Cooperative interactions in the tandem of oligonucleotide derivatives arranged at complementary target. Quantitative estimates and contribution of the target secondary structure

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Received 19 June 1995

Abstract **The intraduplex reaction of the alkylating reagent ClRCH$_2$NHpd(TTCCCA) (X, ClR is p-(*N*-2-chloroethyl-*N*-methylaminophenyl) residue) with the target 26-mer d(TTGCCTTGAATGGGAAGAGGGTCATT) (P) in the presence of effectors was studied. The effectors used were Phn-L-pd(TTCAAGGC)p-L-Phn (E_1) and Phn-L-pd(TGACCCTC)p-L-Phn (E_2), where Phn is *N*-(2-hydroxyethyl)-phenazinium residue and L is NHCH$_2$CH$_2$NH spacer. The dependence of the alkylation extent of the target on the reagent concentration was treated using the equation derived earlier for the two-component system (reagent + target) to calculate association constants of X with P, PE_1, PE_2 and PE_1E_2. The latters were found to be $K_{xe1} = 6.75 \cdot 10^5$ M^{-1}, $K_{xe2} = 4.15 \cdot 10^4$ M^{-1} and $K_{xe12} = 5.87 \cdot 10^6$ M^{-1} as compared with the affinity of X to P $K_x = 2.16 \cdot 10^4$ M^{-1} in the absence of effectors. Taking into account the internal structure of the target, co-operativity parameters describing interactions in the tandem $E_1 \cdot X \cdot E_2$ arranged at the target were calculated as $\alpha_1 = 16$, $\alpha_2 = 10$ and $\alpha_{12} = 139$ for the duplexes PXE_1, PXE_2 and PXE_1E_2.**

Key words: Oligonucleotide; Alkylating derivative; Effector; Association constant

1. Introduction

Earlier the equations were derived for the time course of the intracomplex modification of a target nucleic acid or oligonucleotide (P) by the reagent (X) consisting of oligonucleotide complementary to definite region of the target and attached reactive group [1,2]. It was shown that the dependence of the limit yield of the modification product *PZ* on the initial concentration x_0 of the reagent permits to calculate association constant K_x of the complex PX formed by P and X. The approach was in particular used for the comparison of the affinities of definite reagent to a set of fragments (from 303-mer to decanucleotide) with the same site for the reagent binding [3]. It was demonstrated that K_x decreases significantly with the increase of the target dimension, most probably due to appearance of internal spatial structures of the larger targets unfavourable for the duplex formation with the reagent.

In the present paper we demonstrate that similar treatment may be used for more complicated system containing additionally two auxiliary oligonucleotides (effectors E_1 and E_2) which are complementary to the sequences of the target adjacent to the reagent site. Such effectors enhance significantly modification yield especially being supplied with duplex-stabilizing phenazinium residues [4,5]. This treatment permits to estimate cooperativity parameters characterizing the interactions of the tandem components. Moreover, it was found that both K_x value and cooperativity parameters obtained by this treatment represent only apparent values, and calculation of the true values requires additional experiments taking into account the existence of alternative secondary structures of the target.

2. Materials and methods

T4-polynucleotide kinase (PK) was purchased from Biopol (Russia). [γ-^{32}P]ATP (>3000 Ci/mmol) was from Biosan (Russia). Acrylamide and *N,N'*-methylene bisacrylamide were from Serva (Germany). All other reagents were from Reakhim (Russia). Oligonucleotides T26, T22 and T10 used as a target (Fig. 1) were prepared by a solid phase phosphiteamide method using automated synthesizer Victoria-6M (Russia), and subjected to 5'-terminal labelling by [γ-^{32}P]ATP in the presence of 10–20 units of PK. Reagent was synthesized from oligonucleotide d(pTTCCCA) prepared by a solid phosphotriester method [6] by attachment of p-(*N*-2-chloroethyl-*N*-methylamino)benzylamine (further ClRCH$_2$NH$_2$) according to [7], and purified by HPLC at the Lichrosorb RP-18 column (Merck, Germany) in the 0–60% concentration gradient of CH$_3$OH in 0.05 M triethylammonium acetate, pH 7.5. Effectors E_1 and E_2 were synthesized starting from respective oligonucleotides prepared by the solid phase phosphiteamide method containing 3'-terminal phosphate [8]. Reaction described in [4,5] was used for attachment of (2-hydroxyethyl)phenazinium residue. First residue was bound directly to 3'-phosphate, the second one to the monosubstituted intermediate after introduction of 5'-phosphate using PK. To quantify oligonucleotide derivatives used the extinction coefficient ε_{260} of the ClRCH$_2$NH-group $1.47 \cdot 10^4$ M^{-1}cm^{-1}, and $\varepsilon_{260} = 1.0 \cdot 10^4$ M^{-1}cm^{-1} for each phenazinium residue were added to ε_{260} values of respective oligonucleotides.

Modification was carried out in the buffer: 0.16 M NaCl, 0.02 M Na$_2$HPO$_4$, 0.1 mM EDTA, pH = 7.5, at 25°C. The time of reaction was equal to 5 half-times of the reaction of ionization of C–Cl bond in the reagent [9], $\tau_{1/2} = 5$ h at 25°C. The reaction mixtures were precipitated with 10-fold excess volumes of 2% LiClO4 in acetone, centrifuged, washed with 85% ethanol, dryed and dissolved in formamide with marker dyes. The products of modification were separated by electrophoresis in 10% PAAG, containing 7 M urea. The band with the electrophoretic mobility lower than that of the non-modified target was referred to adducts of the reagent to the target. Gels were dried, and exposed to RM-1 film at −20°C. The autoradiograms of the gels were quantified using laser densitometer UltroScan XL (LKB, Sweden). The limit modification extents were calculated as the ratios of integral intensities of the bands corresponding to the modification products to the sum of integral intensities of the bands on the lane. For the estimation of the quantitative parameters of modification the data were treated using nonlinear regression program.

3. Results

The 26-meric oligonucleotide T26 was chosen as a main target (Fig. 1). It seems reasonable to suggest that T26 partially

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SSDI 0014-5793(95)00733-4

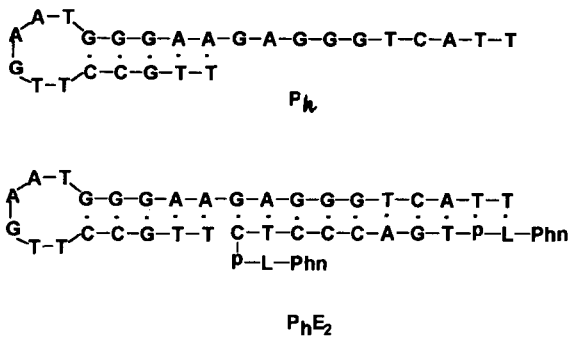

Fig. 1. (a) The structure of the complex T26 · X · E_1 · E_2. (b) The structure of the complex T22 · X · E_2.

exists in a form of hairpin structure with one mismatch (Fig. 2). Therefore, it may serve as a convenient model to study the role of alternative secondary structures in the modification process. Alkylating reagent X is capable of binding with the sequence 11–16 of T26. Bis-(2-hydroxyethylphenazinium) derivatives of oligonucleotides complementary to regions 3–10 and 17–24 of the target were used as effectors. Tandem structure $E_1 \cdot X \cdot E_2$ in the complex with T26 is presented in Fig. 1. This structure corresponds to linear conformation of the target. Haipin T26[(h)] can not interact with X since the part of the reagent site 12–16 participates in the formation of the hairpin stem. To elucidate the role of this conformaiton the target T22 lacking six 5′-terminal residues of T26 was also investigated. The 3′-end of the shortened model was deprived of two G residues inherent to T26 to escape possible complications due to interguanine interactions. Oligonucleotide T22 contains the sites for binding of X and E_2, but does not contain the site for binding of E_1. Target T10 dp(TGGGAAGAGT) was also used which contains only the reagent site.

The dependences of the limit alkylation extent $[PZ]_\infty/p_o$ on the initial reagent concentration x_o was studied for the complexes T26 · X, T26 · X · E_1, T26 · X · E_2, T26 · X · E_1 · E_2, T22 · X, T22 · X · E_2, and T10 · X. As it was already told in Section 1, these denpendences permit to calculate association constant of the reagent with the template. In the presence of one or two effectors taken in the saturating concentrations the

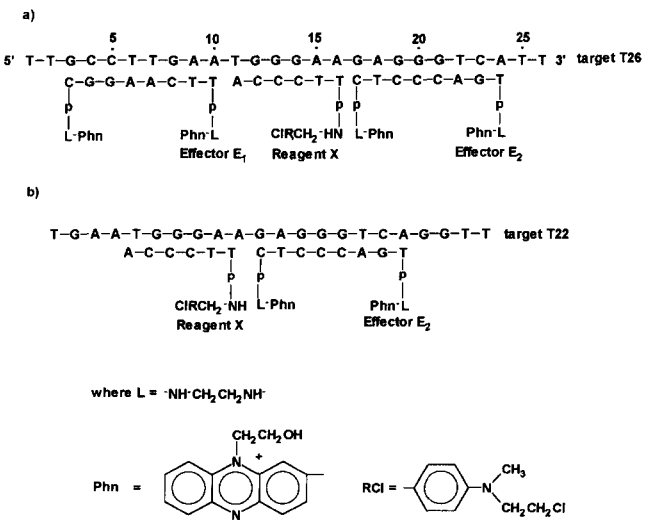

Fig. 2. The hairpin structure of T26 and its complex with E_2.

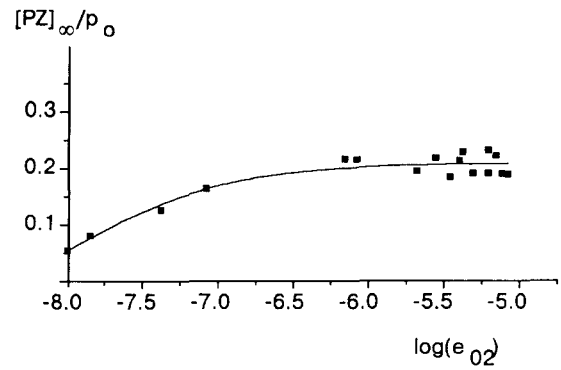

Fig. 3. The dependencies of the limit extent (at $t \to \infty$) of the target P modification, $[PZ]_\infty/p_o$, on the logarithm of the initial concentration of the effector E_2, $\lg(e_{02})$ ($x_o = 5 \cdot 10^{-6}$ M, $p_o = 5 \cdot 10^{-9}$ M, 25°C).

values obtained characterize the affinity of the reagent towards the target saturated with this effector (or effectors). Therefore, all these dependences should be described by the same equation:

$$[PZ]_\infty/p_o = 1 - \exp[-\gamma K_x x_o/(1 + K_x x_o)], \quad (1)$$

where γ corresponds to the efficiency of the intracomplex reaction and is close to unity in all cases under consideration, and K are association constants for X with T (K_x), T · E_1 (K_{xe1}), T · E_2(K_{xe2}) and T · E_1 · E_2(K_{xe12}).

In the preliminary experiments it was demonstrated that at given x_o value the limit yield as a function of the effector concentration reaches plateau value (Fig. 3). The position of this plateau permits to estimate saturating concentration of the effector. The dependencies of the limit modification product yield on x_o recorded in the absence of any effector, in the presence of either E_1 or E_2, and in the presence of both effectors, in all cases at saturating concentrations of effectors, are presented in Fig. 4. The association constants are presented in Table 1. The values for interaction of X with T22 and T10 are identical. This means that these targets may be considered as linear. The data clearly demonstrate that the apparent affinity of X increases in the presence of each of effectors or both of them. This enhancement as will shown below is reasonable to consider as apparent one. The apparent cooperativity parameters $\beta_1 = 31.2$ for E_1, $\beta_2 = 1.92$ for E_2, and $\beta_{12} = 272$ in the presence of both effectors.

Table 1
Association constant values

Complex	Effector concentration	Association constants, M^{-1}
T26 · X	–	$K_x = (2.16 \pm 0.38)\ 10^4$
T26 · E_1 · X	$e_{01} = 1 \cdot 10^{-5}$ M, $e_{02} = 0$	$K_{xe1} = (6.75 \pm 0.12) \cdot 10^5$
T26 · X · E_2	$e_{02} = 1{,}38 \cdot 10^{-5}$ M, $e_{01} = 0$	$K_{xe2} = (4.15 \pm 0.29) \cdot 10^4$
T26 · E_1 · X · E_2	$e_{01} = 1 \cdot 10^{-5}$ M, $e_{02} = 1{,}38 \cdot 10^{-5}$ M	$K_{xe12} = (5.87 \pm 1.00) \cdot 10^6$ M^{-1}
T22 · X, T10 · X	–	$K_x^{(1)} = (4.21 \pm 0.65) \cdot 10^4$
T22 · X · E_2	$e_{02} = 1{,}38 \cdot 10^{-5}$ M	$K_{xe2}^{(1)} = (4.16 \pm 0.72) \cdot 10^5$

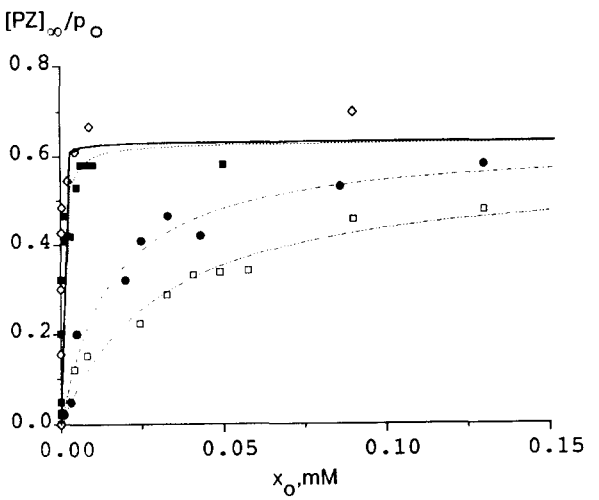

Fig. 4. The dependencies of the limit extent (at $t \to \infty$) of the target P modification, $[PZ]_\infty/p_o$, on the logarithm of the initial concentration of the reagent x_o: curve 1 (—) $-e_{01} = 1 \cdot 10^{-5}$ M, $e_{02} = 1.38 \cdot 10^{-5}$ M (◇); curve 2 (···) $-e_{01} = 1 \cdot 10^{-5}$ M, $e_{02} = 0$ (■); curve 3 (·—·—) $-e_{02} = 1.38 \cdot 10^{-5}$ M, $e_{01} = 0$ (●); curve 4 (---) $-e_{01} = 0$, $e_{02} = 0$ (□), ($p_o = 5 \cdot 10^{-9}$ M, 25°C).

4. Discussion

The data presented also demonstrate that K_x values for T26 is two times lower than for T22. This may be reasonably referred to the presence of $T26^{(h)}$ structure which is absent in the case of T22. Therefore, to estimate the true value of affinity of X to T26 two equilibria must be taken into account:

$$T26^{(l)} \leftrightarrow T26^{(h)} (K_h), \qquad T26^{(l)} + X \leftrightarrow T26 \cdot X (K_x^{(l)}).$$

This permits easily to connect true association constant of the linear structure of the target $K_x^{(l)}$ taken from the value for T22 and T10 with the apparent value K_x for T26.

$$K_x = K_x^{(l)}/(1 + K_h). \tag{2}$$

Using this equation and the values presented in the Table one easily finds that $K_h = 0.95 \pm 0.04$. Consequently, the true cooperativity coefficient value for interaction of E_1 with X may be calculated as the ratio $K_{xe1}/K_x^{(l)} = 16.0 \pm 2.2$.

Similar consideration of K_{xe2} values for T26 (apparent value) and for T22 (true value for linear structure) permits K_{h2} to find for the equilibrium

$$T26^{(l)}E_2 \leftrightarrow T26^{(h)}E_2 \ (K_{h2}),$$

$$K_{xe2} = K_{xe2}^{(l)}/(1 + K_{h2}). \tag{3}$$

Taking K_{xe2} and $K_{xe2}^{(l)}$ from respective values for T26 and T22 (T10) we receive $K_{h2} = 9.0 \pm 1.0$, and the true cooperativity coefficient $\alpha_2 = K_{xe2}/K_x^{(l)} = 10.0$.

The same consideration for K_{xe12} value results for the true cooperativity coefficient $\alpha_{12} = K_{xe12}/K_x^{(l)} = 139 \pm 2$. This value within the error limits is rather close to the product $\alpha_1 \cdot \alpha_2 = 158 \pm 24$. This means that both effectors operate independently.

Acknowledgements: This work is supported in part by Russian Fond of Fundamental Sciences (Grant 95-04-11980a) and Russian Ministry of Sciences (Program 'New Methods in Bioengineering', Part: Gene-Directed Biologically Active Compounds).

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