α -DNA X: α and β tetrathymidilates covalently linked to oxazolopyridocarbazolium (OPC): comparative stabilization of oligo β -[dT]:oligo β -[dA] and oligo α -[dT]:oligo β -[dA] duplexes by the intercalating agent

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ABSTRACT

The influence of the intercalating oxazolopyridocarbazolium (HOPC) on the stabilization of modified oligonucleotides: α -T₄c₅OPC or β -T₄c₅OPC associated to β -oligo (dA) was studied. It appears that the situation is different from what has been observed for the interaction of these modified oligonucleotides with poly (rA). The higher free energy of formation of the α -T₄c₅OPC : β -oligo(dA), when compared to β -T₄c₅OPC, is essentially due to the overall stability added to this system by the intercalator. This enhanced stability comes from a higher number of binding sites of HOPC for the α : β duplex together with a lower van't Hoff energy of formation of the α : β duplex.

INTRODUCTION

In procaryotic cells and bacteria, gene regulation proceeds at the transcriptional level by specific interactions between nucleic acids and proteins (1,2). However, the rules involved in the selective recognition between polypeptidic chains and DNA regulatory sequences are complex and not yet completely elucidated. In procaryotes, antisense RNAs complementary to mRNA sequences have been described as naturally occurring translation inhibitors (3). The interaction of a single stranded nucleotidic chain with its complementary mRNA sequence has been used to inhibit procaryotic (4), eukaryotic (5,6), viral (7) and oncogenic (8,9) protein synthesis at the translational level. In order to distinguish between these two strategies, the translation inhibitors were called 'antisense' when they consist of complementary biologically synthesized RNA (see ref 10 for a review) and 'anti-messengers' when they consist of synthetic oligodeoxynucleotides (see ref. 11 for a review). The efficiency of these anti-messenger oligodeoxynucleotides to regulate translation may be altered mostly at four levels: thermodynamic stability of the duplex, resistance towards nucleases, cellular permeability and activity of RNase H on the hybrid formed between the modified DNA and mRNA, by means of chemical modifications of the phosphodiester bond: methylphosphonates (12,13), phosphorothioates (14) or change in the anomeric configuration of the sugar moiety (15).

As an alternative approach, oligonucleotides covalently linked to intercalating dyes: acridines (16), oxazolopyridocarbazole (HOPC) (17) or to poly(L-lysine) (18) have been experienced. The ability of these molecules to block protein synthesis in vitro (19–21) and proliferation of cultured viruses (18,22) or parasites (23) have been evidenced.

The set of experiments presented here concerns α -T₄c₅OPC and β -T₄c₅OPC, taken as models of such oligonucleotides covalently linked to intercalating agents. They are built from an α - or β -tetrathymidilate covalently linked through a five carbon polymethylene linker to the intercalating OPC. A previous study (17) has shown that for these molecules, the specificity of the A-T base pairs is conserved and the thermal stability of the duplex

Figure 1. Chemical structures of studied compounds.

$\text{T}_4\text{C}_5\text{OPC}:\text{poly}(\text{rA})$ is increased when compared to β -d[(Tp)₃T]:poly(rA). Degradation of the conjugated oligonucleotide with purified enzymes indicates that protection against 3'-exonucleases (24) occurs. Moreover, since it was observed that OPC enhances the cell permeability to oligonucleotides (results not shown), ellipticine derivatives linked to

Figure 2. Changes in absorption spectra of α -T₄C₅OPC (A and B) and β -T₄C₅OPC (C and D) in the presence of increasing concentration of the complementary sequences β -d[Gp(Ap)_nG] at 2°C in 10 mM sodium cacodylate pH 7 and 0.1 M NaCl. Initial concentration is 12 μ M. The values represent ratios of adenine over thymine concentrations.

A and C : Complementary strand is β -d[Gp(Ap)₄G]

B and D : Complementary strand is β -d[Gp(Ap)₁₂G]

Insets: Titration curves are obtained by plotting relative absorbance versus A/T ratio. The initial absorbance is normalized to 1. —■— 310 nm, —●— 270 nm

oligodeoxynucleotides are expected to be potent anti-messengers. In this report we compare the gain in stability induced by the OPC ring, when the oligonucleotide moiety is an α or β -deoxytetrathymidilate. Since oligoribonucleotides of precise length are not readily available, we have studied the annealing of these molecules with complementary β -d[Gp(Ap)_nG] sequences (the flanking G being used in order to avoid catenation). As our goal is to elucidate the role of the intercalating agent in the stabilization of the duplex, we also report the binding of OPC to the short double-stranded α -d[(Tp)₁₁T]: β -d[Gp(Ap)₁₂G] and β -d[(Tp)₁₁T]: β -d[Gp(Ap)₁₂G] helices.

MATERIALS AND METHODS

Synthesis

α - and β -T₄C₅OPC (figure 1) were synthesized as previously described (17) and purified by reverse phase HPLC. Purity was checked using mass spectroscopy.

Concentrations were determined by UV absorbance at 271 nm for α -T₄C₅OPC ($\epsilon_{271} = 46500 \pm 500 \text{ M}^{-1} \cdot \text{cm}^{-1}$) and at 270 nm for β -T₄C₅OPC ($\epsilon_{270} = 47800 \pm 500 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Table 1. Melting temperature of the duplexes. Experimental conditions are 0.01 M cacodylate buffer, pH 7 and 0.1 M NaCl.

First strand → Second strand ↓	α -d(Tp) ₃ T *260 nm	α -T ₄ C ₅ OPC *260 nm 310 nm	β -d(Tp) ₃ T **260 nm	β -T ₄ C ₅ OPC **260 nm 310 nm
β -d(Ap) ₃ A	< 0°C	10.0°C	—	< 0°C
β -d[Gp(Ap) ₄ G]	< 0°C	10.0°C	11.5°C	< 0°C
β -d[Gp(Ap) ₁₂ G]	< 0°C	21.0°C	24.3°C	10.0°C

* [A+T] concentration is 24 μ M.

** [A+T] concentration is 60 μ M.

HOPC: Trimethyl-7,10,12-6H-[1,3] oxazolo[5,4-c] pyrido[3,4-g] carbazole, an ellipticine derivative was synthesised as previously described (25) and purified by reverse phase HPLC. Concentrations were determined by absorbance at 326 nm ($\epsilon_{326} = 6440 \pm 60 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

β -oligodeoxynucleotides were synthesized on an Applied Biosystems Model 380 DNA synthesizer and purified by reverse phase HPLC. Concentrations of β -d[(Ap)_{n-1}A] and β -d[(Tp)_{n-1}T] were measured by UV absorbance using the molar extinction coefficients published by Cassini et al (26).

α -oligodeoxynucleotides were synthesised as previously described (27). Extinction coefficients of α -oligodeoxynucleotides were determined after digestion by 3'-exonuclease

Figure 3. Mixing curve (continuous variation method) corresponding to the interaction of β -T₄C₅OPC with β -d[Gp(Ap)₁₂G] in 10 mM sodium cacodylate pH 7 and 0.1 M NaCl at a temperature of 4°C. Total strand concentration [A+T] = $9.6 \cdot 10^{-4}$ M

A—Fluorescence ($\lambda_{exc} = 330 \text{ nm}$, $\lambda_{em} = 520 \text{ nm}$)

B—Absorbance at 257 nm (maximum absorbance wavelength of β -d[Gp(Ap)₁₂G]). The same curve is obtained at other wavelengths, particularly at the maximum absorbance wavelength of the chromophore (310 nm).

Figure 4. Derivatives of the melting curves. r is the ratio of the bound OPC concentration over the nucleotide concentration.

Figure 5. Changes in the absorption spectra of HOPC with increasing concentrations of double-stranded DNAs in 10 mM sodium cacodylate pH 7, 0.1 M NaCl, at a temperature of 20 $^{\circ}C$. The values represent the ratios of nucleotide concentrations over the HOPC concentrations. (For sake of comparison, the concentration of guanine residues is not taken into account when expressing β -d[Gp(Ap)_nG] concentrations).

A—DNA is α -d[(Tp)₁₁T]: β -d[Gp(Ap)₁₂G].

B—DNA is β -d[(Tp)₁₁T]: β -d[Gp(Ap)₁₂G]. (The initial concentration of HOPC is 5 μ M).

C—DNA is calf thymus DNA. (The initial concentration of HOPC is 10 μ M).

from *Crotalus Durissus* (Boehringer). Total degradation was checked by 5'-end labelling and migration on 20% polyacrylamide sequencing gels.

General methods

UV experiments were carried out on a Uvikon 810 (Kontron) spectrophotometer. Melting curves were obtained using the same spectrophotometer interfaced with an IBM PC compatible microcomputer. The temperature control was through a Huber PD415 temperature programmer connected to a refrigerated water bath (Huber Ministat). Cuvettes were 1 cm pathlength quartz cells and nitrogen was continuously circulated through the cuvette compartment.

Fluorescence experiments were carried out on a SFM25 (Kontron) spectrofluorimeter. At low temperature, nitrogen was continuously circulated through the cuvette compartment. All the experiments reported therein were performed in cacodylate buffer 0.01M, pH 7; NaCl 0.1M. Temperatures were as indicated in the text.

Binding parameters determination were performed according to previously published method (28), using excitation and emission wavelength set up at 330 nm and 520 nm respectively.

RESULTS

α -T₄C₅OPC and β -T₄C₅OPC annealing with β -d[Gp(Ap)_nG]

On figure 2 are shown the UV spectra of α -T₄C₅OPC and β -T₄C₅OPC in the presence of increasing concentrations of the complementary β -d[Gp(Ap)₄G] and β -d[Gp(Ap)₁₂G] sequences. The broad band with a $\lambda_{\max}$ at 270 nm is mostly due to the absorbance of the tetrathymidylate moiety. The peaks located at 317 and 310 nm correspond to the maximum absorbance wavelength of the α - or β -T₄C₅OPC monomer and dimer respectively. Indeed, self-association is a common feature of ellipticine derivatives (29,30). In previous reports, we showed that a bulky substituent such as a tetrathymidylate does not prevent stacking interactions and that the self-association constant is nearly the same for the two anomeric forms (31). As far as β -T₄C₅OPC is concerned, 2D-NMR data suggested (32) that the chromophore is folded on the nearest thymine at low concentration. At high concentration, however, two OPC rings are self-associated head-to-tail leading to a stacked dimer of β -T₄C₅OPC.

The figure 2A shows that the addition of β -d[Gp(Ap)₄G] to α -T₄C₅OPC in cacodylate buffer at 2°C leads to hypochromic effect due to the interaction of the modified oligonucleotide with the short complementary hexanucleotide. This effect is observed at the wavelengths corresponding to both the oligonucleotide moiety (270 nm) and the intercalating agent (310 and 317 nm). Since the band which corresponds to the self associated form (310 nm) disappears first, we conclude that annealing of the modified oligonucleotide disrupts its self-association. The plot of the relative absorbance as a function of the A/T ratio (inset of figure 2A) leads to a titration curve with a saturation obtained for a ratio A/T = 1 whatever the wavelength studied. This indicates that the four thymines of the α -T₄C₅OPC are annealed with the four adenine residues of the β -d[Gp(Ap)₄G]. Moreover, the melting temperature of the duplex formed is equal to 10°C. In the same experimental conditions, only weak hypochromic effect is observed when the unsubstituted α -d[(Tp)₃Tp] is added to β -d[Gp(Ap)₄G], and the melting temperature of this complex is lower than 0°C. We conclude from these observations that the OPC covalently linked to an α -tetrathymidylate induces an increase in stability. On the other hand, the addition of β -d[Gp(Ap)₄G] to β -T₄C₅OPC in the same conditions leads to a slight variation of the

Figure 6. A—Fluorescence excitation spectra of free (solid line) and bound HOPC (dashed line): $r = 0.17$. The DNA is α -d[Gp(Ap)₁₂G]: β -d[(Tp)₁₁T].

B—Scatchard Plot corresponding to the binding of HOPC to d[β]:d[β] (●) and d[α]:d[β] (▲) duplexes (2.4×10^{-5} M in nucleotides) in 10 mM sodium cacodylate pH 7, 0.1 M NaCl at 20°C. Small volumes of a 2×10^{-5} M HOPC in the same buffer were added and rapidly mixed to obtain the various binding ratios.

spectrum (figure 2C) and the melting temperature of the complex is lower than 0°C thus preventing the calculation of the stoichiometry of the association. Figures 2B and 2D show the changes in the absorbance spectra when β -d[Gp(Ap)₁₂G] is added to α -T₄C₅OPC and β -T₄C₅OPC respectively, in [A+T] concentrations which are the same as those used in the experiments with β -d[Gp(Ap)₄G]. In both cases, the hypochromic effect is greater and the melting temperature is increased (results are summarized in table I). It appears that the annealing of α - or β -T₄C₅OPC with their complementary sequence is cooperative. Furthermore, with α -T₄C₅OPC, the duplex is stable at 2°C and the stoichiometry can be derived from the titration curve. The saturation is obtained for A/T = 1 whatever the wavelength is, thus indicating that the three sites theoretically available on β -d[Gp(Ap)₁₂G] are actually occupied. In order to appreciate more accurately the stoichiometry with β -T₄C₅OPC, we performed a continuous variation experiment. Figure 3 shows the plot obtained when following the absorbance at 257 nm and the fluorescence of the OPC moiety. A transition is observed for T/A+T = 2/3 which is in favor of triple-stranded helix formation.

We conclude, from this set of experiments that the complex formed between β -T₄C₅OPC and its complementary sequence is less stable than the one formed, in the same conditions between α -T₄C₅OPC and the same complementary sequence. If we assume that the linker does not play any role in the binding process, the free energy of binding of the modified oligonucleotide (ONBI for OligoNucleotide Bridge Intercalator) may be expressed as:

$$\Delta G_{\text{ONBI}} = \Delta G_{\text{ON}} + \Delta G_{\text{I}} - T\Delta S_{\text{m}}$$

where ΔS_{m} is an entropy positive term which takes into account the restricted configurational space available to the intercalating agent when it is covalently linked to the oligonucleotide.

Therefore the better gain of stability observed for α -T₄C₅OPC as compared to β -T₄C₅OPC can be due to a difference in the stabilities of the α -d[(Tp)_{n-1}T]: β -d[(Ap)_{n-1}A] and β -d[(Tp)_{n-1}T]: β -d[(Ap)_{n-1}A] duplexes or alternatively, to a differential affinity of OPC for these duplexes or to both effects.

Gain in thermal stabilities induced by HOPC on α : β and β : β duplexes

In order to check the role of the intercalator in the stability of the α - or β -T₄C₅OPC with their complementary sequence, we studied the binding of HOPC to α -d[(Tp)_nT]: β -d[Gp(Ap)_nG] and to β -d[(Tp)_nT]: β -d[Gp(Ap)_nG] (these duplexes will be referred to as d[α]:d[β] and d[β]:d[β] respectively). A length of 12 residues was chosen with respect to three considerations:

(i) the duplex must be long enough to be entirely annealed at the temperature used for the binding experiments.

(ii) it must be short enough to avoid cooperative effects met with long homopolymers and which could not be representative of what is observed with α or β -d[(Tp)₃T]:d[Gp(Ap)₄G].

(iii) n should be a multiple of 4 to allow the comparison with α or β -T₄C₅OPC in the same experimental conditions.

Before considering the interaction of HOPC with these duplexes, we first studied the annealing of d[α]:d[β] and d[β]:d[β] respectively. The continuous variation plots obtained (data not shown) using either UV spectroscopy or fluorescence of ethidium bromide show that, for both complexes, we obtain a stoichiometry 1A/1T. Therefore in our experimental conditions of concentration and ionic strength, the annealed oligonucleotides are double-stranded.

We then measured the melting temperature of the d[α]:d[β] and d[β]:d[β] duplexes in the presence and in the absence of HOPC. the derivatives of the melting plots are shown in figure 4. It appears that the thermal stability is little dependent on the anomeric configuration of the thymines since T_m = 28°C for d[α]:d[β] and T_m = 32°C for d[β]:d[β] at a strand concentration of 5 μ M. However the thermal stability enhancement induced by the binding of HOPC is more important when the duplex is d[α]:d[β] than d[β]:d[β]. This result is consistent with the better gain in stability observed with α -T₄C₅OPC compared to β -T₄C₅OPC. According to the theoretical treatment of Crothers (33), at saturation of bound ligand, the ligand induced shift in the T_m can be derived from:

$$1/T_{m0} - 1/T_m = (R \cdot n / \Delta H) \cdot \ln(1 + K \cdot a_l)$$

where T_{m0} and T_m are the Kelvin temperatures corresponding to the midpoints of the transition in the absence and in the presence of drug respectively. ΔH is the enthalpy change corresponding to the melting of a base pair; K and n are the binding constant and the number of sites for the ligand, and a_l is the activity of the ligand (33).

As it can be seen from the differentiated melting curves corresponding to the duplexes without any ligand, the width of the curve at the half-height is larger for d[α]:d[β] duplex than for the corresponding d[β]:d[β] duplex. Since this width is inversely proportional to the van't Hoff transition enthalpy, we can conclude that the enthalpy of the transition is lower for the d[α]:d[β] duplex than for the d[β]:d[β] duplex (34). However the difference in enthalpy is not sufficient to explain the observed differential thermal stabilisation induced by the ligand, and a difference in the respective bindings of HOPC to d[α]:d[β] and d[β]:d[β] should be expected.

Table 2. Binding parameters of HOPC to d[α]:d[β] and d[β]:d[β]. Experimental conditions are 0.01 M cacodylate buffer, pH 7 and 0.1 M NaCl. Temperature is 20°C. V represents the ratio between the bound OPC fluorescence and the free OPC fluorescence.

Duplex	d[α]:d[β]	d[β]:d[β]
Maximum absorbance wavelength	323 nm	324 nm
Maximum excitation wavelength (em:520 nm)	318 nm	317 nm
V	30	28
n	0.25	0.16
K $\times 10^{-5}$ (M $^{-1}$)	1.40	1.25

Binding of HOPC to d[α]:d[β] and d[β]:d[β] duplexes

UV spectra of HOPC mixed with increasing concentrations of d[α]:d[β] or d[β]:d[β] duplexes are shown on figure 5. As pointed out, ellipticine derivatives are able to self-associate. The monomeric and the dimeric forms exhibit absorption spectra whose wavelength of the maxima are 312 and 304 nm respectively. The band which corresponds to the dimeric form of HOPC disappears first indicating that the binding of HOPC to the duplex disrupts its self-association. The binding of HOPC to DNA induces a bathochromic effect leading to a maximum in UV absorbance at 324 nm for bound HOPC (25, 35). When HOPC is bound to d[α]:d[β] and d[β]:d[β], the maxima of UV absorbance correspond to 323 nm and 324 nm respectively. On figure 6A, we show the fluorescence spectra of HOPC bound to d[α]:d[β] duplex. The changes in excitation spectra are the same whatever the DNA studied. Scatchard plots corresponding to the binding of HOPC to d[α]:d[β] and d[β]:d[β] are shown on figure 6B and the results are summarized in table 2. It appears that the affinity of HOPC for d[α]:d[β] is about the same as for d[β]:d[β]. However the number of sites differs, more sites being available on the d[α]:d[β] helix than on the d[β]:d[β]. We can then assume that HOPC is more efficient in stabilizing d[α]:d[β] helix than d[β]:d[β] helix.

DISCUSSION

In the comparison of ONBI molecules made from unnatural analogs of nucleotides such as α -deoxyribonucleotides linked to an intercalator with molecules built from natural nucleotides, two parameters should be taken into account: the intrinsic stability of the new type of duplex formed (in our case α - β compared to β - β) and a difference in the binding of the intercalator to the helix. In a previous work (17), it was reported that α -T₄C₅OPC interacts more strongly with poly (rA) than β -T₄C₅OPC and this difference could be explained by a stability of the duplex α -oligo d(T):poly (rA) higher than the one corresponding to the duplex β -oligo d(T):poly(rA) (36). Thus in this particular case, the free energy of duplex formation is the major parameter responsible for the observed difference in stability. Here, we report the influence of the intercalator (HOPC), when the complementary sequence is a short oligodeoxy-adenylate. In this latter case, the difference in the free energy of formation of the α : β duplex compared to the β : β duplex ($\Delta T_m > 10^\circ\text{C}$) cannot explain the observed difference between the stabilities of α -

T₄c₅OPC:oligo d(A) and β -T₄c₅OPC:oligo d(A) duplexes since in this case the free energies of formation of the duplexes are about the same ($T_m = 28^\circ\text{C}$ for d[α]:d[β] and 32°C for d[β]:d[β] (37)).

In this case, the binding of the intercalator should be the main factor influencing the stability of the duplex. If HOPC presents the same affinity for d[α]:d[β] duplex than for d[β]:d[β] duplex, however the number of binding sites is different and in favor of the d[α]:d[β] duplex. Moreover, examination of the differential temperature plots of the duplexes indicates that the van't Hoff transition enthalpy of the duplex is lower for d[α]-d[β] than for d[β]:d[β]. This parameter, together with the number of binding sites could explain the higher stability of the α -T₄c₅OPC: β -oligo d(A) duplex when compared to β -T₄c₅OPC: β -oligo d(A). The set of experiments presented here shows that a major factor responsible for the thermodynamic parameters of the hybrid formed between α -ONBI and natural oligonucleotides is the interaction of the intercalating agent (number of binding sites, binding constant) with this particular duplex. This property should be taken into account when designing α -ONBI.

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