Quantitative parameters of cooperative interactions of oligonucleotides within tandem complexes

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Quantitative parameters of cooperative interactions of deoxyribooligonucleotides within perfect complementary complexes with a nick in one strand and imperfect complexes containing one mismatched base pair in the nick were obtained. One complementary strand was represented by 22-mer oligonucleotides, while the other, by two short 8-mer oligonucleotides forming a tandem complex with the central part of the 22-mer. In the tandem complexes, the 8-mers form contacts of the following types: 5´-Py*pPy-3´, 5´-Pu*pPy-3´, and 5´-Pu*pPu-3´, where p is phosphate, Py and Pu are pyrimidine and purine nucleosides, respectively, and * stands for a nick. In each incompletely complementary complex, the mismatched base pair in the nick is formed by the 3´-end nucleoside of the 8-mer oligonucleotide and by the nucleoside located in the middle of the 22-mer oligonucleotide. The alkylating {4-[*N*-(2-chloroethyl)-*N*-methylamino]phenyl}methylamino group (RCl) is linked through the 5´-end phosphate of the 8-mers (reagents) close to 3´-ends of the 22-mers. The dependences of the limit extents of alkylation of 22-mers (targets) at zero and saturating concentrations of the neighbor oligonucleotides (effectors) on the initial concentration of RCl-derivatives of oligonucleotides (reagents) were used to calculate the association constants K_X of the reagent with the target. The ratio of these constants was used to determine the parameters of contact cooperativity α, which characterize the interactions at the junction of two oligonucleotides within the tandem complexes.

Key words: oligonucleotides, cooperativity, alkylating derivatives of oligonucleotides

The efficiency of chemical action of reactive oligonucleotide derivatives on nucleic acids can be increased by auxiliary oligonucleotides, that is, effectors, which bind to neighboring sequences of nucleic acids (targets) and thus increase the association constants of oligonucleotides due to the cooperative effect.[1,2] The cooperative effect is caused by stacking of heterocyclic bases of neighboring nucleotides at the junction of neighboring oligonucleotides. The oligonucleotides functioning as effectors develop energetic or conformational advantages for the reagent on binding to the target. Study of the parameters of cooperative interactions currently attracts considerable attention, as tandems of short oligonucleotides can be used to identify point mutations or to perform directed action on nucleic acids.

Previously,[2—8] we studied the cooperative interactions between oligonucleotides containing modifying groups at the nick. The parameters of cooperativity were used as quantitative characteristics of cooperative interactions. This parameter is defined as the ratio of the equilibrium association constants of an oligonucleotide with a single-stranded DNA in the presence and in the absence of another oligonucleotide bound to a neighboring sequence of the target. The association constants of oligonucleotides were determined using a method developed at the Novosibirsk Institute of Bioorganic Chemistry of the Siberian Branch of the Russian Academy of Sciences based on affinity modification,[9,10] which later came to carry the name 'Complementary Addressed Modification Titration' (CAMT).[8] In this method, the equilibrium association constants of reactive oligonucleotide derivatives with nucleic acids are determined from the dependence of the extent of modification of nucleic acids on the concentration of these derivatives (modification isotherms). The theoretical bases of this method have been reported in a number of publications.[11—13] This approach has successfully been used to modify the target by alkylating oligonucleotide derivatives,[14—21] iron-porphyrin derivatives (oxidative modification),[22] and photoactivated aryl-azide oligonucleotide derivatives.[23—24] We have used CAMT to obtain the quantitative parameters of cooperative interactions in nucleic acids. The targets were modified

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using alkylating oligonucleotide derivatives bearing a {4-[*N*-(2-chloroethyl)-*N*-methylamino]phenyl}methylamino group (RCl).

Recently, thermodynamic parameters for all the 16 possible types of coaxial stacking (or cooperative interactions) of oligonucleotides upon continuous stacking hybridization in solution, obtained using melting curves[25,26] and immobilized oligonucleotides[27] have been reported. In addition to junctions containing matched pairs, those of mismatched base pairs on the 3′- side of the nick were also studied.[27] The influence of phosphate groups and modifying groups on the thermodynamic parameters of cooperative interactions was also investigated.

The purpose of the present study is to determine, using the CAMT method, the parameter of cooperative interactions in fully complementary complexes and complexes with a mismatched base pair in the nick and to study the effect of the terminal phosphate group at the junction on the equilibrium association constants and parameters of cooperative interactions. The obtained data would supplement the information on the quantitative characteristics of cooperative interactions of oligonucleotides; they could be of interest for studies on molecular hybridization of oligonucleotides and for practical applications of the method.

Experimental

Commercial acrylamide and *N*,*N*′-methylenebisacrylamide (SERVA Electrophoresis GmbH, Germany) and urea, triphenylphosphine, 4-dimethylaminopyridine (DMAP), and 2,2′-dipyridyl disulfide (Sigma—Aldrich Inc., USA) were used. Other compounds were "special-purity" grade commercial reagents (Reachim, Russia). All solutions were prepared using twice-distilled water.

Oligonucleotides were synthesized on a Cyclone Plus automated DNA synthesizer (MilliGen/Biosearch, USA) using the standard phosphoramidite method. The products were purified by HPLC on a 4.6×250 mm Nucleosil 100-5 SB ion exchange column followed by reversed-phase chromatography on a 4.6×250 mm Nucleosil 100-7 C_{18} column (Macherey-Nagel GmbH, Germany).

^{32}P-labeling at the 5′-end of 22-mer oligonucleotides was carried out by a standard procedure.[28]

Alkylating oligonucleotide derivatives were synthesized by a previously described[29] procedure; the products were isolated by reversed-phase HPLC on a LiChrosorb RP-18 column (Merck KGaA, Germany) using MeCN gradient in 0.05 *M* triethylammonium acetate buffer, pH 7.5 as the eluent. The molar extinction coefficients at $\lambda = 260$ nm were calculated as the sum of contributions of the oligonucleotide moiety[30] and the aromatic fragment of the RCl group, the latter being equal to $1.47 \cdot 10^4$ L mol^{-1} cm^{-1} (see Ref. 31).

Modification of the 22-mer targets was carried out in a buffer with the composition 0.16 *M* NaCl, 0.02 *M* Na_2HPO_4, and 0.1 *mM* EDTA, pH = 7.5, at 25 °C for 25 h, *i.e.*, about five times longer than the half-life of the reagent in solution.[32] It was assumed in calculations that $t \to \infty$. In experiments with nontandem duplexes and with duplexes containing phosphate in the contact region, the concentration of the target was 10^{-8} mol L^{-1}, and the concentrations of the reagents were varied from 10^{-7} to 10^{-4} mol L^{-1}. In experiments with duplexes without phosphate at the junction, the concentration of the target was 10^{-9} mol L^{-1}, and the reagent concentration varied from 10^{-8} to 10^{-6} mol L^{-1}. The modification products were separated by electrophoresis in 20% polyacrylamide gel (PAAG) containing 7 *M* urea. Prior to loading onto denaturing 20% PAAG, the samples were dissolved in 5 μL of 7 *M* urea containing 0.1% Bromophenol Blue and 0.1% Xylene Cyanol FF. Electrophoresis was carried out at 50 V cm^{-1}. The gels were autoradiographed on an Agfa CP-BU New film (Agfa-Gevaert N.V., Belgium) for 8—24 h at −10 °C. The exposure time was pre-adjusted in such a way that the intensities of bands corresponding to the products to be separated fitted within the linear range of the calibration absorption curve.

The extent of modification was determined by processing the gel autoradiogram, subjected preliminarily to densitometric analysis, in terms of the Gel-Pro Analyzer program package (Media Cybernetics, L.P., USA). The electrophoretic mobilities of the modified products corresponding to the adducts formed by the reagent with the target were lower than that of the nonmodified target. The limit extents of modification were calculated as the ratios of the integral intensities of bands corresponding to modified products to the sum of the integral intensities of bands corresponding to the products and the band corresponding to the initial target. The error of determination of the extent of modification normally did not exceed 20%.

The quantitative parameters of modification were determined by nonlinear regression method using the Origin 7.0 program package (OriginLab, Corp., USA).

Hydrolysis of alkylating derivatives of oligonucleotides was carried out in a buffer with the composition: 0.16 *M* NaCl, 0.02 *M* Na_2HPO_4, and 0.1 *mM* EDTA, pH 7.5, at 25 °C. The hydrolysis time was 25 h and the concentration of alkylating derivatives of oligonucleotides was ~2×10^{-4} mol L^{-1}.

Results and Discussion

Complexes

The structure of the oligonucleotide complexes studied here can be represented in the following way:

$$(5')N_1N_2N_3N_4 \ldots N_{10}\text{—}\mathbf{X}\text{—}\mathbf{Y}\text{—}N_{13} \ldots N_{19}N_{20}N_{21}N_{22}(3')$$

$$(3')N_8 \ldots N_2\text{—}Zp^*W\text{—}N_7 \ldots N_1pNHCH_2RCl(5')$$

where N is the nucleotide residue, 5′-$N_1N_2N_3..N_{10}$—**X**—**Y**—$N_{13}..N_{20}N_{21}N_{22}$-3′ is the oligonucleotide target, 5′-Z—N_2.....N_8 and 5′-N_1.....N_7—W are the oligonucleotide effector and oligonucleotide reagent, respectively.

Deoxyoligoribonucleotides comprising 22-nucleotides were employed as single-stranded DNA targets. The reactive derivatives of four oligonucleotides containing the

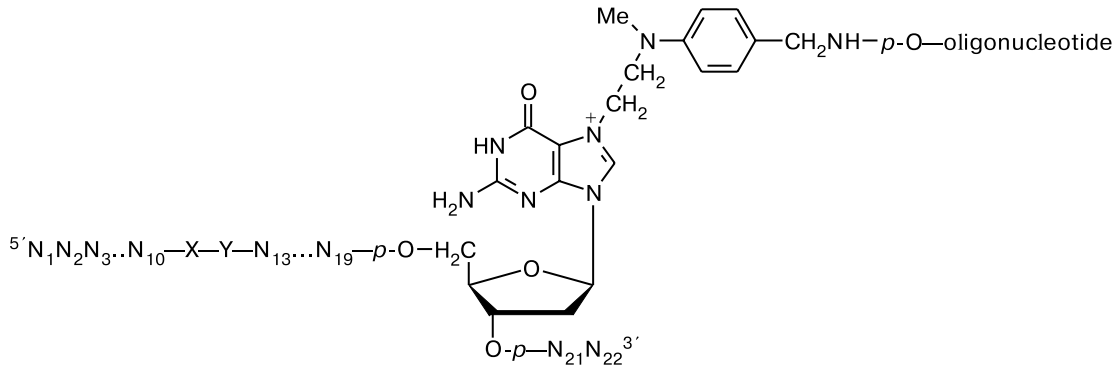


Fig. 1. Structure of the product formed upon modification of 22-mers with RCl-derivatives of oligonucleotides.

ClR′CH_2NH— groups linked covalently to their 5′-phosphates were used as the reagents. They formed complementary complexes with the N_{12}—N_{19} sequence of nucleotides of the targets. Five octanucleotides (effectors) could bind to the N_4—N_{11} nucleotide sequences of the targets, one of them being devoid of the phosphate group at the 5′-end (below referred to as the phosphate-free effector); one octanucleotide could bind to the N_2—N_9 nucleotide sequences of the targets, *i.e.*, it was shifted by two nucleotides from the nick (below referred to as shifted effector).

Targets*

1 3 5 7 9 11 12 14 16 18 20 22

d(pTTTGCCTTGA**A**—**T**GGGAAGAGTT) T′22^{AT}
d(pTTTGCCTTGA**A**—**A**GGGAAGAGTT) T′22^{AA}
d(pTTTGCCTTGA**A**—**C**GGGAAGAGTT) T′22^{AC}
d(pTTTGCCTTGA**A**—**G**GGGAAGAGTT) T′22^{AG}
d(pTTTGCCTTGA**T**—**C**GGGAAGAGTT) T′22^{TC}
d(pTTTGCCTTGA**C**—**T**GGGAAGAGTT) T′22^{CT}
d(pTTTGCCTTGA**C**—**C**GGGAAGAGTT) T′22^{CC}
d(pTTTGCCTTGA**G**—**G**GGGAAGAGTT) T′22^{GG}

Effectors:

d(pTTCAAGGC) E(t)
d(pATCAAGGC) E(a)
d(pGTCAAGGC) E(g)
d(pCTCAAGGC) E(c)
d(GTCAAGGC)** E*(g)
d(pCAAGGCAA)*** DE

Reagents:

ClR′CH_2NHd(pTCTTCCCA) RClX8^{TA}
ClR′CH_2NHd(pTCTTCCCT) RClX8^{TT}
ClR′CH_2NHd(pTCTTCCCC) RClX8^{TC}
ClR′CH_2NHd(pTCTTCCCG) RClX8^{TG}

* The sites of binding of effectors are singly underlined and the sites of reagent binding are doubly underlined.
** Phosphate-free effector.
*** "Shifted" effector.

Thus, we studied 18 usual duplexes, 18 tandem complexes containing phosphate at the junction, and 4 tandem complexes without phosphate at the junction. In all cases, the 5′-end nucleoside of the oligonucleotide effectors formed the Watson—Crick pairs with the nucleoside of the targets, whereas the 3′-end nucleoside of the oligonucleotide reagents formed both the canonical Watson—Crick pairs and noncanonical pairs with nucleosides of the target.

Previously, it was found[7] that the concentration of the effector required for complete saturation of the target is no lower than 10^{-4} mol L^{-1}; therefore, the effector concentration used in all experiments was $3 \cdot 10^{-4}$ mol L^{-1}. The equilibrium binding constants of the alkylating oligonucleotide derivatives (K_X) were calculated from the dependence of the extent of alkylation of single-stranded DNA (ζ_∞) on the concentration of alkylating oligonucleotide derivatives (x_0) at the time $t \to \infty$.[13] Alkylation of the targets yielded covalent adducts, mainly at the N(7) atom of guanine (see Ref. 33 and references cited therein). The alkylation proceeded at the G_{20} residue (Fig. 1).

The modification of the targets resulted in products having lower electrophoretic mobilities.

The dependences of the limit modification extent of the target at $t \to \infty$ on the reagent concentrations were hyperbola-shaped reaching a plateau. The typical modification isotherms with and without an effector are presented in Fig. 2. With the effector, the limit extents of modification were achieved at lower reagent concentrations than without the effector, which indicated a positive cooperativity.

The alkylation by the reagent containing a 2-chloroethylamino group (RCl) follows the S_N1 mechanism with the formation of the highly reactive ethyleneimmonium cation (R^+) in the limiting step.[33] In the solution, R^+ reacts with water to give nonreactive species ROH. The set of processes involved in the alkylation can be represented by Scheme 1.[13]

If only inactivation of the alkylating group takes place in the product of the reagent transformation in solution S,

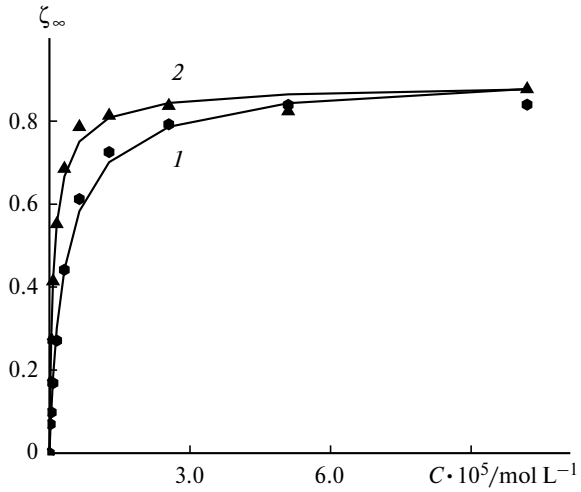
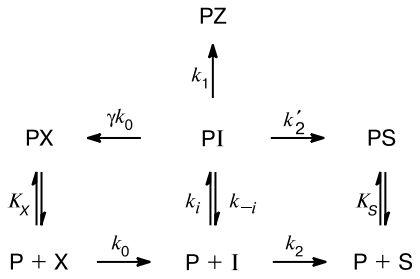


Fig. 2. Limit extent of modification of the T´22^{CT} target at t→∞ *vs.* concentration of the RClX8^{TT} reagent without an effector (*1*) and in the presence of effector E(*g*) (*2*).

Scheme 1



P is the target, X is the reagent, I is the intermediate compound, PZ is the product of the reaction within the complex, S is the product of reagent transformation in the solution.

while the oligonucleotide fragment is not changed, then it retains its affinity for the target and competes with the reagent for binding to the target, thus acting as a competitive inhibitor.

The equation describing the accumulation of the product of biopolymer modification can be written as follows:[13]

$$\zeta = 1 - \left[\frac{1 + K_X x_0}{1 + K_S x_0 + (K_X - K_S) x_0 \exp(-k_0 t)}\right]^{\frac{\gamma_{eff} K_X}{K_S - K_X}}, \qquad (1)$$

where ζ is the extent of modification of the target, equal to the ratio of the concentration of the modification product to the initial concentration of the target; x_0 is the initial concentration of the reagent; K_X is the equilibrium association constant of the reagent with the DNA target; K_S is the equilibrium association constant of reagent transformation products with the DNA target; k_0 is the first-order rate constant for the formation of ethyleneimmonium cation R^+, and γ_{eff} is the effectivity coefficient for the modification of the target complexed with the reagent.

The major contribution to the stability of duplexes formed by the reagents with DNA targets should be made by the oligonucleotide moieties of the reagents rather than by the RCl and ROH groups. Previously, it was shown[34] that oligonucleotide derivatives containing RCl and ROH groups interact with complementary sequences to form duplexes with similar thermodynamic stability parameters. Hence, the condition $K_X = K_S$ should hold and Eq. (1) assumes the form:

$$\zeta = 1 - \exp\left\{-\gamma_{eff} \frac{K_X x_0 [1 - \exp(-k_0 t)]}{1 + K_X x_0}\right\}. \qquad (2)$$

Usually, Eq. (2) describes adequately the kinetics of modification.

In some cases, hydrolysis of the reagent in the solution was found[6] to be accompanied by damage of the oligonucleotide moiety of the reagent. This decreases the affinity of the product S to the target; therefore, the general equation (1) should be used to process experimental data.

Study of the kinetic features of modification

The first stage of our study was to find out how much the values of K_X and K_S differ from each other. The equilibrium association constant (K_S) of the reagent transformation product as the inhibitor S was calculated from the dependence of the extent of modification of the DNA target by the reagent (ζ'_∞) on the concentration of the inhibitor S, the reagent concentration being constant and equal to ~$2 \cdot 10^{-5}$ mol L^{-1}. We studied the complexes T´22^{AT} · RClX8^{TA} · DE, T´22^{AA} · RClX8^{TT} · DE, T´22^{AC} · RClX8^{TG} · DE, T´22^{AG} · RClX8^{TC} · DE, where no contact cooperativity (interaction of the neighboring DNA bases of different oligonucleotides) was involved, because they contained a "shifted" effector. A typical dependence of the extent of modification of the DNA target on the concentration of the inhibitor at a constant concentration of the reagent is presented in Fig. 3.

The limit extent of DNA modification in the presence of an inhibitor is described by the equation[14]

$$\zeta_\infty = 1 - \left[\frac{1 + K_X x_0 + K_S s_0}{1 + K_S (x_0 + s_0)}\right]^{\gamma_{eff} \frac{K_X}{K_S - K_X}}, \qquad (3)$$

where x_0 and s_0 are the initial concentrations of the reagent and the inhibitor, respectively.

The kinetic equation (3) contains three unknown values, K_X, K_S, and γ_{eff}. They cannot be determined simultaneously from one kinetic curve. Therefore, one of these values, in particular K_X, was derived from the dependence of the extent of the DNA target modification during early

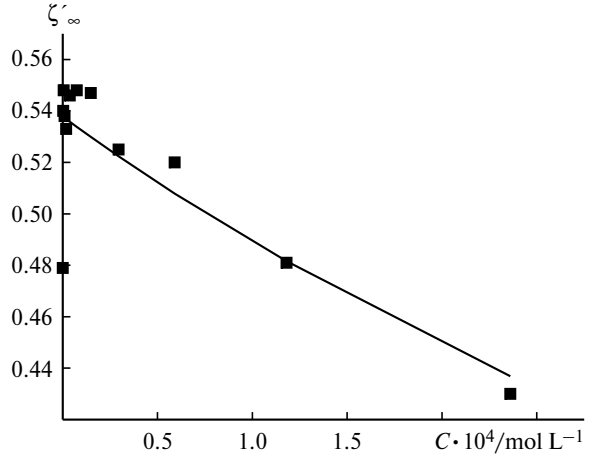


Fig. 3. Extent of modification of the T′22^{AT} target *vs* concentration of the inhibitor at a constant concentration of the RClX8^{TA} reagent in the presence of the DE effector.

transformation times (ζ_t) on the reagent concentration at 25 °C. The transformation time corresponded to 20% of the time needed for complete reaction (1 h 35 min). For short transformation times of the reagent where the formation of product S can be neglected, the extent of the target modification is well described by the equation

$$\zeta_t = \beta K_X x_0/(1 + K_X x_0), \qquad (4)$$

where β is the proportionality coefficient.

The equilibrium binding constants of the inhibitor (K_S) derived from the dependence of the extent of DNA target modification on the inhibitor concentration with the reagent concentration remaining invariable are presented in Table 1, together with the K_X/K_S ratios. It can be seen that the inhibitor has a lower affinity to the target than the initial reagent. The equilibrium association constants of the inhibitor are much lower than the association constants of the reagent for all four complexes we studied. The K_X/K_S ratio determines the extent to what the reagent affinity to the target in the solution decreases after transformation into the inactive product S. The obtained difference between the K_X and K_S values attests to damage of the oligonucleotide moiety of the reagent during the hydrolysis of the RCl group. The damage may be due to an attack of the ethyleneimmonium cation R^+ on the heterocyclic base of the own oligonucleotide chain. Thus, we found that $K_X \neq K_S$; therefore, Eq. (1) should be used for describing the kinetics of the alkylation of the target.

Table 1. Structures of the target—reagent complexes in the presence of "shifted" effectors, nucleotide pairs at the ends of duplexes, equilibrium binding constants of the inhibitor (K_S) determined from the dependence of the extent of modification of the DNA target on the concentration of the inhibitor at a constant reagent concentration, and K_X/K_S ratios

Complex	Type of junction	$K_S \cdot 10^{-3}$ /mol L^{-1}	K_X/K_S
T′22^{AT}·RClX8^{TA}·DE	5′-**GAA**—**T**-3′ 3′-**Cp** **A**-5′	2.22±0.38	50.0
T′22^{AA}·RClX8^{TT}·DE	5′-**GAA**—**A**-3′ 3′-**Cp** **T**-5′	7.16±1.62	43.7
T′22^{AC}·RClX8^{TG}·DE	5′-**GAA**—**C**-3′ 3′-**Cp** **C**-5′	8.84±1.58	14.4
T′22^{AG}·RClX8^{TC}·DE	5′-**GAA**—**G**-3′ 3′-**Cp** **C**-5′	(1.44±0.34)·10^2	14.3

Determination of the equilibrium constants of binding of the reagents to the targets and discrimination parameters in complexes without effectors

In view of the decrease in the reagent affinity to the target in the solution, the equilibrium association constants of the alkylating oligonucleotide derivatives (K_X) were calculated from the dependence of the extent of alkylation of single-stranded DNA (ξ_∞) on the concentration of the alkylating oligonucleotide derivatives (x_0) in terms of Eq. (1), which has the following form for t→∞:

$$\zeta_\infty = 1 - \left(\frac{1 + K_X x_0}{1 + K_S x_0}\right)^{\gamma_{eff}\frac{K_X}{K_S - K_X}}. \qquad (5)$$

One can assume that the K_X/K_S ratio is invariable for a particular reagent in all complexes considered because the transformation of the reagent does not depend on the presence of other oligonucleotides in the solution, and the K_S values for the reagents listed in Table 1 are different.

The equilibrium association constants of the reagents with the targets without an effector are given in Table 2. It can be seen that the association constants of the reagents with the targets decrease if the duplex end carries a noncomplementary pair. For example, in the case of the RClX8^{TT} reagent, upon the replacement of A_{12} of the T′22^{AA} target by thymidine, guanosine, and cytidine, the association constant decreases 2.6-, 8.1-, and 1.8-fold, respectively. The most pronounced changes take place upon the replacement of the GC pair by noncanonical Py—Py pair. Similar data have been reported[35] for the thermodynamics of imperfect RNA duplexes containing a noncanonical pair at the duplex end.

It is known[36] that the formation of imperfect duplexes between the oligonucleotide address and sites of the tar-

Table 2. Structures of the target—reagent complexes, nucleotide pairs at the end of duplexes, equilibrium binding constants of reagents (K_X), and discrimination factors δ

Complex	Type of junction	$K_X \cdot 10^{-6}$ /mol L^{-1}	δ
T′22^{AA} · RClX8^{TT}	5′-**A**—**A**-3′ 3′ **T**-5′	4.89±0.39	—
T′22^{AG} · RClX8^{TC}	5′-**A**—**G**-3′ 3′ **C**-5′	9.63±2.04	—
T′22^{AT} · RClX8^{TA}	5′-**A**—**T**-3′ 3′ **A**-5′	(6.39±1.15)*	—
T′22^{CC} · RClX8^{TG}	5′-**C**—**C**-3′ 3′ **G**-5′	4.56±0.34	—
T′22^{AC} · RClX8^{TG}	5′-**A**—**C**-3′ 3′ **G**-5′	(2.31±0.29) · 10	—
T′22^{GG} · RClX8^{TC}	5′-**G**—**G**-3′ 3′ **C**-5′	9.02±1.03	—
T′22^{CT} · RClX8^{TA}	5′-**C**—**T**-3′ 3′ **A**-5′	(2.77±0.25) · 10	—
T′22^{TC} · RClX8^{TG}	5′-**T**—**C**-3′ 3′ **G**-5′	2.08±0.68	—
T′22^{AT} · RClX8^{TT}	5′-**A**—**T**-3′ 3′ **T**-5′	(1.89±0.23)*	4.0
T′22^{AT} · RClX8^{TC}	5′-**A**—**T**-3′ 3′ **C**-5′	(1.55±0.39) · 10^{-1}	40.3
T′22^{AA} · RClX8^{TC}	5′-**A**—**A**-3′ 3′ **C**-5′	1.48±0.29	2.7
T′22^{AC} · RClX8^{TC}	5′-**A**—**C**-3′ 3′ **C**-5′	(3.12±0.24) · 10^{-1}	74.0
T′22^{AC} · RClX8^{TT}	5′-**A**—**C**-3′ 3′ **T**-5′	2.22±0.34	10.4
T′22^{CC} · RClX8^{TA}	5′-**C**—**C**-3′ 3′ **A**-5′	4.50±0.26	1
T′22^{CC} · RClX8^{TT}	5′-**C**—**C**-3′ 3′ **T**-5′	(8.07±1.45) · 10^{-1}	5.7
T′22^{CT} · RClX8^{TT}	5′-**C**—**T**-3′ 3′ **T**-5′	(4.64±0.58) · 10^{-1}	59.7
T′22^{CT} · RClX8^{TG}	5′-**C**—**T**-3′ 3′ **G**-5′	(7.35±0.57) · 10^{-1}	37.7
T′22^{AG} · RClX8^{TT}	5′-**A**—**G**-3′ 3′ **T**-5′	(4.99±0.62) · 10^{-1}	19.3

* See Refs. 7, 8.

get partly complementary to the address are among the key factors decreasing the selectivity of addressed modification. The most pronounced contribution to the nonspecific modification is expected from complexes containing a noncanonical pair at the end, as it does not lead to significant destabilization of the duplex.[36,37] To avoid the formation of imperfect duplexes, one should know how much the mismatched pair at the end destabilizes the duplex. This destabilization can be described quantitatively (see Table 2) by introducing the discrimination factor δ, equal to the ratio of the K_X constants of the matched and mismatched duplexes

$$\delta = K_X^{mat}/K_X^{mis}. \quad (6)$$

In comparison with the fully matched complex, the mismatch base was located in the reagent (see Table 2).

It can be seen from the data of Table 2 that the equilibrium association constants in matched complexes are larger than in complexes with mismatches. An exception is the complex T′22^{CC} · RClX8^{TA}, whose discrimination factor is equal to unity. Apparently, this may be due to the fact that the AC pair is a wobble pair,[38] in which the bases are paired and incorporated in the double helix.

Determination of the parameters of cooperative interactions

To obtain the parameters of cooperative interactions, we studied the variations of the stability of the target—reagent complex caused by the presence of effectors. These variations were expressed quantitatively in terms of the parameters of cooperativity $\alpha = K_X^{\prime}/K_X$, where $K_X^{\prime}$ is the association constant of the reagent with the preformed target—effector complex.

The equilibrium binding constants of the reagents with the targets in the presence of effectors are summarized in Table 3. For the 5′-Py*p-Py-3′ contact, the cooperativity parameters of the complexes **1—12** are close to unity.* Hence, in these cases, no cooperativity is involved in the formation of either matched or mismatched tandem duplexes. For contacts of the 5′-Pu*pPy-3′ and 5′-Pu*pPu-3′ types, the cooperativity parameters are greater than unity, *i.e.*, positive cooperative interaction is present for these contacts.

* The asterisk (*) marks the nick in the nucleotide sequence, *i.e.*, the contact site of neighboring oligonucleotides.

Table 3. Structures of tandem complexes, nucleotide pairs in the nick, equilibrium binding constants of the reagents to the target—effector complexes (K_X^t), parameters of cooperativity α, and discrimination factors δ

Complex	Type of junction	$K_X^t \cdot 10^{-6}$/mol L^{-1}	α	δ
T′22^{AA} · RClX8^{TT} · E(t) (**1**)	5′-**A**—**A**-3′ 3′-**TpT**-5′	5.35±0.68	1.3	—
T′22^{AG} · RClX8^{TC} · E(t) (**2**)	5′-**A**—**G**-3′ 3′-**TpC**-5′	(1.11±0.1) · 10	1.2	—
T′22^{AT} · RClX8^{TA} · E(t) (**3**)	5′-**A**—**T**-3′ 3′-**TpA**-5′	(2.32±0.21) · 10	3.6*	—
T′22^{CC} · RClX8^{TG} · E(g) (**4**)	5′-**C**—**C**-3′ 3′-**GpG**-5′	(1.71±0.20) · 10^2	37.5	—
T′22^{TC} · RClX8^{TG} · E(a) (**5**)	5′-**T**—**C**-3′ 3′-**ApG**-5′	(1.29±0.35) · 10	6.2	—
T′22^{AA} · RClX8^{TC} · E(t) (**6**)	5′-**A**—**A**-3′ 3′-**TpC**-5′	2.00±0.15	1.4	2.7
T′22^{AG} · RClX8^{TT} · E(t) (**7**)	5′-**A**—**G**-3′ 3′-**TpT**-5′	(8.50±1.95) · 10^{-1}	1.7	13.1
T′22^{AT} · RClX8^{TT} · E(t) (**8**)	5′-**A**—**T**-3′ 3′-**TpT**-5′	(1.70±0.17)*	1.1*	13.4*
T′22^{CC} · RClX8^{TT} · E(g) (**9**)	5′-**C**—**C**-3′ 3′-**GpC**-5′	1.92±0.25	2.4	89.1
T′22^{CC} · RClX8^{TC} · E(g) (**10**)	5′-**C**—**C**-3′ 3′-**GpT**-5′	(7.77±1.55) · 10^{-1}	—	220.1
T′22^{CT} · RClX8^{TT} · E(g) (**11**)	5′-**C**—**T**-3′ 3′-**GpT**-5′	2.08±0.20	4.5	26.8
T′22^{CT} · RClX8^{TG} · E(g) (**12**)	5′-**C**—**T**-3′ 3′-**GpG**-5′	(1.12±0.17) · 10	15.2	5.0
T′22^{CT} · RClX8^{TA} · E(g) (**13**)	5′-**C**—**T**-3′ 3′-**GpA**-5′	(5.57±0.69) · 10	2.0	—
T′22^{AC} · RClX8^{TG} · E(g) (**14**)	5′-**A**—**C**-3′ 3′-**TpG**-5′	(2.01±0.37) · 10^2	8.7	—
T′22^{AC} · RClX8^{TC} · E(t) (**15**)	5′-**A**—**C**-3′ 3′-**TpC**-5′	(8.81±1.24) · 10^{-1}	2.8	228.1
T′22^{AC} · RClX8^{TT} · E(t) (**16**)	5′-**A**—**C**-3′ 3′-**TpT**-5′	1.24±0.52	0.6	162.1
T′22^{AT} · RClX8^{TC} · E(t) (**17**)	5′-**A**—**T**-3′ 3′-**TpC**-5′	(5.05±0.32) · 10^{-1}*	3.3*	45.0*
T′22^{CC} · RClX8^{TA} · E(g) (**18**)	5′-**C**—**C**-3′ 3′-**GpA**-5′	(1.41±0.25) · 10	3.1	12.1

* See Refs. 7, 8.

According to our results, the parameters of cooperativity vary in the following sequence: 5′-Py*pPy-3′ < < 5′-Pu*pPy-3′ < 5′-Pu*pPu-3′. The greatest parameters were found for the 5′-G*p-G-3′ contact in the perfect and imperfect complexes.

Discrimination factors of the mismatched bases in the tandem complexes are larger than those in usual complexes (exceptions are complexes 6, 7, 11, and 12, see Table 3). Thus, in most cases, the use of oligonucleotide tandems for identification of point mutations may be more efficient. These data are consistent with the results obtained previously.[8,39]

The discrimination factors found for the complexes $T'22^{CC} \cdot RClX8^{TC} \cdot E(g)$, $T'22^{AC} \cdot RClX8^{TC} \cdot E(t)$, and $T'22^{AC} \cdot RClX8^{TT} \cdot E(t)$ can be very high. Among other reasons, this is due to the fact that the CC and CT mismatched pairs are open, have low ΔS and ΔH values, and are not incorporated in the double helix in the duplex.[38]

Study of the effect of the terminal phosphate in the nick in tandem complexes on the equilibrium binding constants

The influence of the terminal phosphate at the junction in tandem complexes on the equilibrium association constants was studied for four contacts devoid of the phosphate group in the contact of neighboring oligonucleotides. The corresponding equilibrium association constants of the alkylating derivatives of oligonucleotides are listed in Table 4. The same Table contains the ratios of the constants determined for the effector with and without the phosphate.

In all four complexes considered, the equilibrium binding constants of the reagents to the target—phosphate-free effector complexes are smaller than the binding constants in the corresponding tandem complexes containing the phosphate at the junction. Thus, it follows from our results that removal of the phosphate from the junction decreases the parameter of cooperativity. Apparently, this can be attributed to the fact that the phosphate group protects the inner hydrophobic region of the DNA helix from the unfavorable hydrophilic surrounding.

Table 4. Structures of tandem complexes, nucleotide pairs in the nick, equilibrium binding constants of reagents to the target—phosphate-free effector complexes ($K_X{}^{I}$*), and $K_X{}^{I}/K_X{}^{I}$* ratios

Complex	Type of junction	$K_X{}^{I}$* /mol L^{-1}	$K_X{}^{I}/K_X{}^{I}$*
$T'22^{CC} \cdot RClX8^{TG} \cdot E^*(g)$	5′-**C**—**C**-3′ 3′-**G** **G**-5′	$(1.14\pm0.40) \cdot 10^8$	1.5
$T'22^{CC} \cdot RClX8^{TA} \cdot E^*(g)$	5′-**C**—**C**-3′ 3′-**G** **A**-5′	$(4.65\pm0.63) \cdot 10^6$	3.0
$T'22^{CC} \cdot RClX8^{TC} \cdot E^*(g)$	5′-**C**—**C**-3′ 3′-**G** **C**-5′	$(3.85\pm0.95) \cdot 10^5$	2.0
$T'22^{CC} \cdot RClX8^{TT} \cdot E^*(g)$	5′-**C**—**C**-3′ 3′-**G** **T**-5′	$(4.11\pm1.06) \cdot 10^5$	4.7

In our study, the parameters of cooperativity for perfect contacts decreased in the series

5′-**C**—**C**-3′ / 3′-**GpG**-5′ > 5′-**C**—**C**-3′ / 3′-**G** **G**-5′ > 5′-**A**—**C**-3′ / 3′-**TpG**-5′ ≈

≈ 5′-**T**—**C**-3′ / 3′-**ApG**-5′ > 5′-**A**—**T**-3′ / 3′-**TpA**-5′ > 5′-**C**—**T**-3′ / 3′-**GpA**-5′ ≈

≈ 5′-**A**—**A**-3′ / 3′-**TpT**-5′ ≈ 5′-**A**—**G**-3′ / 3′-**TpC**-5′

This series differs from that given in previous publications,[25,26] where it was found that the efficiency of cooperative contacts in groups with the same 3′-base (X*pG/CZ′, X*pA/TZ′, X*pT/AZ′, X*pC/GZ′) expressed as the change in the free energy ΔG_{12}° on binding of an oligonucleotide in the vicinity of another oligonucleotide on a complementary sequence usually increases upon the change in 5′-X in the series C < T < A <G and correlates well with the corresponding overlap areas of bases involved in coaxial stacking. Meanwhile, in the groups of contacts with an identical 5′-base (C*pY/ZG, T*pY/ZA, A*pY/ZT, G*pY/ZC), more complex dependences were obtained, in particular, the overlap efficiency is correlated with the overlap area of the corresponding bases only for the C*pY/ZG and T*pY/ZA contacts in which the coaxial stacking is enhanced in the sequence Py*pA < Py*pG < Py*pT < Py*pC. For the A*pY/ZT and G*pY/ZC contacts within each group, the order of variation is different: Pu*pG < Pu*pT < Pu*pA < Py*pC. Thus, the latter sequence is partly in variance with the results of our study where the greatest cooperativity parameter was found for the G*pG contact.

For duplexes containing mismatched pairs on the 3′-side of the nick, the following sequence was derived:

5′-**C**—**T**-3′ / 3′-**GpG**-5′ > 5′-**C**—**T**-3′ / 3′-**GpT**-5′ ≈ 5′-**A**—**T**-3′ / 3′-**TpC**-5′ ≈

≈ 5′-**C**—**C**-3′ / 3′-**GpA**-5′ ≈ 5′-**A**—**C**-3′ / 3′-**TpC**-5′ ≈

≈ 5′-**C**—**C**-3′ / 3′-**GpT**-5′ > 5′-**A**—**G**-3′ / 3′-**TpT**-5′ ≈ 5′-**A**—**A**-3′ / 3′-**TpC**-5′ ≈

≈ 5′-**A**—**T**-3′ / 3′-**TpT**-5′ ≈ 5′-**A**—**C**-3′ / 3′-**TpT**-5′

For noncanonical pairs in the contact region, the maximum parameter of cooperativity is found for G*pG, although it is only half that for a contact with canonical pairs. The 5′-Pu*pPy-3′ contacts are usually characterized by greater parameters of cooperativity than 5′-Py*p-Py-3′. For these, the parameters of cooperativity are smaller and in most cases, they are close to unity, which implies the absence of cooperativity. One can conclude than the presence of an effector, which provides the 5′-Py*p-Py-3′ contact, does not stabilize the tandem complex. This can be explained as follows. It was shown[40] that in the vast majority of cases, the dangling ends stabilize the duplexes; stabilization by 5′ dangling ends is the same as that by the 3′-ends or stronger. The complexes without effectors that we studied contain 5′-dangling ends. One can suggest that the stabilizing contribution of the effectors in tandem complexes containing the 5′-Py*p-Py-3′ contacts is comparable with the stabilizing contribution of the dangling ends in the corresponding complexes without effectors.

Cooperativity was studied for oligonucleotide decamers immobilized on a microchip in the polyacrylamide gel.[27] These were hybridized with 17-mer oligonucleotides in the presence of auxiliary pentanucleotides. As a result, duplexes are formed with a nick in one chain having both canonical base pairs and noncanonical, modified, and overlapping pairs as well as gaps on the 5′-side. In addition, a phosphate and/or hydroxy groups were located at the 5′- and 3′-ends of the nick. The key conclusion made[27] is that the coaxial stacking of 3′-adenine with any 5′-base is stronger than that between any other bases. Among the mismatched bases, the greatest stacking occurs between guanine at the 5′-end of the nick and all 3′-end bases.

Thus, the quantitative characteristics of cooperative interactions obtained previously[27] differ from the results of our present study. However, in our study, the mismatched base was located in imperfect tandem duplexes at the 3′-end of the stacking, whereas in the previous paper,[27] this was at the 5′-end; the phosphate group was at the 5′ stacking end in the duplexes we studied, whereas in the previous study,[27] this position of the phosphate group was not considered. Therefore, thermodynamic parameters of cooperative interactions obtained in our study and in the previous one[27] can be compared only for perfect tandem complexes of oligonucleotides.

It follows from reported data[25–27] that the efficiency and the area of base stacking in the nick region are not always strictly correlated. Probably, the theoretical base stacking area differs from the real one, which depends on the type of the nearest environment, as it can influence the local structure of the duplex at the junction. The same can be responsible for different effects of the phosphate groups located in the nick region.

The results we obtained supplement the information reported in the literature concerning the quantitative characteristics of the cooperative interactions in oligonucleotide duplexes and are of practical interest for the medical diagnostics using the molecular hybridization method.

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