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Catalytic site-specific cleavage of a DNA-target by an oligonucleotide carrying bleomycin A5

Dmitry S. Sergeyev, Tatjana S. Godovikova and Valentina F. Zarytova*

Institute of Bioorganic Chemistry, Siberian Division of the Russian Academy of Sciences, Lavrentjev pr. 8, Novosibirsk 630090, Russia

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ABSTRACT

Oligonucleotide reagents have been created which are capable of catalytic site-specific cleavage of DNA-targets. The oligonucleotide reagent Bim-R-pd(CCAA-ACA) bearing the bleomycin A5 (Bim-RH) residue was used to degrade the DNA-target pd(TGTTTGGCGAAG-GA). It has been shown that at equimolar reagent: target concentration the bleomycin oligonucleotide derivative can repeatedly cleave the target at G9, G7, T5, T4 and T3 in site-specific manner. This paper demonstrates that with a 10-fold excess of the DNA-target relative to the reagent 30% degradation of the target was observed primarily at a single position G7. The paper also shows that one reagent molecule containing bleomycin A5 residue was capable to degrade three molecules of the DNA-target. The catalytic activity of Bim-R-pd(CCAAACA) was the highest in the temperature range close to the melting temperature of the reagent-target complex, that is under conditions where the oligonucleotide reagent can form a complementary complex and easily dissociate to interact with the next molecule of the target. The number of target molecules degraded by the bleomycin reagent is limited by the degradation of the antibiotic residue itself.

INTRODUCTION

Reactive oligonucleotide derivatives can selectively recognize defined sites of a DNA target and site-specifically modify the DNA molecule (1–4). The efficiency of nucleic acid modification is largely determined by the ability of the reagent's oligonucleotide moiety to form complementary complexes with a target and by the reactivity of the functional group introduced into the oligonucleotide. The most effective reagents shall be those bearing active groups capable of repeated reactions with DNA. Such groups may theoretically include compounds chelating transition metals, for example, antitumor antibiotics, bleomycins (5) or EDTA, 1,10-phenanthroline and porphyrines (6), imitating the action of the bleomycins. They are capable of producing active oxygen-containing species in a catalytic reaction in the presence of oxidants (6–10). These groups are bound with oligonucleotides and the reagents obtained can site-specifically modify the DNA-target (11–15). But no catalytic process of degradation of the nucleic acids with the oligonucleotide derivatives bearing reactive groups including EDTA, 1,10-phenanthroline and porphyrines has been demonstrated yet. The most promising group may be bleomycin which can cause single- and double-stranded cleavage of DNA (16–18). The bleomycin-induced DNA degradation mechanism includes linking an antibiotic molecule with DNA, chelating Fe(II) ion in its metal-binding center and further Fe(II) interaction with molecular oxygen. 'Activated bleomycin' formed then selectively oxidizes the 4′-carbon atom of deoxyribose that results in degradation of the DNA backbone (5,18,19). Base damage is only a minor reaction for bleomycin (20).

After oxidation of deoxyribose, bleomycin can re-bind with a new DNA site and oxidize the DNA backbone again after the reduction of Fe ion in the active center. In the case of extended DNA it has been shown that one molecule of antibiotic causes the formation of several molecules of free bases and base propenals (21). The ability of a single bleomycin molecule to degrade several DNA molecules has been demonstrated for oligonucleotides used as a target (22). To create effective site-specific reagents on the basis of oligonucleotides, bleomycin should be coupled with oligonucleotides.

To be able to damage the DNA target site-specifically, the bleomycin–oligonucleotide derivatives should preserve the cleaving capacity of bleomycin and not lose the ability of the oligonucleotide tail to form complementary complexes. Earlier we have suggested a method of conjugating bleomycin A5 with oligonucleotides. The oligonucleotides and antibiotic were connected by a phosphoramide bond between the primary amino group of the spermidine residue of bleomycin A5 and the 5′-phosphate group of the oligonucleotides (23). It has previously been shown that the bulky antibiotic covalently attached to oligonucleotides is far from preventing the formation of complementary complexes, but additionally stabilizes such complexes (24,25). As already shown, 12 or 14mer oligonucleotides conjugated to bleomycin and complementary to a single region in DNA fragment (302mer) cleave at the positions near the oligonucleotide binding sites. Degradation of DNA target by free bleomycin or bleomycin conjugated to a noncomplementary oligonucleotide takes place at the same sites which are, however, different from those cleaved by bleomycin coupled with the

* To whom correspondence should be addressed

complementary oligonucleotides (26). These results prove that bleomycin oligonucleotide derivatives can damage DNA-target sites specifically.

The paper presents results demonstrating the possibility of catalytic degradation of a DNA-target by a complementary bleomycin-oligonucleotide derivative.

MATERIALS AND METHODS

Oligonucleotides and their derivatives

Unmodified oligonucleotides pd(CCAAACA), pd(TGTTTGGCGAAGGA) and pd(TCCTTCG) were synthesized by the triester method (27). Upon complete removal of the protective groups, the unblocked oligonucleotides were isolated by HPLC with a Beckman-332 (USA) system on 10 × 250 mm Partisil 10SAX (10 mm) anion-exchange and 10 × 250 mm LiChroprep RP-18 (5–20 mm) reverse-phase columns.

Bleomycin A5 was purchased at a pilot plant of the Institute of Organic Synthesis, Latvia. The antibiotic was attached to activated 5′-phosphate of the oligonucleotide pd(CCAAACA) through a spermidine amino group of bleomycin A5. The 5′-phosphate had been activated with a mixture of triphenylphosphine and 2,2′-dipyridyldisulfide. The details of this synthesis have previously been described (25). After reaction, the bleomycin oligonucleotide derivative was isolated by reverse-phase liquid chromatography on 4.6 × 250 mm LiChrosorb RP-18 (10 mm) column (Merck, Germany) with 0–30% linear gradient of acetonitrile in 0.05 M $LiClO_4$.

Spectrophotometric measurements

The concentrations of oligonucleotides and their derivatives were measured spectrophotometrically on a Specord M40 (Carl Zeiss, Jena) in 200 mM LiCl, 10 mM Tris-HCl (pH 7.5) buffer. The molar extinction coefficients were estimated at 260 nm for pd(TGTTTGGCGAAGGA) (140 000 $M^{-1}.cm^{-1}$) and pd(TCCTTCG) (57 000 $M^{-1}.cm^{-1}$) according to Cantor and Tinoco (28). After complete hydrolysis by venom nuclease (29) the molar extinction coefficient of unmodified oligonucleotide pd(CCAAACA) (66 000 $M^{-1}.cm^{-1}$) was determined. The radioisotope method (30) has been used to determine the molar extinction coefficient of bleomycin oligonucleotide derivative Blm-R-pd(CCAAACA) (81 000 $M^{-1}.cm^{-1}$).

Preparation of radiolabeled oligonucleotides

The oligonucleotides pd(TGTTTGGCGAAGGA) (1 nmol) and pd(CCAAACA) (10 nmol) were 5′-end labeled using T4-polynucleotide kinase, [γ-^{32}P]ATP (>3000 Ci/mmol) and ADP. After purification on 20% polyacrylamide gels under denaturing conditions (8 M urea, 89 mM Tris-H_3BO_3, ~40°C), oligonucleotide [^{32}P]-d(TGTTTGGCGAAGGA) was used as a target in cleavage experiments. Oligonucleotide pd(CCAAACA) was purified by reverse-phase HPLC and the bleomycin oligonucleotide derivative containing radiolabeled 5′-phosphate was synthesized. The latter was further used for studies of reagent self-degradation and for determination of the molar extinction coefficient (30).

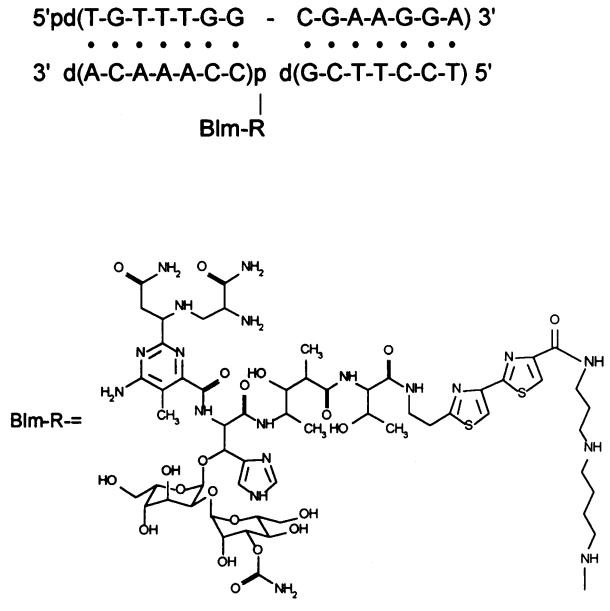


Figure 1. Model system for studying the catalytic cleavage of 14mer DNA-target by a complementary oligonucleotide reagent carrying the bleomycin A5 residue.

Analysis of target cleavage extent

After DNA-target degradation by bleomycin oligonucleotide reagent, the cleavage products were separated on 20% denaturing PAGE and visualized. In the case of 10-fold target excess the bands were excised and counted in water by Cherenkov effect in a scintillation counter (LKB), and the extent of target cleavage after subtracting the background was calculated. The accuracy of determining the total cleavage extent at one temperature relative to the other was 5% (Fig. 6). The overall error of determining the total cleavage extent including background cleavage of the target, oligonucleotide concentration measurements, taking aliquots and radioactivity measurements did not exceed 25% (Figs 5 and 6). In the case of DNA-target degradation at equimolar target:reagent ratio an Ultrascan laser densitometer (LKB) was used to quantify cleavage products (Figs 2 and 3).

RESULTS

Site-specific catalytic activity of bleomycin oligonucleotide derivatives can be revealed either by repeated cleavage of DNA target by the reagent within complementary complexes or in site specific destruction of several DNA-target molecules by one reagent molecule. Both possibilities are demonstrated in this paper.

The catalytic properties of bleomycin oligonucleotide derivative were studied using the model system (Fig. 1) which was investigated earlier to demonstrate site-specific cleavage of the DNA-target by this reagent (24,25).

Antibiotic bleomycin is known to cleave single- and double-stranded DNA with different efficiency (31–33). Single-stranded DNA is cleaved predominantly at the secondary structure regions (34). Taking this into consideration, a model comprising a 14mer DNA-target and two complementary heptanucleotides with one of them bearing the bleomycin A5 residue was chosen (Fig. 1).

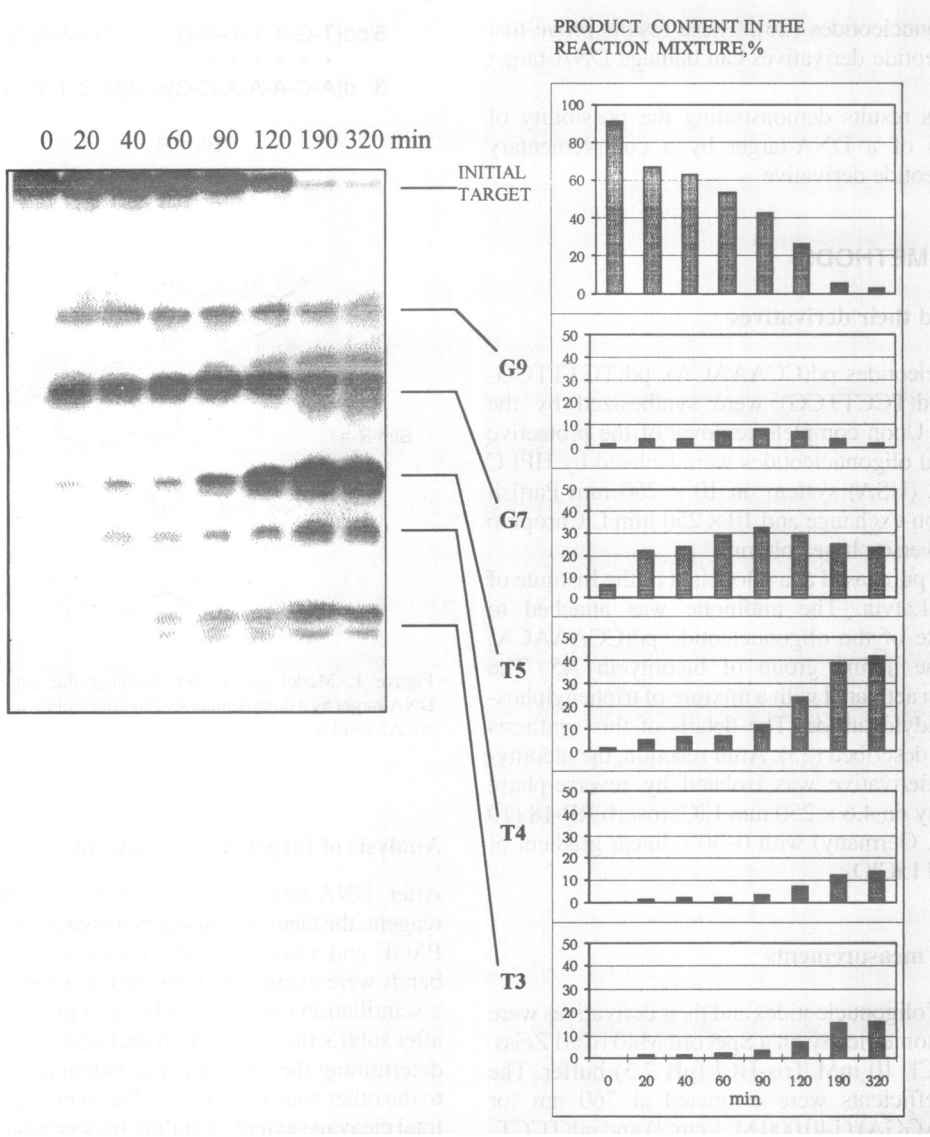


Figure 2. Time dependence of the DNA-target degradation by bleomycin oligonucleotide derivative at equimolar target:reagent ratio. The reaction mixture (total volume 50 µl) contained 10 µM 5′-^{32}P-labeled target, 10 µM heptanucleotide pd(TCCTTCG) and 10 µM reagent in solution of LiCl (200 mM), Tris-HCl, pH 7.5 (10 mM) and 2-mercaptoethanol (50 mM). Cleavage reactions were initiated by addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ (100 µM) and proceeded at 20°C. At certain intervals aliquots were taken, the cleavage reaction was stopped by adding EDTA (1 mM) and subsequent precipitation with 2% $LiClO_4$ in acetone. The probes were treated with 10% piperidine (95°C, 30 min) and the cleavage products were analyzed on a 20% denaturing PAGE. The bar graphs depict quantitative densitometric evaluation of target degradation and cleavage products accumulation.

As shown earlier (25), the oligonucleotide reagent cleaved the DNA-target both in the presence and absence of the heptanucleotide pd(TCCTTCG) complementary to 3′-end of the target. The oligonucleotide reagent substantially lost DNA-damage activity in the presence of oligonucleotides complementary to the DNA-target and capable to compete for the same binding site. The products of target degradation by free antibiotic and bleomycin tethered to the complementary oligonucleotide were different. Additionally, both the non-complementary oligonucleotide reagents and the free antibiotic attack the DNA-target at the same sites but less efficiently than the complementary oligonucleotide reagent. According to results with an extended DNA-target (26), the effective cleavage of the 14mer DNA target by bleomycin oligonucleotide derivatives took place only in a complementary complex (25).

Repeated cleavage of DNA-target

Under conditions of equimolar reagent:target ratio a wide variety of target cleavage products were formed (G9, G7, T5–T3). Modification occured at the positions (T5–T3) located rather far from the attachment site of the antibiotic residue (Fig. 1).

A time dependence study of tetradecanucleotide degradation at 20°C showed that G7 and, to a lessser degree, G9 and T5 residues were the first to be affected (Fig. 2). Later, short target fragments appeared, which were formed by destruction at T5, T4 and T3 sites. This may have resulted from repeated cleavage of the DNA-target by the bleomycin oligonucleotide derivative.

The emergence of T5–T3 cleavage sites remote from the place of antibiotic attachment raises the problem whether the cleavage occurs within the complementary complex or is caused by the

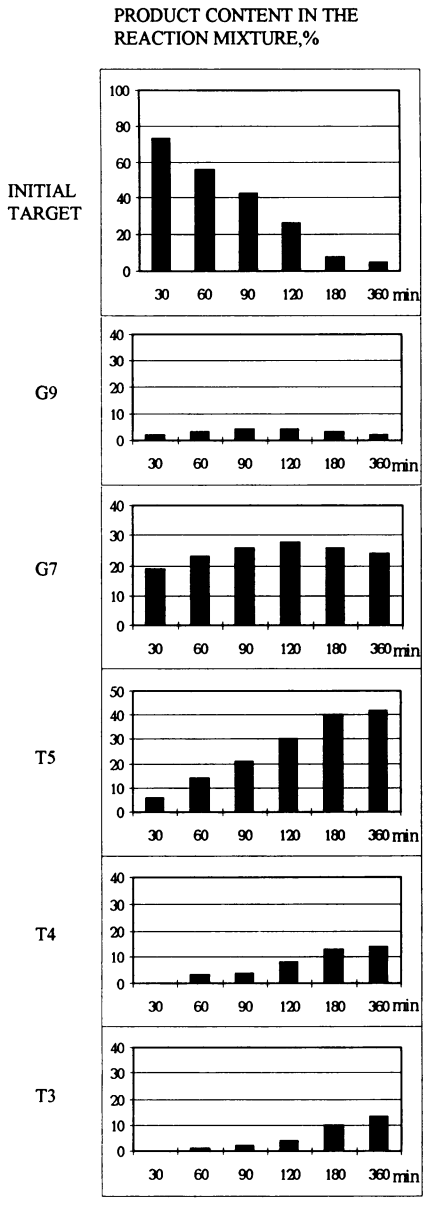


Figure 3. Densitograms of DNA-target degradation by bleomycin oligonucleotide derivative and accumulation of cleavage products with time. The reaction conditions were the same as in Figure 2. Reaction mixture contained additionally double-stranded plasmid DNA with 10-fold excess (per nucleotide) relative to the DNA-target.

antibiotic residue without Watson–Crick interactions. Addition of an excess of double-stranded DNA to the reaction mixture should inhibit target cleavage occurring without complementary complex formation. However, the distribution of cleavage products and time dependence of tetradecanucleotide degradation in the presence of a 10-fold excess (per nucleotide) of double-stranded plasmid DNA practically did not change (compare Figs 2 and 3). This indicates that the cleavage at T5–T3 sites resulted from site specific interactions.

Only the first attack of the bleomycin reagent takes place at the G7 site at 37°C (Fig. 4). Cleavage of the DNA-target backbone or formation of an apurinic site at the G7 residue should lead to the reduction of stability of the reagent-target complex. Raising the

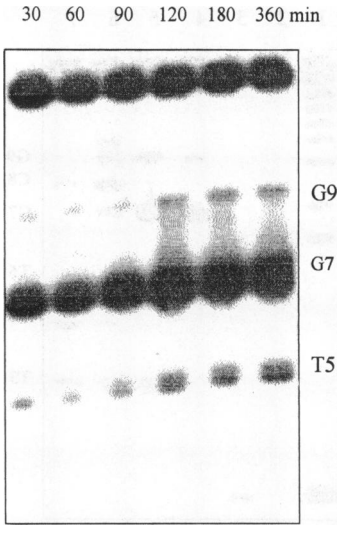


Figure 4. Time dependence of the DNA-target degradation by bleomycin oligonucleotide derivative at 37°C. The reaction conditions were the same as in Figure 2.

reaction temperature to 37°C suppressed the formation of short cleavage products (at T5–T3 residues) thus showing the necessity of a complementary duplex for effective cleavage. The products formed after attack at G7 position of the target cannot form a strong complementary complex with the reagent at 37°C and higher, and further degradation at T5–T3 sites ceases. Since this temperature exceeds the melting temperature of the reagent-target complex, the total extent of target cleavage also drops (Fig. 4). Thus the cleavage of G9, G7 and T5–T3 sites by bleomycin oligonucleotide derivative takes place only in complementary duplexes.

With an increase in incubation period of the reaction mixture at 20°C the quantity of G7 cleavage product ceased to grow and began to drop. Analogous changes occured with the G9 cleavage product (Figs 2 and 3). At the same time short products (T5–T3 sites) accumulated. This is direct evidence that one and the same target molecule is attacked site-specifically several times by the oligonucleotide reagent.

Destruction of several DNA-target molecules by one reagent molecule

To demonstrate the catalytic ability of the bleomycin oligonucleotide derivative the following conditions were chosen. First, the DNA-target was used in excess relative to the oligonucleotide reagent. The temperature interval was then determined that the reagent could form the complementary complex for target destruction and simultaneously could easily dissociate. Accordingly, the model system presented in Figure 1 contained the reagent with a concentration 10-fold smaller than that of the target and the heptanucleotide pd(TCCTTCG). It has been found that 20% of the target underwent direct cleavage at 30°C (Fig. 5, lane 4), and a further 10% of the alkaline-labile sites formed were revealed by the subsequent piperidine treatment (Fig. 5, lane 5). Under chosen conditions, free bleomycin cleaved the DNA-target only to 10% (Fig. 5, lane 6) that makes up only one-third of the cleavage of bleomycin reagent. According to data in the literature, the association constant of bleomycin with double-stranded DNA

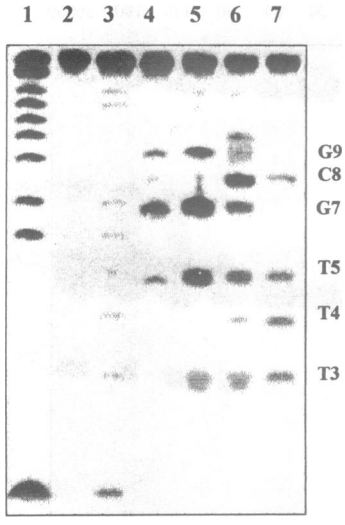


Figure 5. Autoradiogram of a 20% sequencing gel showing the DNA-target cleavage by the bleomycin oligonucleotide derivative with 10-fold target excess. The 5′-^{32}P-labeled target (10 μM) and the unlabeled heptanucleotide pd(TCCTTCG) (10 μM) were incubated with oligonucleotide reagent (1 μM) or with bleomycin A5 and oligonucleotide d(CCAAACA) (both 1 μM) in the buffer containing 200 mM LiCl, 10 mM Tris-HCl (pH 7.5), 50 mM 2-mercaptoethanol. Cleavage reactions were initiated by the addition of $Fe(NH_4)_2(SO_4)_2.6H_2O$ up to a final concentration of 100 μM and proceeded for 6 h at 30°C. After the incubation the reactions were stopped by precipitation with 2% $LiClO_4$ in acetone and the cleavage products were analyzed. Lanes: 1, 7, A+G and T+C Maxam–Gilbert markers, respectively; lanes 2, 3, the target alone under reaction conditions; lanes 4, 5, target degradation by the oligonucleotide reagent; lane 6, target degradation by free bleomycin A5; lanes 3, 5, 6, 10% piperidine treatment (95°C, 30 min) after reaction.

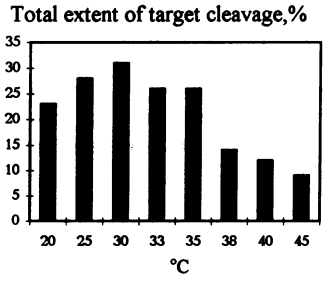


Figure 6. Temperature dependence of the total extent of target cleavage by the bleomycin oligonucleotide reagent with 10-fold target excess. The reaction conditions were the same as in the legend to Figure 5 except the reaction mixture temperatures. After the precipitation the reaction mixtures were incubated with 10% piperidine (95°C, 30 min). The cleavage products were analyzed on a 20% denaturing PAGE and the extent of target cleavage was calculated as described in Materials and Methods.

is ~10^5 M^{-1} (19). At a DNA-target concentration of 10 μM and at bleomycin concentration of 1 μM the low cleavage efficiency can be explained by weak binding of the latter with the target. It is thus necessary to show the difference in position of cleavage performed by the oligonucleotide reagent and free antibiotic. The former carries out the cleavage chiefly at G7 residue (60–70%) while the cleavage by free bleomycin is distributed more uniformly along some sites with a predominant cleavage at C8

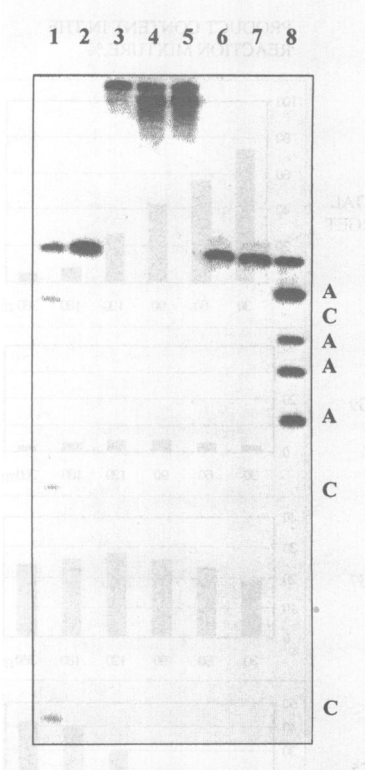


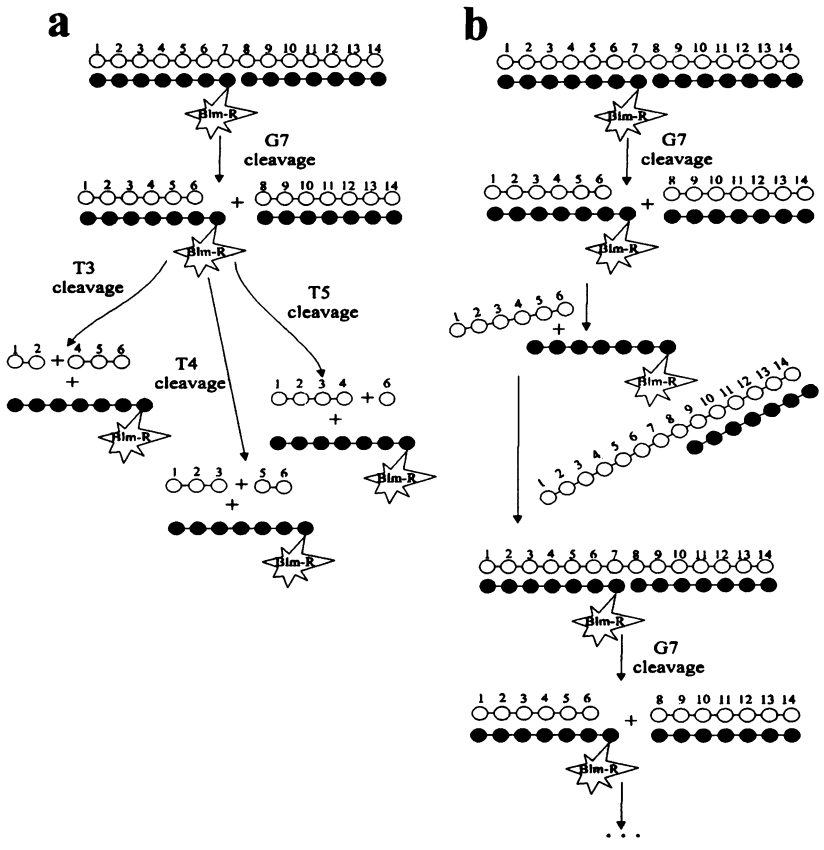
Figure 7. 20% denaturing PAGE analysis of the 5′-^{32}P-labeled oligonucleotide reagent self-degradation after reaction with the unlabeled target. The reaction conditions were the same as in Figure 5. Lanes 1, 8, T+C and A+G Maxam–Gilbert markers, respectively; lane 2, oligonucleotide pd(CCAAA-CA); lane 3, intact reagent; lane 4, the reagent alone under reaction conditions; lane 5, the reagent after degradation of the target; lane 6, the reagent incubated after the reaction under phosphoramide bond hydrolysis conditions (pH 3.5, 37°C, 16 h) to remove the antibiotic residue; lane 7, the same as in lane 6 with 10% piperidine treatment (95°C, 30 min) after hydrolysis.

(Fig. 5), as is observed in the case of bleomycin derivatives of oligonucleotides non-complementary to the DNA-target.

The catalytic activity of DNA degradation requires the transition from one target molecule to another, both for the free bleomycin and the bleomycin oligonucleotide conjugate. In the case of the oligonucleotide reagent, these transitions are due to the dissociation of the complementary complex. The maximum extent of the target cleavage was observed between 25 and 35°C (Fig. 6), which includes the melting temperature of the reagent-target complex (25). Lower reaction temperature lead to a lower cleavage extent, which may result from the non-optimal conditions for catalytic activity of the reagent, for example, the difficulty of the reagent to transit from one to another target molecule. Higher temperature also reduced the cleavage extent (Fig. 6) which may be due to the lower concentration of the complementary complex. Degradation of ~30% of tetradecanucleotide by the bleomycin reagent with a 10-fold excess of the target indicates that on average one molecule of the reagent can damage three target molecules.

Bleomycin reagent self-degradation

The limited efficiency of the reagent in the presence of excess Fe(II) ions, reducing agents and molecular oxygen may be caused by the degradation of either the oligonucleotide part of the reagent



Scheme 1. (**a**) Repeated cleavage of the DNA-target (Equimolar reagent:target ratio). (**b**) Destruction of a number of the target molecules (10-fold target excess over the reagent).

or of the antibiotic residue itself. To elucidate this question the 5′-labeled bleomycin reagent was synthesized and the DNA-target degradation experiments were performed using a 10-fold excess of the unlabeled target (Fig. 7). The appearance of more mobile products (lanes 4 and 5) than the starting bleomycin reagent (lane 3) leads to the conclusion that during catalytic cleavage of the target, the bleomycin reagent underwent substantial degradation both in the presence of the DNA-target (lane 5) and in its absence (lane 4). The stability of the oligonucleotide moiety was demonstrated by hydrolysis of the phosphoramide bond between the antibiotic residue and the oligonucleotide reagent (pH 3.5, 37°C, 16 h) (25), yielding the intact 5′-labeled oligonucleotide (lane 6). Additional piperidine treatment of the latter did not reveal any degradation of the oligonucleotide (lane 7). Thus, the limited catalytic activity of the reagent seems to be due to the destruction of the antibiotic residue itself.

It is worth mentioning that the antibiotic residue attached to the oligonucleotide pd(CCAAACA) cleaved only the DNA-target (Fig. 1). Neither the heptanucleotide pd(TCCTTCG) complementary to the target 3′-end, nor the oligonucleotide part of the reagent were cleaved by the antibiotic residue. The relative resistance of these oligonucleotides may be due to the absence of dinucleotide sequences GT and GC preferentially cleaved by the antibiotic (35,36). The absence of the heptanucleotide pd(TCCTTCG) in the system did not influence the efficiency and practically did not change the position of cleavage by the oligonucleotide reagent. The cleavage at G9 position was only lowered (data not shown).

DISCUSSION

The results presented in Figures 2 and 5 indicate that there are different ways a bleomycin residue covalently attached to an oligonucleotide can react. The cleavage activity of the antibiotic residue may be directed to either one and the same target molecule or a number of target molecules. In both cases the first site of modification is the G7 residue. The next events depend on the conditions chosen. It is illustrated in scheme 1.

The possibility of target cleavage at a distance, significantly different from the place of attachment of the antibiotic residue to the phosphate group of the oligonucleotide (Scheme 1a) does not contradict the data available in the literature. Data on binding of a Zn(II)-bleomycin A5 complex with a self-complementary octanucleotide duplex have recently been obtained by two-dimensional NMR methods (37). Extended by molecular dynamics calculations, an optimal structure was presented. The antibiotic molecule is placed in the minor groove, overlapping ~5 base pairs. In our case the bleomycin residue preserves significant mobility in the conjugate obtained and is capable of modifying relatively remote sites (Fig. 1).

Cleavage efficiency of 10-fold excess DNA-target by the oligonucleotide reagent has a maximum in a definite temperature range (Fig. 6). This is caused by a number of processes, and, first of all, by reagent dissociation from the reagent-target complex. On one hand, higher temperature facilitates the transition of the reagent from one target molecule to another which is necessary for catalytic activity. In such a case however the fraction of

complementary complex decreases and results in diminished cleavage. This is especially noticeable at temperatures >35°C exceeding the melting temperature of the reagent-target complex. Another phenomenon may contribute to the process (Fig. 6), including the intrinsically decreasing cleavage efficiency of antibiotic with rising temperature. However, the cleavage efficiency of DNA by bleomycin changes only slightly between 0 and 50°C (38). The cleavage efficiency of the DNA-target studied with free antibiotic decreases only by 15–20% between 20 and 45°C (data not shown), which does not explain the sharp reduction of DNA-target cleavage by oligonucleotide reagent with temperature raising from 30 to 45°C. The temperature dependence in Figure 6 thus reflects mainly the equilibrium processes of Watson–Crick pairing.

Destruction of the reactive residue in the oligonucleotide reagent is typical of redox-active groups of chelating transition metals. EDTA and porphyrine-containing oligonucleotides under conditions of DNA-target cleavage are shown to be subjected to fast self-destruction (39,40). The oxidation of Fe(II)-bleomycin complex by oxygen in the absence of DNA is known to be accompanied by inactivation of a part of the antibiotic molecules with a loss of ability to induce DNA cleavage (41–43). DNA addition inhibits the inactivation process, because the active oxygen-containing species, formed by oxidation of Fe(II) in a metal-binding center, are consumed for DNA oxidation (44,45). Even with a large excess of Fe(II) ions and reducing agents and in the presence of DNA the number of events of oxidative destruction of DNA is, however, limited. The ratio of the sum of thymine and cytosine bases and their base propenals liberated by the cleavage of a long DNA by bleomycin to the antibiotic was 2.4–2.6 (21), and in the case of oligonucleotides the same ratio varied from 3 to 10 (22). As mentioned above, in the case of 10-fold target excess one oligonucleotide reagent molecule can damage ~3 molecules of DNA-target. The efficiency of catalytic DNA degradation by the oligonucleotide reagent correlates with the quantitative data demonstrating catalytic DNA degradation by the free antibiotic (21,22). There is, however, no reason to consider the obtained cleavage efficiency as a limit for catalytic activity of bleomycin-oligonucleotide reagent. The target base sequence, double helical structure and the conditions promoting reagent transition between target molecules shall play a significant role here.

ACKNOWLEDGEMENTS

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