Kinetics and thermodynamics of i-DNA formation: phosphodiester versus modified oligodeoxynucleotides

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ABSTRACT

At slightly acidic or even neutral pH, oligodeoxynucleotides that include a stretch of cytidines have been shown to form a tetrameric structure in which two parallel-stranded duplexes have their hemiprotonated C.C$^+$ base pairs face to face and fully intercalated, in a so-called i-motif. Cytosine-rich pyrimidine oligodeoxynucleotides can form an intramolecular i-motif. We have studied the ability of several DNA analogs to fold into this structure. Evidence for folding was provided by thermal denaturation. We have shown that phosphorothioate and phosphodiester oligodeoxynucleotides, but not methylphosphonate or PNA oligomers, may form the i-motif. Four different PS oligodeoxynucleotides were compared with their PO counterparts. In all cases, the melting temperature ($T_m$) of the phosphorothioate oligomer was equal or slightly inferior (by 2–3°C) to the $T_m$ of the natural oligodeoxynucleotide. For long oligodeoxynucleotides, a small change of pH leads to a completely different melting profile: the curves are reversible at pH 6.4 or lower, and a hysteresis is obtained at pH 6.8 or higher; cooling and heating curves were not superimposed, allowing us to determine the rate constants of association ($k_{on}$) and dissociation ($k_{off}$) as a function of the temperature: these rate constants give linear Arrhenius plots, in agreement with the prediction of the two-state model of association–dissociation. The activation energy $E_A$ is strongly negative and, at neutral pH, the phosphorothioate associates and dissociates nine times faster than the phosphodiester oligodeoxynucleotide of identical sequence.

INTRODUCTION

It is well known that cytosine-rich oligodeoxynucleotides or polynucleotides may adopt a non-B structure at acidic pH. The structure and stoichiometry of the complex formed by the hexamer d(TCCCCC) has been described recently (1). i-Motif formation has been demonstrated so far by NMR and crystallography for a large number of sequences (1–9). The i-motif has a specific circular dichroism (10) and Raman (11) signature. This so-called i-motif is a tetramer of equivalent strands, which presents the novel feature of intercalated C.C$^+$ base pairs of two parallel-stranded duplexes. Individual parallel-stranded duplexes are right-handed and underwound, and the two duplexes are ‘zipped together’ in an antiparallel fashion. The molecule is flat with two very wide grooves and two very narrow grooves. The molecules twist in a right-handed manner, and the distance between the nearest base pairs is only 3.1 Å. A strand carrying four copies of the cytosine-rich telomeric repeat may form an intramolecular i-motif (3,10,12). Other biologically relevant sequences may also form this motif (13,14).

Oligodeoxynucleotides have been used widely in the antisense or triple helix strategy. The i-motif could interfere with these strategies, by trapping the oligodeoxynucleotide in a stable folded conformation (15). Phosphorothioate oligodeoxynucleotides, in which one of the non-bridging oxygen atoms in each internucleotide phosphate linkage is replaced by a sulfur atom, are commonly used as antisense agents. However, oligodeoxycytidine phosphorothioates [d(C$_n$); $n$ ≥ 20] exhibit virus inhibition activity by a non-antisense mechanism (16,17). Little is known about the ability of cytosine-rich oligophosphorothioates to form the i-motif and potential competition with triplex formation. In this study, we have determined the stability of the folded form(s) of different single-stranded oligodeoxynucleotides, with a phosphodiester or phosphorothioate backbone, 16–29 bases in length. The formation of an intermolecular i-motif with short phosphorothioate cytosine oligomers was recently shown by CD spectroscopy and UV absorption (18). We thus wanted to measure the stability of an intramolecularFS–i-motif, and evaluate the formation of this structure with other oligodeoxynucleotide analogs.

MATERIALS AND METHODS

Oligodeoxynucleotides

All oligodeoxynucleotides (phosphodiester, phosphorothioate and methylphosphonate) were synthesized by Eurogentec (Belgium) on the 0.2 µmol scale, and treated as described previously (19). The PNA 16mer was synthesized by PerspectiveBiosystems. All concentrations were expressed in strand molarity, using the absorption coefficients given by Cantor and Warshaw (20) for the unfolded species.

UV absorption spectrophotometry

Absorbance versus temperature heating and cooling curves were obtained using a KONTRON-UVIKON 940 spectrophotometer, as described (19). K, the equilibrium association constant, can be written as $K = k_{on}/k_{off} = \theta/(1 – \theta)$ for an intramolecular
phenomenon. $\theta$ is the fraction of the oligomer engaged in i-motif formation.

**Analysis of an hysteresis**

A prerequisite for the recovery of thermodynamic, i.e. equilibrium, parameters from these curves is that they are true equilibrium curves. This implies that the sample must be heated or cooled at a rate which must be slow with respect to the rate of the association–dissociation reaction under study. A simple and useful criterion for this is the coincidence of the heating and cooling curves, whatever their rates of heating and cooling. This is not always the case, and we have observed in many cases that, at near neutral pH, the thermal dissociation (heating) curves of the i-motif are largely shifted towards higher temperatures as compared with the association (cooling) curves (3,19). Such behavior, which is the result of slow association and dissociation kinetics, has already been described for triple helix formation (21). This hysteresis was concentration independent, indicating a slow intramolecular process. Quantitative analysis of the kinetic parameters of i-motif formation was performed based on the results of Rougé et al. (21), with little modifications (the equilibrium is, in our case, intramolecular).

**Gel filtration**

The size, and hence stoichiometry of the oligodeoxynucleotides was determined by high pressure gel filtration chromatography performed at room temperature with Beckman equipment. The column was a Synchropack GPC 100 (250 x 4.6 mm I.D.) from Interchim, calibrated with oligodeoxynucleotides as described previously (19).

**Nuclear magnetic resonance (NMR)**

The 18(O) and 18(S) samples were prepared by lowering the pH of a 6.9 mM solution from neutrality to pH 6.4 with aliquots of HCl. The NMR experiments were performed on a 360 MHz home-built spectrometer as described previously (19).

**RESULTS**

**The 18(O) and 18 (S) oligodeoxynucleotides**

Dissociation of the i-motif leads to an hyperchromism at 265 nm (3,10,19). An example of a denaturation/renaturation profile at pH 6.4, obtained for the 18(O) and 18(S) pyrimidine oligodeoxynucleotides d-CCTTTCCCTTTACCTTTCC is presented in Figure 1. A denaturation profile is characterized by a sharp increase of absorbance at 265 nm (Fig. 1A), whereas a hypochromic transition is observed at 295 (Fig. 1B) (19) or 305 nm (18). Both curves could well be analysed as an all-or-none intramolecular phenomenon. Thermodynamic parameters determined from the profiles at 265 and 295 nm were identical within experimental error. The difference between the absorbance spectra of cytosine and protonated cytosine explains the inverted denaturation curves obtained at 295 nm (Fig. 1B). The shorter wavelength (265 nm, close to the isosbestic point of the pH titration) gives information which is independent of the protonation state of the cytosines. The longer wavelength (295 nm) should tell us whether the formation of a structure requires protonation/deprotonation of some cytosines: at this wavelength, the difference between protonated and non-protonated cytosine is maximum, as inferred from absorbance spectra at different pH values (19). The comparison of the 18(O) and 18(S) oligodeoxynucleotides showed almost no difference in melting temperature $T_m = 22\degree$ C for 18(S) and 23\degree C for 18(O). Nevertheless, the shape of the melting profiles is strikingly different [the melting of the 18(S) oligodeoxynucleotide seems 'less cooperative']. In both cases, the $T_m$ was concentration independent, showing that the folding of both oligodeoxynucleotides was intramolecular (not shown). From the melting profiles, one could determine the fraction ($\theta$) of oligodeoxynucleotide engaged in i-motif formation. Thus, the equilibrium association constant $K$ of the i-motif, can be plotted against 1/T. As shown in Figure 1C, an excellent linear fit can be derived from the experimental points ($r > 0.997$), showing that the data is in excellent agreement with an all-or-none simple intramolecular equilibrium. However, one cannot fail to notice that the slopes of the curves are different, thus leading to different $\Delta H^\circ$ values [$\Delta H^\circ = -60$ kcal/mol for the 18(O) oligodeoxynucleotide and $\Delta H^\circ = -48$ kcal/mol for the 18(S) oligodeoxynucleotide]. Thus, i-motif formation with a phosphorothioate oligodeoxynucleotide is less enthalpy driven ($\Delta H^\circ$ is less negative) than for unmodified DNA. On the other hand, there is a partial entropy compensation for the 18(S) oligodeoxynucleotide [$\Delta S = -160$ cal/mol/K for the 18(S) and $\Delta S = -205$ cal/mol/K for the 18(O)], leading to melting temperatures in the same temperature range.

To analyze the folded forms of the phosphodiester and phosphorothioate oligomers, the 18(S) and 18(O) oligodeoxynucleotides were both studied by 1D NMR under identical experimental conditions (pH 6.4; $T = 5\degree$ C; 6.9 mM strand concentration). Preliminary 1D NMR spectra of the 18(S) oligodeoxynucleotide were strikingly similar to the spectra of the previously published 18(O) oligodeoxynucleotide (22), but with much broader peaks, probably as a result of the complex overlap of the proton signals of the P-chiral diastereoisomers (not shown). The quasi-perfect superimposition of the C.C$^+$ N3H and thymine N3H peak positions strongly supported that the folded form of the 18(S) oligodeoxynucleotide was very similar to the well-known i-motif formed by the 18(O) oligodeoxynucleotide. Four C.C$^+$ base pairs were formed in each case. The i-motif offers the same energetic advantages for phosphorothioate and phosphodiester oligodeoxynucleotides: better stacking interactions, extensive sugar van der Waals’ contacts and systematic intermolecular C-H...O hydrogen bonding network between the deoxyribose sugar moieties (23,24). Molecular dynamics studies suggest that the central core of an i-DNA molecule is exceptionally stable (25). These results, obtained on long phosphorothioate oligodeoxynucleotides are in excellent agreement with the recent work on short phosphorothioate oligomers (18).

**DNA backbone modifications and stability of the i-motif**

The 16 (O), 16(S), 16(M) and 16 (P) oligomers. Intramolecular formation of the i-motif was shown previously for the 16(O) DNA oligodeoxynucleotide (d-TCCCTCTTTTACCTTT) (19). We synthesized DNA analogs having the same primary sequence, with a different backbone. These oligomers were tested for their ability to form an i-DNA motif by UV-spectroscopy. As shown in Figure 2, only the 16(O) and 16(S) oligodeoxynucleotides showed a cooperative melting behavior at pH 5.6. In this case, the $T_m$ values for the two oligodeoxynucleotides were identical within experimental error ($T_m = 36\degree$ C). These two oligodeoxynucleotides were...
Figure 1. Denaturation profiles obtained in a 10 mM sodium cacodylate pH 6.4 buffer for the 18(O) (open circles) and 18(S) (black triangles) d(CC TT TA CC TT) oligodeoxynucleotides (6 µM strand concentration). The cooling and heating profiles were superimposable at this pH. (A) 265 nm, (B) 295 nm. (C) Thermodynamic analysis of the denaturation profile: Van’t Hoff plot of ln(K) versus 1/T [18(O): open circles; 18(S): black triangles]. K is the equilibrium association constant. The fraction of oligodeoxynucleotide engaged in i-motif formation was determined from the profiles at 295 nm.

Also exhibited similar if not identical, melting temperatures at pH 6.4, 6.8 and 7.2 (Table 1). No variation of absorbance at 295 nm (as well as at 265 nm, not shown) was obtained with the 16(M) and 16(P) oligomers. Lowering or increasing the pH (in the range pH 4.5–7.5) did not lead to a melting process for the 16(P) and 16(M) oligomers. At pH 6 or higher, 16(P) and 16(M) exhibited an absorption spectrum characteristic of unprotonated cytosines, in agreement with an unfolded state (not shown).

The 20(O) and 20(S) oligomers. A quick overview of the literature on antisense revealed that several of the studied oligodeoxynucleotides are potentially able to form the i-motif. For example, several antisense oligodeoxynucleotides directed against HIV complementary to gp120 mRNA, such as ISIS 3466, have a sequence compatible with i-motif formation: d(CA CC CC CC TTGG CC CCC AC). Such an oligodeoxynucleotide was synthesized in the phosphodiester [20(O)] and phosphorothioate series [20(S)]. Both oligodeoxynucleotides showed a melting transition around 50°C at pH 5.6. This melting profile was concentration independent but pH dependent (Tm = 33°C at pH 6.4 and 17°C at pH 7.2; Table 1). The primary sequence of the oligomer, which contains four stretches of two or three cytosines, allows intramolecular i-motif formation, and the melting behavior of the oligomer is in good agreement with the formation of this structure.

The 29(O) and 29(S) oligomers. Intramolecular formation of the i-motif was shown recently for the 29(O) DNA oligodeoxynucleotide d(CC CCTT TCCCCC TTTTTTTCCC CCC CCC) (19). The stability of this i-motif oligodeoxynucleotide was extremely high (Tm = 60°C at pH 6.0) and i-motif formation was still observed at neutral pH. We synthesized its phosphorothioate equivalent which has the same primary sequence [29(S)]. These oligomers were tested for their ability to form an i-DNA. At pH 6.4, the 29(O) and 29(S) oligodeoxynucleotides melt in the same temperature range (Fig. 3A, Tm = 52°C). It should be noted that the melting behavior of the 29(S) oligodeoxynucleotide was completely reversible, whereas a slight but reproducible shift between the heating and cooling curves for the 29(O) oligodeoxynucleotide was observed. At pH 6.0 or lower, both oligodeoxynucleotides gave completely reversible denaturation profiles (not shown). In both cases, the melting temperatures were concentration independent, showing that the structure was unimolecular. This experiment was confirmed by gel filtration studies, which showed that at acidic pH (4.7), both 29(S) and 29(O) oligodeoxynucleotides eluted as a single peak, with a retention time in agreement with an intramolecular form (not shown).
Table 1. List of the oligodeoxynucleotides

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Type</th>
<th>Tm pH 6.4</th>
<th>pH 6.8</th>
<th>pH 7.2</th>
<th>ΔH° kcal/mol</th>
<th>ΔS° cal/mol/K</th>
</tr>
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<tbody>
<tr>
<td>TCCTCCTTTTTCCTCTT</td>
<td>16(O)</td>
<td>24</td>
<td>16</td>
<td>9</td>
<td>−50</td>
<td>−169</td>
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<tr>
<td>16(S)</td>
<td></td>
<td>23</td>
<td>16</td>
<td>7</td>
<td>−35</td>
<td>−118</td>
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<tr>
<td>CTTTTTCTTAATTTTTC</td>
<td>18(O)</td>
<td>23</td>
<td>18</td>
<td>10</td>
<td>−60</td>
<td>−205</td>
</tr>
<tr>
<td>18(S)</td>
<td></td>
<td>22</td>
<td>17</td>
<td>9</td>
<td>−48</td>
<td>−160</td>
</tr>
<tr>
<td>CACCCGCTTGCGCTCCAC</td>
<td>20(O)</td>
<td>33</td>
<td>26</td>
<td>17</td>
<td>−45</td>
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<td>20(S)</td>
<td></td>
<td>32</td>
<td>25</td>
<td>15</td>
<td>−37</td>
<td>−120</td>
</tr>
<tr>
<td>CCCCCTTTCCTTCCCCCTTCTTCCCCC</td>
<td>29(O)</td>
<td>52</td>
<td>40</td>
<td>28</td>
<td>−110</td>
<td>−338</td>
</tr>
<tr>
<td>29(S)</td>
<td></td>
<td>52</td>
<td>38</td>
<td>28</td>
<td>−80</td>
<td>−246</td>
</tr>
</tbody>
</table>

Tm values of oligodeoxynucleotides presented in this study. Tm values are given at ±1°C at pH 6.4 and 6.8 and ±2°C at pH 7.2. ΔH° values at ±4 kcal/mol; ΔS° values at ±10 cal/mol/K. (Average of two to three measurements). Folding was shown to be intramolecular from concentration-independent denaturation profiles (in the range 0.5–15 μM). All values were calculated from the denaturation profiles at 295 nm. Identical results were obtained from the denaturation curves recorded at 265 nm. We adopted the following convention for all the oligodeoxynucleotides: the number indicates the length of the oligodeoxynucleotide, and the letter allows differentiation between phosphodiester DNA (O) and phosphorothioate (S) oligomers. No transition was obtained with the methyl phosphonate 16(M) and PNA 16(P) oligomers. ΔH° and ΔS° values were determined at pH 6.4 for all other oligodeoxynucleotides.

 Effect of pH increase: analysis of non reversible curves

As shown in Figure 3, the thermal stability of the 29mer structure was pH dependent. The melting temperature was maximum at a pH value close to the pKa of cytosine (4.8 in a low-salt buffer) (19). In the pH range 5.2–7.2, the Tm of the structure was a linear function of pH, as observed previously for a telomere oligodeoxynucleotide. An increase in pH of one unit led to a large decrease (>20°C) of the melting temperature. Such pH dependence was observed previously for cytosine-rich polynucleotides and polydeoxyribonucleotides, as well as for cytosine-rich oligodeoxynucleotides (3).

The pH value also had an impact on the kinetics of intramolecular folding. At neutral pH (6.8–7.2), the heating and cooling profiles for the 29(O) and 29(S) oligodeoxynucleotides were not superimposed. Successive heating and cooling cycles gave identical results (all heating curves were superimposed, all cooling curves were superimposed, but the latter were shifted towards lower temperatures as compared with the former). Therefore, a chemically reversible reaction such as the folding/unfolding of an i-DNA may be thermodynamically irreversible (as shown by a hysteresis). Confusion could therefore arise from the ‘reversible’ or ‘irreversible’ terms. Such a reproducible phenomenon was reported previously for the folding of the C-rich strand of human telomeres, and was indicative of slow kinetics of folding at neutral pH (3).

To test whether this hysteresis was not artefactual, we performed the same experiment at two strand concentrations, using two different heating and cooling temperatures for the 29(O) oligodeoxynucleotide (0.05 and 0.25°C/min). The hysteresis was concentration independent, in agreement with an expected intramolecular process, but it was dependent upon the rate of heating and cooling: the faster the experiment, the greater the separation between the heating and cooling curves. Identical results were obtained for the 29(S) oligodeoxynucleotide (not shown).

The values of kon and koff determined from the hysteresis cycles at pH 7.2, such as those presented in Figure 3C, were plotted in the form of Arrhenius plots ln(kon) and ln(koff) versus 1/T in Figure 4, as described previously (21). In the temperature range where these values were obtained with some confidence, straight lines are obtained with a positive slope for kon and a negative one for koff. One may notice that less experimental points are given for the 29(S) oligomer, as the hysteresis is smaller: the reaction is completed over a narrower temperature range. The intersections of the straight lines give the concentration-independent Tm values, as the process is intramolecular (at Tm, θ = 1/2, kon = koff).

In both cases [29(O) and 29(S)], the Tm was determined to be 28°C. The activation energy Eon for the association is negative [Eon = −43 kcal/mol for 29(O) and Eon = −43.5 kcal/mol for 29(S)]. The slopes of the activation energy curves for the 29(O) and 29(S) oligomers are extremely positive, showing that i-motif formation is strongly accelerated by a modest decrease of temperature. The Eoff values are identical within experimental error, showing that the same temperature dependence of kon is obtained with the 29(S) and 29(O) oligodeoxynucleotides (the two line fits are almost parallel). The fact that the kon curve for the 29(S) oligodeoxynucleotide is shifted towards higher values as compared with 29(O) illustrates the fact that the association of the 29(S) oligodeoxynucleotide is nine times faster than the association of the 29(O) oligomer. Concerning the activation energy Eoff for the dissociation, both calculated values are positive [Eoff = +67 kcal/mol for 29(O) and Eoff = +33 kcal/mol for 29(S)] but significantly different. The standard molar enthalpy change for the formation of the i-motif is simply ΔH° = Eon − Eoff, thus, ΔH° = −110 kcal/mol for the 29(O) oligomer, and −76.5 kcal/mol for the 29(S) oligomer. Therefore, a modest decrease of temperature stabilizes the i-motif for two additive reasons: kon would be increased whereas koff would be decreased.

The Arrhenius plot of Figure 4 gives important clues to the folding of an i-motif: the negative values we have obtained for Eon are a strong argument in favor of the nucleation-zipping
Figure 3. Denaturation profiles of 29(O) (open circles) and 29(S) (black triangles) recorded at 265 nm [sequence: d(CCCCCCTTT)3CCCCC] in a 10 mM cacodylate buffer, at three different pH values: (A) pH 6.4; (B) pH 6.8; (C) pH 7.2. The profile at pH 6.4 was nearly reversible for the 29(S) oligodeoxynucleotide, as shown by the quasi-perfect superimposition of the heating and cooling profiles. A slight hysteresis was observed for the 29(O) oligomer in the same conditions. At pH 6.8 or higher, the heating profile was shifted toward higher temperatures (the direction of temperature variation is indicated by arrows). Oligodeoxynucleotide strand concentration is 3.5 µM.

model. The ‘V’ shape of the Arrhenius plot confirms that the absolute value of $E_{on}$ is in the same order as $E_{off}$. In the case of the 29(S) oligomer, $E_{on}$ is even larger than $E_{off}$. One has thus to imagine that the nucleation involves at least one-third to one-half of the total base pairs. Figure 5 presents the reconstitution of the melting curves for the 29(O) (Fig. 5A) and 29(S) (Fig. 5B), with the parameters calculated from the Arrhenius plot. A good agreement is reached between the calculated and experimental points, showing that the model accurately describes the melting of both oligodeoxynucleotides. Finally, Figure 5C presents the calculated equilibrium curves, which would have been experimentally recorded with a infinitely slow heating and cooling rate. The $T_m$s for the 29(O) and 29(S) oligodeoxynucleotides were identical ($T_m = 28^\circ$C), and the different shapes of the curves again reflect the difference of $\Delta H^\circ [\Delta H^\circ = -110 \text{ kcal/mol and } -76.5 \text{ kcal/mol for the 29(O) and 29(S) oligomers at pH 7.2, respectively}].$ As previously measured for phosphodiester (O) oligodeoxynucleotides, the formation of each C.C+ base pair leads to a decrease of the $\Delta H^\circ$ of $-9$ to $-12$ kcal/mol (19). Therefore, the $\Delta H^\circ$ value of $-110$ kcal/mol for the 29(O) oligodeoxynucleotide is in excellent agreement with this estimation, as the folding of this oligodeoxynucleotide should involve the formation of 10 C.C+ base pairs. A significantly lower value may be estimated from the analysis of the 18(S), 20(S) and 29(S) phosphorothioate oligodeoxynucleotides: in this case, each C.C+ base pair leads to a $-6$ to $-8 \text{ kcal/mol decrease in } \Delta H^\circ$.

**DISCUSSION**

In a previous study, we showed that many cytosine-rich oligodeoxynucleotides could adopt a folded conformation at slightly acidic or neutral pH, as shown by thermal denaturation and non denaturing gel electrophoresis experiments. Surprisingly, RNA oligoribonucleotides were unable to form this motif, as compared with DNA oligodeoxynucleotides of corresponding sequence (15,26,27). This observation led us to investigate several other routinely used DNA modifications. Uncharged DNA analogs, such as methylphosphonates or PNA oligomers, were unable to fold into an i-motif. In this case, the formation of hemiprotonated C.C+ base pairs, instead of partially neutralizing the negative charges of the DNA backbone, creates a net positive
Figure 5. Reconstruction of the melting profiles and comparison with the experimental points. From the $E_{\text{on}}$ and $E_{\text{off}}$ activation energies determined from the Arrhenius plot presented in Figure 4, one can reconstruct a theoretical denaturation or renaturation profile, using the Runge–Kutta algorithm. Theoretical points are presented with open symbols (circles, simulation of a heating curve; triangles, simulation of a cooling curve; line, theoretical equilibrium curve). Experimental points are given for comparison (black symbols). 

(A) 29(O) oligodeoxynucleotide; (B) 29(S) oligodeoxynucleotide. 

(C) Having determined $k_{\text{on}}$ and $k_{\text{off}}$ at each temperature, one can determine the equilibrium constant ($k_{\text{on}}k_{\text{off}}$) at each temperature, and thus the fraction of folded oligodeoxynucleotide at equilibrium as a function of T: circles, 29(O); triangles, 29(S). These equilibrium curves would only be experimentally reached using an ‘infinitely’ slow temperature gradient.

At neutral pH, the denaturation and renaturation profiles of several cytosine-rich oligodeoxynucleotides were not superimposable (3,19,28). An estimation of the $T_m$ of an oligodeoxynucleotide judging only from one heating or cooling experiment could lead to an important overestimation (for heating) or underestimation (for cooling) of the melting temperature. For the 29(O) oligodeoxynucleotide at pH 7.2, one would determine a $T_m$ of 38°C from the heating experiment, and 16°C from the cooling profile, a difference of 22°C as compared with the calculated equilibrium $T_m$ of 28°C. As shown by the Arrhenius plot, 29(S) does indeed associate and dissociate eight to 10 times faster than 29(O). As a result, the stability of the i-motif is nearly identical (both $k_{\text{off}}$ and $k_{\text{on}}$ are affected by the same amount). This result can qualitatively be deduced from the melting profiles in Figure 3B and C: the heating and cooling profiles of the 29(S) oligo are sandwiched between the profiles for the 29(O), in agreement with faster kinetics but with similar equilibrium affinity constants ($K$). An explanation of this behavior could be that the higher hydrophobicity of the 29(S) oligo facilitates the initial binding steps, but has a destabilizing (steric?) effect on the folded structure, therefore increasing both $k_{\text{on}}$ and $k_{\text{off}}$.

Insights into the mechanism of oligodeoxynucleotide association are provided by the characteristic features of their kinetics. For DNA duplex formation, the reaction begins with two or three bases pairing and unpairing in rapid but unfavorable equilibrium. Then, when the critical intermediate adds one more base pair, a helix nucleus is formed which zips up to form the fully bonded helix (29,30). In the case of the i-motif formed by the 29(O) oligomer, roughly half of the base pairs appear to be involved in the nucleation process. All kinetic data presented here were indeed obtained with long oligonucleotides, having four runs of five cytosines. We have performed a similar analysis with shorter PO oligonucleotides (not shown): d-CCCCTTTTCCCCTTTTCC-.
cytosines, were always reversible, even at neutral pH (with a very low $T_m$, generally $<10^\circ C$). It was thus impossible to extract kinetic data from the melting profile of the 17mer. Therefore, this model is valid at least for oligodeoxynucleotides containing runs of three cytosines or more.

Modified oligodeoxynucleotides are routinely used in the antisense strategy; therefore, the differential capacity for unexpected folding of these modified oligodeoxynucleotides might have an impact on the uptake, stability, cellular localization and effect of the molecule, and formation of a competing structure should be taken into account for triplex and antisense applications.

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