, autoradiogram of the same gel. Lanes 1–4 of both panels, aliquots of the reaction mixture taken after 10, 20, 30, and 40 min of irradiation at 340 nm, respectively; lanes 5–8, aliquots of a similar reaction mixture which was kept in the dark. a, unmodified Eco RV methyltransferase; b, methyltransferase covalently attached to one strand of the dodecamer; c, methyltransferase covalently attached to both strands of the dodecamer.

became progressively less (Fig. 1). An identical effect was observed with d(GACGA[^{4S}T]ATCGTC) (not shown).

Similar crosslinking experiments were carried out between the methyltransferase and the 6-thiodeoxyguanosine-containing oligodeoxynucleotides d(GAC[^{6S}G]ATATCGTC) and d(GACTAC[^{6S}G]TAGTC). In these cases the levels of photocrosslinking were much less than those observed with the 4-thiothymidine-containing oligodeoxynucleotides. Only one new protein band, corresponding to attachment of one of the oligodeoxynucleotide strands to the enzyme could be detected by silver staining. Furthermore, both d(GATATC)- and non-d(GATATC)-containing substrates became crosslinked to the methyltransferase and the levels of crosslinking were similar in either case (Fig. 1 and Table 1). Interestingly, the crosslinked products formed from d(GAC[^{6S}G]ATATCGTC) and d(GACTAC[^{6S}G]TAGTC) had slightly different mobilities on denaturing gel electrophoresis. Similar controls to those arried out with 4-thiothymidine-containing oligodeoxynucleotides were performed and showed that, 1) crosslinking required irradiation with 340−360 nm light; 2) the same competitive twenty-mer mentioned above decreased the level of crosslinking with both d(GAC[^{6S}G]ATATCGTC) and d(GACTAC[^{6S}G]TAGTC).

The photoaffinity labelling of the Eco RV methyltransferase was carried out either in the presence of either S-adenosyl-L-methionine (AdoMet), the cofactor required for methylation, or sinefungin, a nonreactive cofactor analog. No difference in the extent of crosslinking and in its specificity was observed.

Photocrosslinking experiments were also carried out with the Eco RV endonuclease. Low but significant levels of crosslinking (about 6%) were seen between this enzyme and the oligodeoxynucleotides d(GACGATA[^{4S}T]CGTC) and d(GAC[^{6S}G]ATATCGTC), both of which contain a photoreactive base within the enzyme's recognition hexamer (data not shown; summarized in Table 1). This crosslinking required the presence of Mg^{2+}, a cofactor for hydrolysis, and only traces of crosslinking were observed in its absence. No crosslinking was observed between the endonuclease and d(GACGA[^{4S}T]ATCGTC). No crosslinking was also observed with two control oligodeoxynucleotides, d(GACTAC[^{6S}G]TAGTC) and d(GAC[^{4S}T]ACGTAGTC), which lack the Eco RV recognition site. Other controls were carried out, as described for the methyltransferase, and it was demonstrated that the presence of the competitive twenty-mer abolished the photocrosslinking (data not shown).

Treatment of the oligodeoxynucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine with CNBr/KCN converted them very rapidly to new products, which we assume contain 4-thiocyanatothymidine and 6-thiocyanatodeoxyguanosine, respectively [24, 25, 31]. A deoxynucleoside composition analysis was carried out on the product obtained from d(GAC[^{4S}T]ACGTAGTC), and the corresponding HPLC trace after complete digestion is shown in Fig. 3. For comparison, the corresponding trace of the starting oligodeoxynucleotide is also given. The last eluting deoxynucleoside, ^{4S}T, which is present in the case of d(GAC[^{4S}T]ACGTAGTC), disappears after the CNBr/KCN treatment and a new, somewhat later eluting product appears, which we assume is ^{4SCN}T. The spectral changes observed during the course of the CNBr/KCN reaction are also consistent with a conversion of 4-thiothymidine or 6-thiodeoxyguanosine to the corresponding thiocyanato derivatives. Thus, the characteristic absorption maxima at about 340 nm of the starting products were shifted to about 320 nm. The following results are also in agreement with the proposed structures of the products. When d(GACGATA[^{4SCN}T]CGTC) was treated with conc. NH_3, it was instantaneously converted to two major and several minor products (not shown). By coelution with suitable standards we proved that the major products obtained are d(GACGATATCGTC) and d(GACGATA[^{5Me}C]CGTC), the corresponding products of hydrolysis and ammonolysis, respectively. When, on the other hand, d(GAC[^{6SCN}G]ATATCGTC) was treated with DTT, it was almost immediately converted back to the starting d(GAC[^{6S}G]ATATCGTC) (not shown). We have previously demonstrated that derivatives of free 4-thiocyanatothymidine [25] and 6-thiocyanatodeoxyguanosine (unpublished results from our laboratory) have identical properties. We are currently investigating the chemical properties

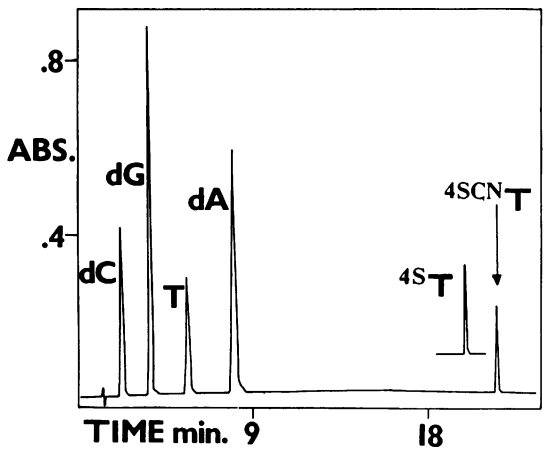

Figure 3. Deoxynucleoside composition analysis of d(GAC[^{4SCN}T]ACGTAGTC). This oligodeoxynucleotide was treated with snake venom phosphodiesterase and alkaline phosphatase and the resulting deoxynucleosides were separated by HPLC. The position of ^{4SCN}T is indicated by an arrow. For comparison, the position of ^{4S}T, obtained by a similar treatment of d(GAC[^{4S}T]ACGTAGTC), is also shown.

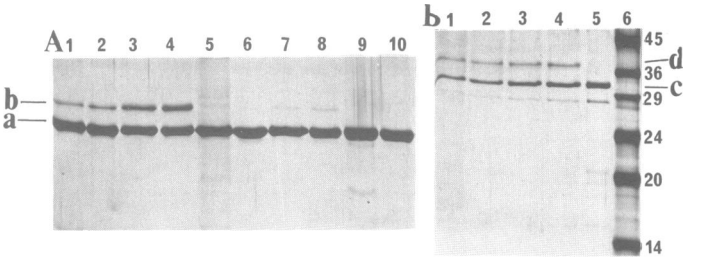

Figure 4. Affinity labelling of Eco RV endonuclease (panel A) and Eco RV methyltransferase (panel B) with an oligodeoxynucleotide containing a 4-thiocyanatothymidine residue. Analysis by SDS-PAGE followed by silver staining. A, lanes 1−4, reaction with d(GACGATA[^{4SCN}T]CGTC), aliquots of the reaction mixture taken after 1, 2, 3, and 4 h of incubation; lanes 5−8, reaction with the same oligodeoxynucleotide, but also in the presence of an excess of the competitive, non-reactive twenty-mer d(CACCCAAGATATCTTGGGTG); lanes 9 and 10, unmodified endonuclease. B, lanes 1−4, reaction with d(GACGATA[^{4SCN}T]CGTC), samples taken after 1, 2, 3, and 4 h reaction, respectively; lane 5, unmodified methyltransferase; lane 6, molecular weight marker (molecular weights in thousands on the right apply to both panels A and B). a, unmodified endonuclease; b, endonuclease attached to one strand of the dodecamer; c, unmodified methyltransferase; d, methyltransferase attached to one strand of the dodecamer.

of oligodeoxynucleotides containing thiocyanato groups in greater detail and the results will be published separately.

Incubation of the Eco RV methyltransferase with d(GACGA[^{4SCN}T]ATCGTC), d(GACGATA[^{4SCN}T]CGTC) or d(GAC[^{4SCN}T]ACGTAGTC) under the conditions described in the experimental section led to the crosslinking of these oligodeoxynucleotides to the protein. This is shown for d(GACGATA[^{4SCN}T]CGTC) in Fig. 4, and the levels of crosslinking observed are summarized in Table 1. In general, the levels of crosslinking are low (5−12%), and with the substrates containing the d(GATATC) Eco RV recognition sequence they are lower than in the case of photochemical crosslinking. When the Eco RV endonuclease was incubated with the above three oligodeoxynucleotides, relatively high levels of crosslinking were obtained as shown in Fig. 4 for d(GACGATA[^{4SCN}T]CGTC) and detailed in Table 1. In contrast to the methyltransferase, higher levels of chemical crosslinking as opposed to photocrosslinking were observed with the endonuclease. Interestingly, with both the endonuclease and methyltransferase similar levels of crosslinking were observed with both specific and non-specific oligodeoxynucleotides. However, in all cases crosslinking could be decreased significantly by the addition of the competitive substrate twenty-mer d(CACCCAAGATATCTTGGGTG) as shown in Fig. 4 for one of the reactions of the endonuclease. No crosslinking was observed between either enzyme and any oligodeoxynucleotide containing a thiocyanatodeoxyguanosine residue.

DISCUSSION

Previous work in this laboratory has focused on the synthesis of base-modified oligodeoxynucleotides for the study of the process of sequence-specific protein-DNA recognition using both Eco RV enzymes as a model [29, 30]. To obtain information on the importance of the 4-keto group in thymidine and of the 6-keto group in deoxyguanosine in this process, substrate oligodeoxynucleotides have been prepared in which 4-thiothymidine and 6-thiodeoxyguanosine have been substituted for thymidine and deoxyguanosine, respectively. These oligodeoxynucleotides were based on d(GACGATATCGTC), a substrate for both proteins, and contained 4-thiothymidine or 6-thiodeoxyguanosine residues within the central d(GATATC) Eco RV recognition sequence. For the endonuclease, both d(GAC[^{6S}G]ATATCGTC) (unpublished observation) and d(GACGATA[^{4S}T]CGTC) [29, 30] were found to be substrates, albeit with much lowered k_{cat}/K_m ratios as compared to the parent dodecamer. The oligodeoxynucleotide d(GACGA[^{4S}T]ATCGTC) was not a substrate for the endonuclease, but we have recently demonstrated that it binds to the active site of the enzyme. All three modified substrate oligodeoxynucleotides are substrates for the methyltransferase [29, 30]. Here again the k_{cat}/K_m values differ from that of the parent oligodeoxynucleotide, being much reduced for d(GAC[^{6S}G]ATATCGTC) and d(GACGA[^{4S}T]ATCGTC), and slightly higher for d(GACGATA[^{4S}T]CGTC).

The main goal of the present work was to find out whether these modified oligodeoxynucleotiodes can be used as photoaffinity labels for the two DNA-modifying enzymes under study. Initial results were promising and in all cases but one (the exception being d(GACGA[^{4S}T]ATCGTC) and the endonuclease) oligonucleotides containing the d(GATATC) sequence and either 4-thiothymidine or 6-thiodeoxyguanosine could be photocrosslinked to both the endonuclease and methyltransferase. The levels of crosslinking are shown in Table 1 and vary between 6% and 35%. These levels of photocrosslinking compare very favourably to those seen with other photoreactive base analogues. A central question in these studies, and indeed in all affinity labelling experiments, is whether or not covalent attachment takes place at the active site. As mentioned above, all the thiobase-containing oligodeoxynucleotides are either substrates, or, in one case, a competitive inhibitor, of both enzymes. This shows that the presence of the modified base does not prevent binding at the active site. A potential method of showing that crosslinking is active site directed is to use the control oligodeoxynucleotides d(GAC[^{4S}T]ACGTAGTC) and d(GACTAC[^{6S}G]TAGTC), which contain the reactive thiobases, but lack the Eco RV recognition site, d(GATATC). Due to the lack of the enzyme's recognition sequence, they should not bind to the enzyme's active site and therefore not give rise to any crosslinked products. In some of our experiments, this is indeed the case, e. g. with the 4-thiothymidine series of oligodeoxynucleotides for both enzymes, and with the 6-thiodeoxyguanosine oligomers for the endonuclease (Table 1). However, in other cases, namely 6-thiodeoxyguanosine-containing oligodeoxynucleotides with the methyltransferase, and the thiocyanato-derivatives mentioned below, the controls and experimental oligodeoxynucleotides crosslink to similar extents. We believe that this is most likely due not to labelling occurring away from the active site, but to the known affinity of almost all DNA-binding proteins for any DNA sequence and not just their specific recognition sites. This was demonstrated for the Eco RV endonuclease in a recent paper by Halford and coworkers [32]. From these results, it is clear that the endonuclease is capable of binding to oligodeoxynucleotides that lack the d(GATATC) site. Although no information is currently available on the Eco RV methyltransferase it is likely that it too shows a similar non-specific binding. Probably the best evidence that photocrosslinking is active site directed comes from the experiments using a non-reactive, cognate substrate twenty-mer, d(CACCCAAGATATCTTGGGGT). In all cases of photocrosslinking with reactive oligodeoxynucleotides the presence of this twenty-mer decreased the extent of covalent bond formation between enzyme and oligomer. This effect was concentration-dependent, as expected for a simple competitive inhibition effect.

The photochemistry of 4-thiothymidine (and of its close analogue, 4-thiouridine) has been extensively studied. Two main mechanisms for photocrosslinking are observed. In the absence of oxygen, compounds become attached to the 6-C atom of the pyrimidine ring with concomitant reduction of the 5,6-double bond. This is explained by a radical mechanism, which involves hydrogen atom abstraction by the excited state of the 4-thiopyrimidine [11, 33, 34]. On irradiation in the presence of oxygen, 4-thiopyrimidines are oxidized to their corresponding 4-sulphonate derivatives [35−37]. These are extremely reactive species and react with nucleophiles with displacement of the entire 4-sulphonate group. Thus, in this case compounds become attached to the C-4 position of the pyrimidine ring and the sulphur is lost. Much less is known about the photochemistry of 6-thiodeoxyguanosine, but it too is suggested to form a sulphonate derivative that can react with nucleophiles [17]. The exact mechanism of photocrosslinking is currently under investigation.

Since we [25] and others [24] have previously noted the high reactivity of a thiocyanato group in the 4-position of thymidine and uridine towards nucleophilic substitution, we reasoned that another possible way of crosslinking the Eco RV enzymes would

be by the introduction of 4-thiocyanatothymidine into the substrate molecules. It is well documented that 4-thiouridine residues in some naturally occurring tRNA species are selectively modified by treatment with CNBr, without affecting any other bases [38]. Thus, treatment of the dodecamers containing 4-thiothymidine or 6-thiodeoxyguanosine with CNBr in the presence of KCN rapidly yielded the corresponding thiocyanato-containing products [24, 25].

Incubation of all the thiocyanatothymidine-containing oligodeoxynucleotides (both those containing the d(GATATC) site and those lacking it) with both enzymes gave rise to crosslinking in good yields. The endonuclease was more efficiently labelled than the methyltransferase. For the reasons mentioned above we believe that the crosslinking seen with the control oligodeoxynucleotides containing a thiocyanatothymidine residue represents a 'non-specific' active site-directed process. Control experiments with the competitive twenty-mer were also carried out and in these cases the extent of affinity labelling was decreased, thus confirming the active site-directed nature of the process.

No crosslinking was observed between either enzyme and any oligodeoxynucleotide containing a thiocyanatodeoxyguanosine residue. This probably reflects the lower intrinsic chemical reactivity of these modified bases compared to the thiocyanatothymidine bases [24, 25, 31].

The formation of covalent crosslinks of oligonucleotides containing thiocyanatothymidine with both Eco RV enzymes can be explained by a reaction pathway involving attack of an enzyme nucleophile at C-4 with substitution of the SCN group. This agrees with the known properties of these derivatives.

To our knowledge, only one similar attempt of affinity labelling of restriction endonucleases has been reported [39]. In this study, the restriction endonucleases Eco RI and Eco RV have been photochemically crosslinked to self-complementary oligodeoxynucleotides containing 5-bromo-2-deoxyuridine residues. The yield of crosslinking was however extremely low, in the case of the Eco RI endonuclease it was estimated to be 0.05%. Furthermore, as in our experiments, crosslinking did occur both with specific and non-specific oligodeoxynucleotides. Despite the low yield of the crosslinking reaction, using a CNBr cleavage of the product obtained, the authors were able to locate the site of attachment to the Eco RI endonuclease as being most probably at or near Met137. Clearly, the significantly higher yields of crosslinked products obtained by us using either the photochemical or chemical approach should make the identification of the modified residue(s) much easier.

In conclusion, we have found that oligodeoxynucleotides containing 4-thiothymidine or 6-thiodeoxyguanosine are very useful photochemical crosslinking reagents for the Eco RV endonuclease and methyltransferase. The favourable properties of these thiobases include a close structural similarity to the parent bases (the only change being substitution of a keto oxygen by sulphur), which is expected to minimize perturbations to substrate binding. The wavelength required for photocrosslinking, 340 nm, is well removed from the absorption maxima of proteins and nucleic acids, thus reducing possible background photodamage. The possibility for conversion into molecules that contain 4-thiocyanatothymidine or 6-thiocyanatodeoxyguanosine, the former of which are very efficient chemical affinity labels, further extends their possible uses. It is anticipated that oligodeoxynucleotides containing these two thiobases will find wide application in the study of DNA-binding proteins and related areas of biochemistry and molecular biology.

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