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The influence of the target structure on the efficiency of alkylation of single-stranded DNA with the reactive derivatives of antisense oligonucleotides

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Site-directed alkylation of three oligonucleotide targets: 41-mer (hairpin structure), 22-mer (loop part of this hairpin) and 10-mer (part of the loop) with 5′-*p*-(*N*-2-chloroethyl-*N*-methylamino)benzylamides of oligonucleotides complementary to the loop region was studied. Thermodynamic parameters of the interaction were estimated using the dependence of the limit modification extent on the reagent concentration at several temperatures. The stability of the complex increases significantly in the set: 302-mer carrying above hairpin, 41-mer, 22-mer, the data for 22-mer and 10-mer being nearly identical. This indicates significant influence of the loop supporting structure on the interaction with antisense reagents.

Antisense oligonucleotide; Nucleic acid structure

1. INTRODUCTION

A set of data dealing with the site-directed modification of single-stranded DNA were obtained using 302-meric fragment of the structure presented in [1]. This fragment according to its primary structure should contain hairpin formed by nucleotide residues 243–283 with the loop containing residues 251–275 [2]. The tentative structure of the hairpin is given in Fig. 1. The central region of the loop was used as the target first of all for alkylation with oligonucleotide derivatives containing *p*-(*N*-2-chloroethyl-*N*-methylamino)-phenyl-group (RCl) [1]. Selective alkylation was achieved with the reagents carrying RCl-group both at 5′- and 3′-ends. However, quantitative treatment of the data resulted in the constant of association of the complex target–reagent being several orders of magnitude lower than expected from the affinity calculated using the data for the respective base pairs [3]. Therefore, in the present paper we subjected to quantitative study the alkylation of the 41-mer representing the hairpin as well as 22-mer containing the main part of the loop (252–269 residues flanked with the $(pdT)_2$) as well as decanucleotide representing sequence 261–269 flanked with 3′-pdT residue. The data obtained in this paper in common with above-mentioned results for 302-mer clearly demonstrate strong influence of the whole structure of the DNA fragment on the affinity towards open target region.

2. MATERIALS AND METHODS

Oligonucleotides d(AATGGAGCGTGCCTTGAATGGGAAGAGGGTCAGGTTCCATT), d(TTTGCCTTGAATGGGAAGAGT-T), d(TGGGAAGAGT) used as targets, were synthesized by a method [4] on automated synthesizer, Victoria-6M; oligonucleotide moieties of the reagents $ClRCH_2NH$-d(pTCTTCCCA) and $ClRCH_2NH$-d(pTCTTCCCATTC) containing 5′-end phosphate groups were synthesized by a solid phase phosphotriester method [5]. Reactive group $ClRCH_2NH$- was attached to 5′-end phosphate groups of the oligonucleotides as in [6]. The separation of the reagents from the rest of unreacted oligonucleotide and the product of hydrolysis of C–Cl bond of the reagent in solution, bearing the HOR-group, was performed by HPLC at the LiChrosorb RP-18 column (Merck, Germany) using a gradient of CH_3OH in 0.05 M triethylammonium-acetate buffer, pH 7.5. Oligonucleotide-effector was kindly submitted by I.V. Kutyavin. 5′-ends of the 41-mer, 22-mer and 10-mer were labelled by ^{32}P using the standard procedure with the help of polynucleotide kinase of phage T4 and [γ-^{32}P]ATP (>3000 mCi/pmol). Concentration of the oligonucleotides and the reactive derivatives were measured spectrophotometrically. The extinction coefficients of oligonucleotides were calculated as in [7]. The extinction coefficients of the reagents were taken as the sum of contributions of the oligonucleotide moieties and of the reactive group $ClRCH_2NH$- (14.7×10^3 $M^{-1} \cdot cm^{-1}$ [8]). Oligonucleotide-effector was kindly submitted by I.V. Kutyavin. Modification was carried out in the buffer: 0.16 M NaCl, 0.02 M Na_2HPO_4, 0.1 mM EDTA, pH = 7.5, at 20, 25, 30 and 35°C. The reaction mixtures were precipitated with 10-fold excess volumes of 2% $LiClO_4$ in acetone, centrifuged, washed with 70% ethanol, dried and dissolved in formamide with marker dyes. The product of modification at 20, 25, 30°C and non-reacted target were resolved by electrophoresis in 10% PAAG, containing 7 M urea. The modification products have lower electrophoretic mobilities than the initial target. In the case of reaction at 35°C the partial apurinization of modification product may occur. Therefore the electrophoresis was carried out after treatment of the reaction mixture in 1 M piperidine at 95°C lasting

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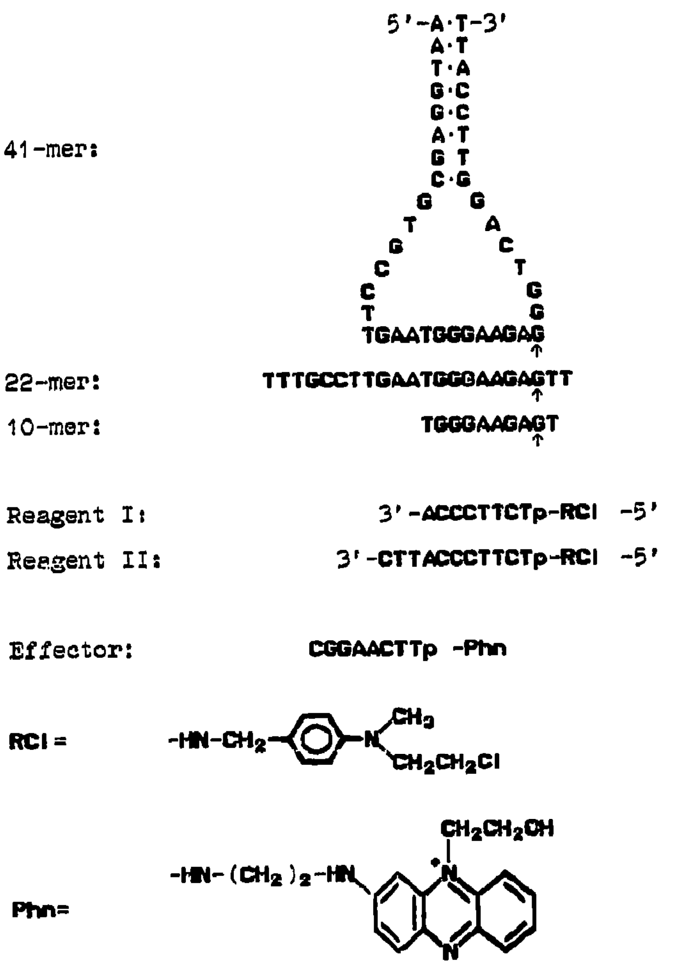

Fig. 1. The structures of the targets and reagents used presented in their appropriate orientation. The arrows indicate the position of modified base.

1 h to cleave oligonucleotides at the position of modified purines [9]. In this case the products have a higher electrophoretic mobility than the target. The autoradiograms of the gels were quantified using laser scanner UltroScan XL (LKB, Sweden). The limit modification extents were calculated as the ratios of integral intensities of the spots corresponding to the modification products to the sum of integral intensities of the targets and the products. For the estimation of the values of γ_{eff} and K_x according to Eqn. 1 the data were treated using non-linear regression programm [10].

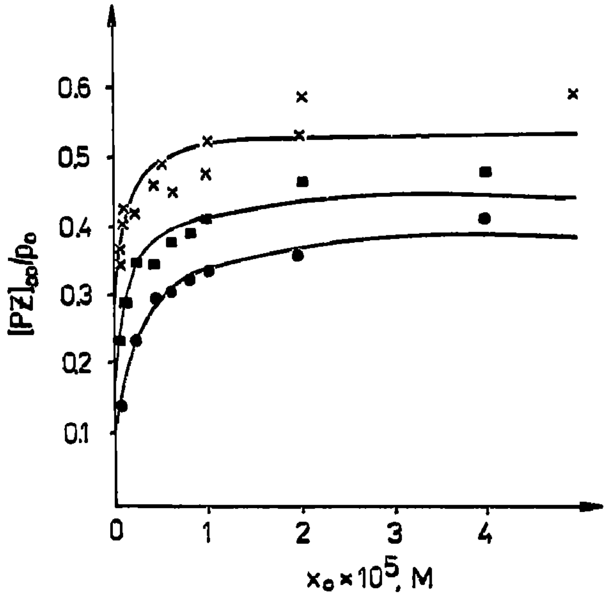

Fig. 2. The dependencies of the limit modification extent, $[PZ]_\infty/p_0$, of 22-mer (1×10^{-8} M) with reagent I on its concentration x_0 at 20°C (×), 25°C (■) and 30°C (●).

3. RESULTS AND DISCUSSION

The 41-mer used in this investigation entirely reproduced the structure of the tentative hairpin of the above-mentioned 302-mer. The 22-mer contained 18 nucleotide residues of the loop which were flanked at both sides with $(pdT)_2$ residues to decrease the number of dG residues which could introduce additional spatial ordering of the fragment not inherent to the hairpin. For the same reason 10-mer contained 3'-flanking pdT residue. The structures of the targets and reagents used in a proper mutual orientation are given in Fig. 1. In all cases dG residue following directly the duplex region was predominantly modified. The limit alkylation extent (at $t \to \infty$) of the target was measured as described in Materials and Methods after 5 half-times of C–Cl bond conversion (97% of the completion of the process) [11]. To found association constants the dependence of the limit modification extent, $[PZ]_\infty/p_0$ (PZ = modification product, p_0 = initial concentration of the target), on the initial concentration of the reagent x_0 was treated according to equation:

$$[PZ]_\infty/p_0 = 1 - \exp[-\gamma_{eff}K_x x_0/(1 + K_x x_0)] \qquad \text{(i)}$$

This equation was derived in [12] under the assumption that target, reagent and their complex are present in quasi-equilibrium concentrations with association constant K_x, the excess reagent is hydrolyzed to respective unreactive ROH derivative with the same affinity to the target and the latter competes with RCl derivative for the target. Both reaction in the complex and hydrolysis were assumed to pass via formation of highly reactive ethyleneimmonium cation in the rate-limiting step. Parameter γ_{eff} represents a combination of the rate constants of the different stages of the process.

Fig. 2 demonstrates one set of the dependencies of $[PZ]_\infty/p_0$ on x_0 for the reaction of 22-mer with reagent I at three different temperatures. Solid curves represent the results obtained from the experimental points by non-linear regression. It is noteworthy that experimental points corresponding to the greatest x_0 value in most cases reside a bit higher than the calculated curves. This may be due to oversimplification of the scheme used for derivation of the Eqn. 1.

The whole set of the association constants as well as

Table I

T (°C)	10-mer		22-mer		41-mer		302-mer
	$K_x \times 10^{-6}$ (M^{-1})	γ_{eff}	$K_x \times 10^{-6}$ (M^{-1})	γ_{eff}	$K_x \times 10^{-6}$ (M^{-1})	γ_{eff}	$K_x \times 10^{-6}$ (M^{-1}) [2]
Reagent I							
20°C	–	–	3.02 ± 0.81	0.74 ± 0.04	0.16 ± 0.04	0.60 ± 0.05	–
25°C	1.15 ± 0.31	0.67 ± 0.04	1.37 ± 0.36	0.58 ± 0.03	0.14 ± 0.04	0.55 ± 0.06	0.002
30°C	0.57 ± 0.10	0.58 ± 0.03	0.50 ± 0.07	0.51 ± 0.02	0.06 ± 0.02	0.56 ± 0.09	–
35°C	0.36 ± 0.13	0.58 ± 0.06	0.36 ± 0.10	0.50 ± 0.05	–	–	0.002
Reagent II							
20°C	–		–		1.21 ± 0.31	0.79 ± 0.06	–
25°C	–		5.87 ± 1.43	1.00 ± 0.08	1.28 ± 0.12	0.59 ± 0.02	–
30°C	–		2.21 ± 0.44	0.90 ± 0.06	–		–
35°C	–		2.92 ± 1.03	0.87 ± 0.11	–		–

γ_{eff} values are presented in Table I. It is seen that K_x values for 10-mer and 22-mer are nearly identical. However, in all cases investigated the K_x values for 41-mer are significantly lower than those for 22-mer. At the same time they significantly exceed K_x given in [2] for alkylation of 302-mer with similar reagent. Thus it is seen that in the presence of the stem supporting the looped structure and especially in the presence of the whole sequence of 302-mer a significant decrease in the affinity of reagents to template occurs.

It may be seen in Fig. 3 that the dependence of K_x obtained for the reaction of the derivative I with the 10-mer and 22-mer on temperature is described by the van't Hoff equation. Therefore ΔH and ΔS values may be calculated. They were found to be $\Delta H = -(23.74 \pm 1.64)$ kcal/mol, $\Delta S = -(51.86 \pm 5.46)$ cal/mol·K.

As may be easily seen from the Eqn. 1, γ_{eff} value predetermines the maximal modification extent which may be achieved at high reagent concentrations:

$$\lim_{x_0 \to \infty} [PZ]_\infty / p_0 = 1 - \exp[-\gamma_{eff}].$$

The higher the γ_{eff} the higher this extent. The results presented in Table I demonstrate that γ_{eff} value increases with the fall of temperature as well as with the increase of the oligonucleotide length. In both cases the increase of γ_{eff} coincides with the rise of the duplex stability. The same result was achieved earlier by addition to the mixture of 22-mer with the reagent I of special effector stabilizing the former complex [13]. This effector used (see Fig. 1) can form a duplex with the region of the same target in tandem with the reagent and carries phenazinium residue which is known to stabilize the duplex [14]. However, in no case does γ_{eff} exceed 1, and therefore, the highest yield of the modification product does not exceed: $1 - \exp(-1) = 0.63$.

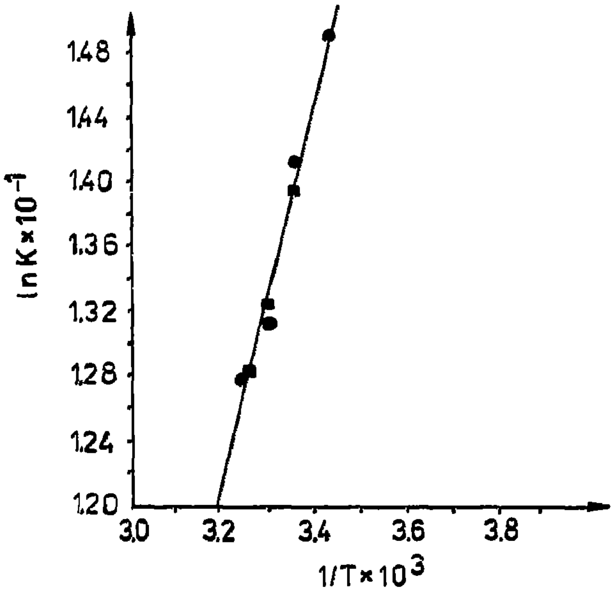

Fig. 3. The dependence of association constants of the reagent I with 10-mer (■) and 22-mer (●) K_x on the temperature T.

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