Circular dichroism studies of an oligodeoxyribonucleotide containing a hairpin loop made of a hexaethylene glycol chain: conformation and stability

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ABSTRACT

An oligodeoxyribonucleotide, d(GCTCACAAT-X-ATTGT-GAGC), where X represents a hexaethylene glycol chain, was studied using circular dichroism spectroscopy. Its conformation and conformational stability were compared to those of compounds where X was replaced by four thymines and to the duplex of same sequence without loop. The compound with the hexaethylene glycol chain can form a hairpin looped structure as well as a bulged duplex structure. In both cases the duplex region of the oligodeoxyribonucleotide exhibits the same conformation. In similar conditions the oligodeoxyribonucleotide with a four thymines loop forms exclusively a hairpin structure. Comparison between the thermodynamic parameters (ΔH,ΔS,ΔG) associated with the formation of the structure of the three compounds are presented. In the case of the compound with the hexaethylene glycol chain it is shown that the large increase in its melting temperature (by about 35°C in our experimental conditions) when compared to the non looped structure is mainly due to the fact that its melting process is intramolecular (monomolecular) whereas the other one is bimolecular.

INTRODUCTION

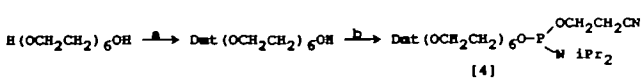
Inverted repeat sequences in DNA (palindromes) capable of forming hairpin structures as well as cruciform structures frequently occur in region known to have a peculiar function such as regulation and promotion sites (1−4). For example, it has been demonstrated that the single strand specific endonuclease S1 cleaves supercoil plasmid in the middle of a palindromic sequence, i.e in a possible loop (3). As a consequence, over the last few years a great number of studies on the formation and the stability of the oligodeoxyribonucleotides having the ability to fold back into hairpin structures have been carried out (5−24). From these studies, the general opinion is that the maximal stability displayed by hairpin structure is obtained when the loop portion of the molecule contains four to five nucleotide residues, and that the base composition of the loop region can also influence the hairpin stability. It is worth noting that it was recently shown that much smaller loops (e.g. two residues) can be remarkedly stable in oligodeoxyribonucleotide with a stem rich in GC base pairs (25−29). In this paper we report circular dichroism results relative to a palindromic sequence d(GCTCACAAT-X-ATTGTGAGC), where X represents a hexaethylene glycol chain, a quasi-palindromic sequence d(GCTCACAAT-T_4-ATTGTG-AGC) and a reference double-stranded sequence d(GCTCAC-AAT):d(ATTGTGAGC) (figure 1). The hexaethylene glycol chain was chosen because it allows a large mobility of the two oligodeoxyribonucleotide parts of the molecule, a potential loop, and because the presence of the oxygen atoms should allow a good solubility of the compound.

MATERIALS AND METHODS

Oligodeoxyribonucleotide synthesis

The synthesis of compounds [1a], [1b] and [3] (fig 1) was achieved in 2.7 μmol scale on a Pharmacia automatic synthesizer using the phosphoramidite chemistry (30).

Introduction of the hexaethylene glycol into the middle of the 18-mer[2] was carried out via the phosphoramidite [4]. This compound was obtained in a two-step process from hexaethylene glycol (scheme 1).



Scheme 1. Dmt: dimethoxytrityl, iPr: isopropyl.
a: dimethoxytri-tylchloride, pyridine.
b: 2-cyanoethyl N,N,N',N'-tetraisopropyl-phosphorodiamidite,tetrazole.

Phosphoramidite [4]: a solution of dimethoxytrityl chloride (0.6 g, 1.8 mmol), hexaethylene glycol (2.5 g, 9 mmol, dried by co-evaporation with dry pyridine) in pyridine (10 ml) was stirred at room temperature for 2h. 50 ml of CH_2Cl_2 was added and the organic phase was extracted with 5% of aqueous $NaHCO_3$ (2×25 ml) then with water. The organic phase was dried over Na_2SO_4, filtered then evaporated. The residue was

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chromatographied on a silicagel column (CH_2Cl_2-methanol). The fractions containing the hexaethylene glycol monodimethoxytritylether were combined and the solvent was evaporated. (Yield≈ 75%); TLC:silicagel 60F254, CH_2CL_2/MeOH (9:1, v/v), Rf≈ 0.45. The compound obtained above (O.7 g, 1.2 mmol) was dried by co-evaporation with dry pyridine and kept under high vacuum overnight then dissolved in CH_2Cl_2 (8ml) containing dimethylethylamine (0.44 g), 2-cyanoethyl-N,N- diisopropylamino-chloro-phosphite (0.7 g, 3 mmol) was added under nitrogen atmosphere with stirring. After 30 min the mixture was poured into 50 ml ethylacetate and extracted with 10% of aqueous $NaHCO_3$ (2×80 ml) then with 20 ml of saturated NaCl. The organic phase was dried over Na_2SO_4, filtered then evaporated. The compound was chromatographied on a silicagel column (ethylacetate-Et_3N). The fractions containing [4] were combined and the solvent was evaporated. (Yield≈ 80%); TLC: silicagel 60F254, ethylacetate/Et_3N (9:1, v/v), Rf≈ 0.75.

18-mer oligodeoxyribonucleotide [2]: assembly of the 18- mer[2] was carried out by the phosphoramidite route using deoxycytidine CPG (2.7 μmol), 23 μmol of phosphoramidite [4] and standard deoxyribonucleoside -3'-(2-cyanoethyl)-diisopropylamino-phosphoramidite per cycle. The yield of the coupling of the phosphoramidite [4] determined by dimethoxytrityl cation release was similar to that obtained with deoxyribonucleoside phosphoramidites (98−99%). After the assembly of the oligomer, the 'trityl on' support was treated with a solution of concentrated ammonia (10 h at 50°C). The 5'-dimethoxytrityl 18- mer[2] was purified by FPLC on a RPC HR5/5 column (Pharmacia) with a linear gradient of acetonitril from 5% to 45% over 30 min, in 0.1 M triethylammonium acetate buffer pH 7 at 1 ml/min. Retention time= 21 min 5 sec. The oligomer obtained above was then detritylated by treatment with 80% acetic acid and purified on a mono P HR5/5 column (Pharmacia) with a linear gradient of NaCl from 0 to 1 M over 25 min, in 0.01 M NaH_2PO_4 (pH 6.8) and 20% acetonitrile buffer at 1 ml/min. Retention time of 18 mer[2]= 17 min 3 sec.

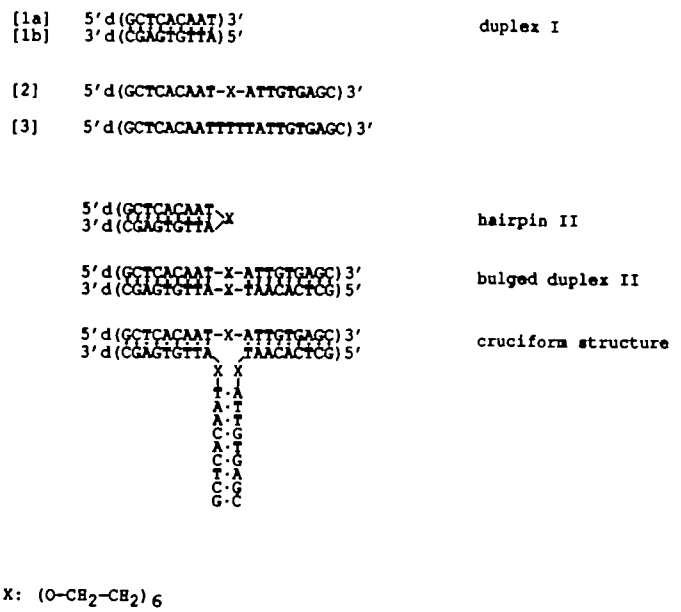

Figure 1. Sequences of oligodeoxyribonucleotides used , [1b], [2] and [3] and some possible secondary structures for compound [2]. The two 9-mers of oligodeoxyribonucleotide [2] are held together by a hexaethylene glycol group X bound via two phosphodiesters. The compound [3] has potentialy the same possibilities that the compound [2] of forming a hairpin III, a bulged duplex III and other concatemer structures.

Solution preparation for dichroism studies

Oligodeoxyribonucleotide solutions were prepared with a buffer containing 0.1 M sodium chloride and 10 mM sodium cacodylate, pH 7.0. The oligodeoxyribonucleotide concentration have been calculated by UV absorption at 260 nm in denaturing conditions, assuming an extinction coefficient of 14000 M^{-1} cm^{-1} for purines and of 7000 M^{-1} cm^{-1} for pyrimidines. The concentration used to calculate the CD amplitude was that of the nucleotide units. Single strands d(GCTCACAAT) and d(ATTGTGAGC)[1b] were mixed in equimolar proportions to give the linear duplex I (figure 1).

Circular dichroism spectroscopy

Circular dichroism measurements were carried out on a Jobin-Yvon Mark III dichrograph. Data acquisition and analysis were performed on a computer interfaced to the spectrometer. Optical cells with pathlengths of O.1, 0.2, 0.5 and 1.0 cm were used to allow melting curve measurements over a range of oligomer concentrations. The temperature of the cell was adjusted with a circulating refrigerated water bath and held constant to ±0.5°C. During the experiments nitrogen was continuously circulated through the cuvette compartment. Dichroism spectra were collected at 2°C intervals in the transition range and at 4°C intervals outside. Each CD spectrum was run at least twice and we checked for possible base line shifts. Each thermal denaturation was performed at least twice.

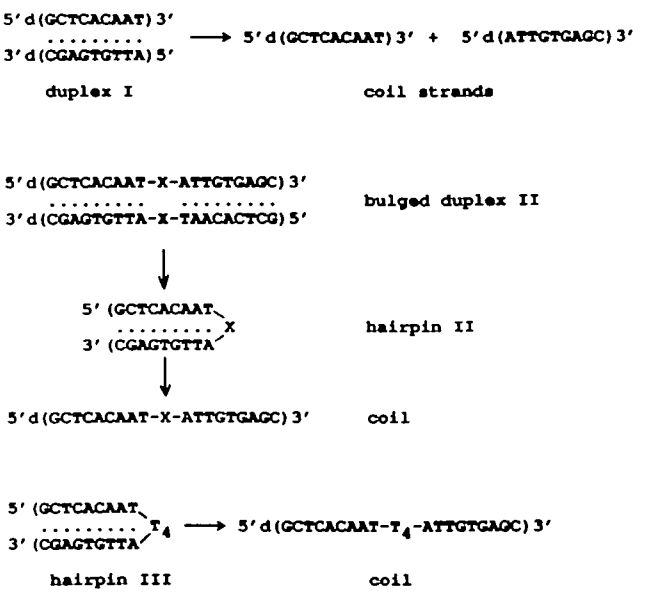

Figure 2. Representation of equilibria occuring with the duplex I (duplex-coil), the oligomer [2] (duplex-hairpin and hairpin-coil) and with the oligomer [3] (hairpin-coil).

Electrophoreris

Besides the circular dichroism method, the structures of the oligomer [2] and [3] at nucleotide concentrations comparable to those used for spectroscopy experiments were explored by polyacrylamide gel electrophoresis. The reference sequence was a double-stranded oligodeoxyribonucleotide of 25 base pairs.

All samples were prepared in solutions containing 10% glycerol and bromophenol blue as a marker. PAGE experiments were carried out for 4 hours on gels obtained by polymerizing a solution containing 10% acrylamide and 0.5% bisacrylamide in TBE buffer. The temperature was maintained at 1°C with a circulating refrigerated water bath. Bands were visualized by staining with ethidium bromide.

Thermodynamic analysis of transition data

As described under results and discussion the helix-coil transitions observed by CD spectroscopy were bimolecular for the hybrided oligodeoxyribonucleotide :[1b](linear duplex I) and monomolecular for the oligodeoxyribonucleotides [2](hairpin II) and [3](hairpin III) (figure 2). In order to estimate the enthalpic, entropic and free energy changes involved in the formation of the duplex I, hairpin II and III, each dichroic melting curve was fit to a two-state transition model.

According to a two-state model the expression of the molar circular dichroism at the temperature T is given by the equation:

$$\Delta\epsilon(T)=\alpha\ \Delta\epsilon_h(T)+(1-\alpha)\ \Delta\epsilon_c$$

In this expression: i) the subscripts h and c of the molar (base unit) circular dichroism, $\Delta\epsilon$, refer to the helical and coil states respectively; ii) α is the fraction of strand in the helix state.

The molar circular dichroism in both helical and coil form were assumed to be linear function of temperature according to:

$$\Delta\epsilon_h=aT+b \text{ and } \Delta\epsilon_c=a'T+b'$$

The above assumptions are consistent on one hand with previous studies of single-stranded and double-stranded oligomer melting (31−35) and on the other hand with the shape of the melting curves shown in figure 6. In the case of the duplex I formed of oligomers and [1b], the molar circular dichroism $\Delta\epsilon_c$ is the mean value of those obtained for the two coil species.

For the bimolecular process involving the duplex I the molar fraction α, is related to the changes in enthalpy (ΔH), and entropy (ΔS) by the relationship:

$$K=\ 2\alpha/[(1-\alpha)^2C_t]=\ \exp(-\Delta H/RT+\Delta S/R)$$

where K is the equilibrium constant, C_t is the total strand concentration and R is the gas constant.

For the monomolecular process involving the hairpins II and III, the relationship is:

$$K=\ \alpha/(1-\alpha)=\ \exp(-\Delta H/RT+\Delta S/R)$$

Using a non linear least-squares method the program fits each experimental melting curve with the first equation, treating ΔH, ΔS, a, b, a′ and b′ as independent variable parameters.

RESULTS AND DISCUSSION

Structure of oligodeoxyribonucleotides [2] and [3]

The 18-mer oligodeoxyribonucleotide [2] and the 22-mer oligodeoxyribonucleotide [3] are shown in figure 1. These palindromic (oligomer [2]) and quasi-palindromic (oligomer [3]) sequences have the ability to form either intramolecular hairpin structures or intermolecular structures recalled in figure 1. Polyacrylamide gel electrophoresis is a suitable technique to distinguish between species differing in size. The figure 3 shows the electrophoretic profiles of the 18-mer [2], the 22-mer [3] and of an equimolar mixture of oligomer and [1b], at 1°C following the procedure described in Materials and Methods. In the experiment shown in the left part of the figure 3, the oligomers [2] and [3] were heated to 90°C and then kept at room temperature for several hours before loading. As shown in lane **b** the 18-mer [2] migrated in two well-resolved bands in accord with the presence of two species differing in size. The slow moving band appears much less intense than the fast moving band. The oligomer [3] running in lane **a** presented an unique migrating band close to but slower than the fast migrating band of lane **b** (oligomer [2]). The equimolar mixture of complementary oligomers and [1b] (lane **c**) migrated as a single band close to the fast migrating species of lane **b**. All bands migrated at a remarkedly faster speed than the reference, a deoxyribonucleotide of 25 base pairs (lane **d**). In the experiment shown in the right part of the figure 3, the oligomers [2] and [3] were heated to 90°C and quickly cooled in an ice bath just prior to loading. Like on the left part of the figure 3 we observed one band for the oligomer [3] (lane **a**) and two bands for the oligomer [2] (lane **b**), but in this later case in contrast with the result shown in left part of the figure 3, we remarked that the duplex form (the upper band) was favored with respect to the hairpin form (the lower band). The comparison of the electrophoretic mobilities illustrated in figure 3 clearly shows under the gel conditions (buffer and temperature) that : i) for the 18-mer [2] there is the presence of an interconversion between a full duplex (corresponding to the slow moving band), and a hairpin structure (corresponding to the fast moving band); the duplex reconstitution being a slow process; ii) for the 22-mer [3] there is only the presence of a hairpin structure; iii) for the equimolar mixture of oligomers and [1b] there is the formation of a full duplex, the duplex I. These results eliminate the formation of cruciform structures like that illustrated in figure 1.

Dichroic properties

The CD spectrum of an equimolar mixture of oligodeoxyribonucleotides 5′d(GCTCACAAT)3′ (oligomer [1a]) and

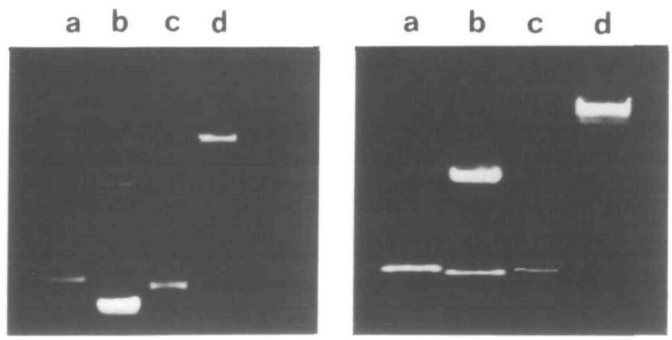

Figure 3. Electrophoretic analysis at 1°C in the experimental conditions described in Materials and Methods of the d(GCTCACAAT- T_4-ATTGTGAGC) [3] (lane **a**), d(GCTCACAAT-X-ATTGTGAGC) [2] (lane **b**), d(GCTCACAAT):d(ATTGTGAGC) (lane **c**) and of a reference fragment DNA of 25 base pairs (lane **d**). left: oligomers [2] and [3] were heated at 90°C and then slowly cooled during several hours before loading. right: oligomers [2] and [3] were heated at 90°C and quickly cooled in an ice bath just prior loading. In lanes **b** the upper band corresponds to the bulged duplex II form and the lower one corresponds to the hairpin II form.

5'd(ATTGTGAGC)3' (oligomer [1b]) at low temperature (0°C) and at the higher concentration used (.81 mM, expressed in nucleotide units) is shown in figure 4. It is characterised by a positive band and a negative band of nearly equal rotational strength, respectively centered at 280 nm and 250 nm. This spectrum differs from the averaged sum of the oligomers and [1b] alone, under the same experimental conditions (data not shown) and is similar to the CD spectrum of DNA-B form. As the concentration used was diminished no significant change in the CD signal was observed. These results revealed that at low temperature and under our buffer conditions no aggregation process occurred and that the duplex I was formed with a degree of stacking in the hybrid close to one.

The comparison of the CD spectra of the 18-mer [2], of the 22-mer [3] and of the duplex I at 0°C (figure 4) shows that, even though weak differences in the magnitude of the CD bands are observed, the spectra exhibit an overall similarity: a positive band centered at 280 nm, a negative band of nearly equal rotational strength centered at 250 nm and a zero crossover point at 262 nm. Qualitatively the spectra of all oligodeoxyribonucleotides are caracteristic of DNA-B form.

On the basis of the electrophoretic experiments previously described, we know that the CD spectrum of 18-mer [2] does not reflect only one pure conformation since contributions from both dimeric form and hairpin form are present. However, the strong similarity between the spectra of the 18-mer [2] and that of the duplex I shows that the environment of the bases are similar in both oligomers. We conclude that there is no significant change in the stacking geometries of the bases between the respective form of the oligomers, that is to say between the bulged duplex II, the hairpin II and the double-stranded structure of the duplex I. This result confirms on one hand the double helical nature of the 18-mer [2] (hairpin or dimeric structure), and on the other hand that the possible interactions of the linker (O- CH_2-$CH_2)_6$ with the flanking bases paired in the stem, either do not exist or do not induce any perceptible dichroic effect.

The weak dichroic differences noticed at low temperature (0°C) between the 22-mer [3] and the reference duplex I are certainly due to the presence of the T_4 sequence in the 22-mer.

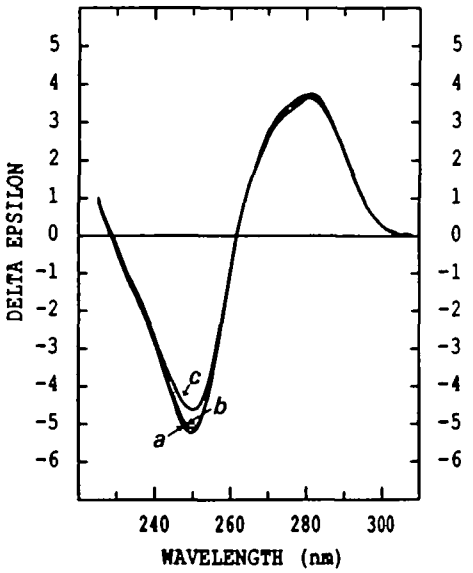

Figure 4. Circular dichroism spectra of duplex I and of oligodeoxyribonucleotide [2] and [3] at 0°C in 0.1 M sodium chloride 0.01 M sodium cacodylate pH 7.0.
a: d(GCTCACAAT):d(ATTGTGAGC); 0.81 mM.
b: d(GCTCACAAT-X-ATTGTGAGC); 0.97 mM.
c: d(GCTCACAAT-T_4-ATTGTGAGC); 1.3 mM.
The concentrations are expressed in nucleotide units.

Thermal stability of oligodeoxyribonucleotides

Upon increasing the temperature of the 18-mer [2], of the 22-mer [3] and of the duplex I we observed a decrease of the CD signal (figure 5). With the duplex I, we verified that at the higher temperatures, whatever the nucleotide concentrations used, the CD spectrum was the average of those of the corresponding

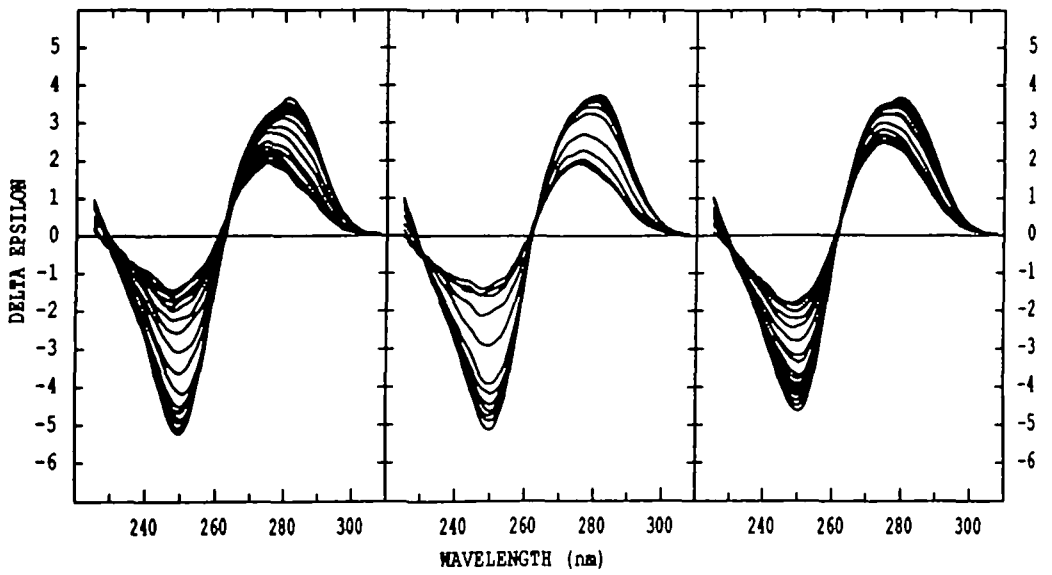

Figure 5. Circular dichroism spectra of oligodeoxyribonucleotides as a function of temperature in 0.1 M sodium chloride 0.01 M sodium cacodylate pH 7.0. left: d(GCTCACAAT):(ATTGTGAGC); 0.81 mM. middle: d(GCTCACAAT-X-ATTGTGAGC); 0.97 mM. right: d(GCTCACAAT-T_4-GCTCACAAT), 1.3 mM. In order of decreasing curve intensity the temperature varied from 0°C to 95°C. The concentrations are expressed in nucleotide units.

oligomers alone under the same conditions (data not shown). We noted that for the 22-mer [3] the variation of magnitude of the decrease of the signal versus temperature was lower than that of the duplex I or that of the 18-mer [2] (38% vs 46%). Presumably, it is essentially due to the lower temperature dependence of the CD signal corresponding to the T_4 loop in the 22-mer [3].

The left part of the figure 6 shows the variation of CD signal of the duplex I at 280 nm versus temperature various strand concentrations ranging from 8.2 to 90 μM. We note that the sigmoidal melting profiles exhibit linear parts before and after the region of the transition and that the midpoint temperature (Tm) moves up as the concentration increases (the Tm ranges from about 33°C to 40°C in our experimental conditions). We concluded to a thermal annealing of the duplex I on the basis of a bimolecular process as illustrated on figure 2.

With the 18-mer [2] and the 22-mer [3], the plots of the CD signal versus temperature were monophasic, whatever the concentration used in our experimental conditions, that is to say from 5.4 to 54 μM (strand units). No concentration effect on the midpoint was observed. The transition ranges were centered at 72°C and 69°C for the 18-mer [2] and the 22-mer [3] respectively (figure 6). For the transitions observed, the concentration independence of the Tm values of both oligodeoxyribonucleotides is in accordance with monomolecular processes.

From electrophoresis studies we know that the 18-mer [2] is present in a mixture of an intramolecular monomer (hairpin) and an intermolecular dimer. Further, our CD melting profiles are not biphasic but monophasic with a linear pretransition range. This result can be explained if one considers that for the oligomer [2] the dimeric structure and the hairpin structure are not significantly different with respect to the circular dichroism, and that the transition from the duplex into the hairpin induces only a reorganization of the base pairs (intermolecular hydrogen bonds being replaced by intramolecular ones), the relative geometries of the bases in the dimeric and hairpin structures being similar.

On the basis of CD and electrophoretic measurements we concluded to: i) a one-step denaturation of the 22-mer [3] consisting in a hairpin-coil transition; ii) a two-step denaturation of the 18-mer [2] consisting in a duplex-hairpin transition followed by a hairpin-coil transition, this transition being the only one observable by circular dichroism. These thermal annealings are illustrated in figure 2.

Many authors have reported that oligodeoxyribonucleotides containing built-in loop, made of nucleotide residues, at the center of the sequence have a much lower tendency to give rise to a dimeric form than to a hairpin form (5−8). So far, our results are consistent with the studies of Haasnoot et al (6,22) on a series of oligodeoxyribonucleotides of sequence d(ATCCTAT$_n$TAGG-AT) (n=0−7), which present an A-T percentage in the stem of the hairpin form close to that of the oligomers studied here. Strong evidence was presented that for $n \geq 4$ the aforementionned oligodeoxyribonucleotides adopt exclusively a hairpin structure (as for the 22-mer [3]), while for $n \leq 3$ there is an equilibrium between the linear duplex and the hairpin form (as for the 18-mer [2]).

Thermodynamic analysis

According to the thermodynamic analysis exposed in Materials and Methods we have estimated the enthalpic, entropic and free energy changes involved in the helix formation for the linear duplex I, a bimolecular process, and for the hairpins II and III, a monomolecular process. The parameters obtained from fit of the individual melting curves with linear sloping base lines are reported in tables I and II.

For the linear duplex I the average results obtained are $\Delta H = -55.6$ kcal/mol and $\Delta S = -155.8$ eu (table I). The value of the free energy change at 25°C is equal to −9.2 kcal/mol. The estimated error is of the order of ±5%.

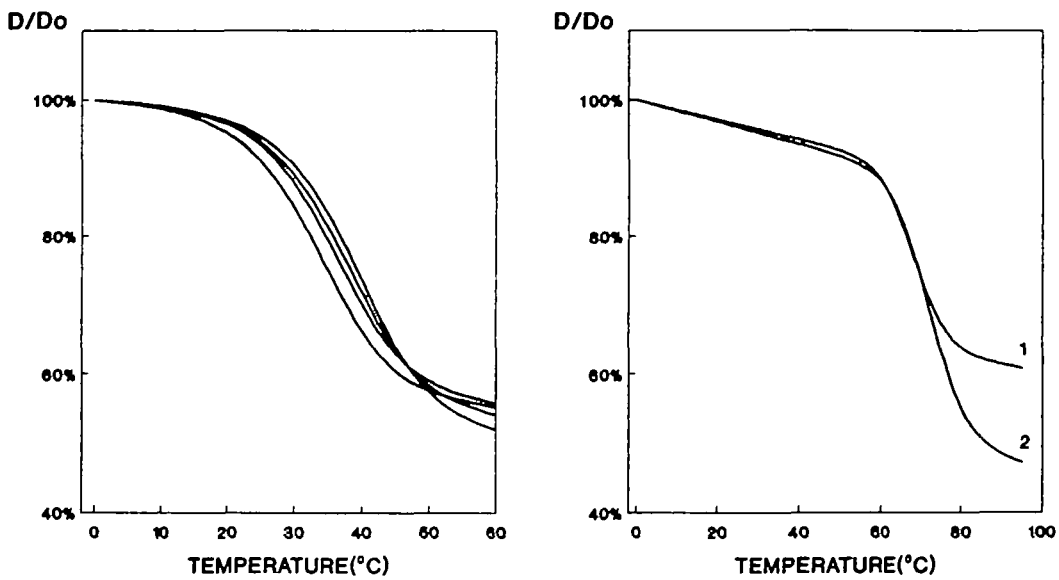

Figure 6. Normalized delta epsilon at 280 nm vs. temperature for the duplex I (left), d(GCTCACAAT-X-ATTGTGAGC) [2] and d(GCTCACAAT-T_4-ATTGTG-AGC) [3] (right). left: the melting curves shift to higher temperatures in order of increasing strand concentration from 8.2 to 90 μM. right: oligomer [2] (curve 2) and oligomer [3] (curve 1). For both oligomers we observed concentration independence of the thermal denaturation in the range of strand concentrations used i.e. from 5.4 to 54 μM. Delta epsilon values were normalized by dividing the delta epsilon value at a given temperature by that at 0°C. All solutions contained 0.1 M sodium chloride, 0.01 M sodium cacodylate, pH 7.0.

strand concentration (μM)	- ΔH (kcal/mol)	- ΔS (eu)
90.0	57.1	161.6
44.3	55.7	156.5
18.6	54.0	151.0
8.2	54.6	151.5
mean	55.6	155.8

Table I: Thermodynamic parameters of duplex I calculated from individual melting profiles. The experimental conditions were 0.1 M sodium chloride, 0.01 M sodium cacodylate, pH 7.0. The estimated errors in ΔH and ΔS are ± 5%.

sample	- ΔH (kcal/mol)	- ΔS (eu)	Tm (°C)
18-mer [2]	55.7	161.5	72
22-mer [3]	61.0	178.6	69

Table II: Thermodynamic parameters for hairpin formation. Values are the mean of parameters obtained from the fits of individual melting profiles measured at molar strand concentrations between 5.4 and 54 μM. The other experimental conditions were 0.1 M chloride sodium, 0.01 M sodium cacodylate, pH 7.0. Tm is determined from the relationship: 1/Tm= ΔH/ ΔS. The estimated errors in ΔH and ΔS are ± 5%. The estimated error in Tm with respect to the fit is ± 1°C.

For the hairpin structures the mean values of ΔH, ΔS and ΔG at 25°C are respectively −55.7 kcal/mol, −161.5 eu, −7.6 kcal/mol for hairpin II and −61.0 kcal/mol, −178.6 eu, −7.8 kcal/mol for hairpin III, with an estimated error of ±5% (table II).

The comparison of the enthalpic changes on the hairpin III formation (−61 kcal/mol) with that of the reference duplex I (−55.6 kcal/mol) suggests that the presence of the T_4 loop stabilizes enthalpically the hairpin structure with respect to the unconstrained stem by −5.4 kcal/mol. This enthalpic stabilization is of the same order of magnitude than those previously observed for different hairpin systems. For example, Xodo et al (21) demonstrated that the sequence d(CGCGCGTTTTTCGCGCG) can form a hairpin structure caracterized by a loop of unpaired nucleotide residues T_5, and that this structure was stabilized enthalpically by −7 kcal/mol with respect to the d(CGCGCG):d(CGCGCG) duplex through the presence of the loop. In the same way, with oligodeoxyribonucleotides d(ATCCTAT$_n$TAGGAT)($n \geq 4$), Haasnoot et al (6,11,22) showed that noncomplementary bases were highly favorable for hairpin formation and that a loop consisting of four thymine residues contributed by −6 kcal/mol to the formation of an ordered hairpin structure. From nuclear Overhauser experiments, it was suggested a stacking of the loop thymines on the stem duplex (11). Senior et al (23) demonstrated that d(CGAACGT$_n$CGTTCG) exhibits higher van't Hoff transition enthalpies than the isolated stem duplex (−39 kcal/mol vs −36 kcal/mol). The origin of this negative enthalpic contribution of the loop was attributed to stacking of the bases of the loop region (single-strand stacking and stem- loop-stacking). On the basis of a proton NMR data analysis by using distance geometry methods, the three dimensional structure of the hairpin d(CGCGTTTTCGCG) was determined (24) and showed that the bases of the loop region are stacked; in particular that the thymine residues in the loop adjacent to the stem stack to the flanking GC base pair in the top of the stem and point inwards one in front of the other, leaving room for a possible hydrogen-bonded pairing. From our CD data we cannot determine the origin of the enthalpic stabilization of the hairpin III with respect to the duplex I, but from the results above reviewed it seems reasonable to think that the origin of this contribution is both a stacking interaction within the thymine residues in the loop and a stacking interaction between thymine residues of the loop and the flanking A·T base pair at the top of the hairpin stem. Besides, the suppression of the 'fraying end' effect for the A·T base pair next to the loop should contribute to the enthalpic stabilization (36).

In contrast with these findings, our studies show clearly that with the 18-mer [2] there is no enthalpic stabilization of the hairpin form with respect to the reference duplex I. This may be attributed to the absence of base residues in the loop. Moreover, the similarity between the CD spectra of the duplex I and of the modified oligomer [2] indicates that the relative geometries of the bases in the reference duplex I and in the hairpin form are overall similar. The hexaethylene glycol linker does not perturb the conformation of the stem. Consequently, the greater stability of the hairpin II with respect to the duplex I cannot be attributed to a difference between the respective structures. We rather think that in the 18-mer [2] the linker hold together the oligodeoxyribonucleotide fragments and [1b] at an 'efficient' distance, increasing by this way the probability of a pairing event between the two fragments, with respect to the free oligodeoxyribonucleotides and [1b] at the same fragment concentration. The concentration at which the duplex I would exhibit the same stability that the hairpin II is 0.28 M expressed in strand units, or 0.7 g/cm^3. In comparison the crystal density of the 'Dickerson' double-stranded dodecamer d(CGCGAATTCGCG) is 1.5 g/cm^3 (37). That means that in solution there is no strand concentration which permits the duplex I to reach the thermal stabilty of the hairpin II. It is worth noting that with an oligodeoxyribonucleotide susceptible to adopt a hairpin form like the self-complementary Dickerson dodecamer, a high tendency to hairpin formation was observed as the dA or dT residues were replaced by the 1,3-propanediol (38). However, the differences with the hairpin form of the 18-mer [2] are the partly abasic loop which contains three bases, and the stem containing only four base pairs.

Hairpin II has a Tm sligthly higher than hairpin III (72°C vs 69°C). The higher stability of hairpin II with respect to hairpin III is entropic in origin (−161.5 eu vs −178.6 eu), the enthalpic change being less favorable (−55.7 kcal/mol vs −61.0 kcal/mol). However, it is noteworthy that at 25°C the free energy changes of hairpin formation in the oligomers [2] and [3] are similar (−7.6 kcal/mol for hairpin II vs −7.8 kcal/mol for hairpin III). There is a compensation between the enthalpic and entropic contributions in the stability of both hairpins.

CONCLUSION

We have reported experimental evidence that the palindromic sequence d(GCTCACAAT-X-ATTGTGAGC), where X is a hexaethylene glycol chain, can exist as a mixture of two species in exchange with each other, a bulged duplex form and a hairpin form, while the d(GCTCACAAT-T_4-ATTGTGAGC) sequence is only in the hairpin form. In this latter oligodeoxyribonucleotide the built-in hairpin loop T_4 at the center of the sequence prevents the formation of a dimeric structure. The analysis of the helix coil transition, in term of a two-state model leads to the following

conclusions: i) the T_4 loop stabilizes enthalpically the hairpin with respect to the unconstrained stem (this stabilization is most likely due to stacking of the thymine residues of the loop region); ii) for the stem duplex studied here, the addition of a no-nucleoside residue loop is an enthalpically neutral process; the enhancement of the duplex pairing between the sequences which are on the both sides of the linker, with respect to the unlinked sequences, reflects a simple concentration effect.

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