Length-dependent energetics of (CTG)$_n$ and (CAG)$_n$ trinucleotide repeats

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ABSTRACT

Trinucleotide repeats are involved in a number of debilitating diseases such as myotonic dystrophy. Twelve to seventy-five base-long (CTG)$_n$ oligodeoxy-nucleotides were analysed using a combination of biophysical [UV-absorbance, circular dichroism and differential scanning calorimetry (DSC)] and biochemical methods (non-denaturing gel electrophoresis and enzymatic footprinting). All oligomers formed stable intramolecular structures under near physiological conditions with a melting temperature that was only weakly dependent on oligomer length. Thermodynamic analysis of the denaturation process by UV-melting and calorimetric experiments revealed an unprecedented length-dependent discrepancy between the enthalpy values deduced from model-dependent (UV-melting) and model-independent (calorimetry) experiments. Evidence for non-zero molar heat capacity changes was also derived from the analysis of the Arrhenius plots and DSC profiles. Such behaviour is analysed in the framework of an intramolecular ‘branching-hairpin’ model, in which long CTG oligomers do not fold into a simple long hairpin–stem intramolecular structure, but allow the formation of several independent folding units of unequal stability. We demonstrate that, for sequences ranging from 12 to 25 CTG repeats, an intramolecular structure with two loops is formed which we will call ‘bis-hairpin’. Similar results were also found for CAG oligomers, suggesting that this observation may be extended to various trinucleotide repeats-containing sequences.

INTRODUCTION

Recent molecular genetic studies have revealed a correlation between spontaneous expansion of several DNA trinucleotide repeats and a variety of debilitating human diseases [for reviews see (1–4)]. This class of diseases was first characterized in Fragile-X syndrome (5) and later in myotonic dystrophy and other disorders. Myotonic dystrophy type 1 (DM1) is caused by the expansion of a (CTG)–(CAG) repeat in the DMPK gene (6). To date, at least nine distinct loci show instability with the same (CAG)–(CTG) repeat. These diseases increase in severity with earlier onset in successive generations and have no cure. Although the pathological states show different characteristics, one common feature among them is that the affected repetitive DNA unit has expanded beyond the number of repeats found in the healthy population. Therefore, the expansion of triplet repeats represents a novel mutational mechanism. More recently, other disorders have been associated with the expansion of non-trinucleotide motifs, such as the CCTG tetranucleotide in myotonic dystrophy type 2 (7).

DNA secondary structures may be considered as a common and causative factor for triplet expansion (8–10) but the molecular mechanisms causing the instability are unknown and remain a subject of intensive study. Even if no therapeutic approach is currently available to prevent or revert repeat expansion, in vitro studies suggest that repeat deletion could be induced by various chemotherapeutic agents (11,12); thus, opening a new field of study aimed at the design of trinucleotide repeat-specific ligands. Preliminary results suggest that selective recognition of trinucleotide repeat structures is possible (S. Amrane, unpublished data), but the rational design of such ligands should be facilitated by knowledge of the structure and energetics of their nucleic acid target.

Repetitive CNG sequences are susceptible to the formation of duplexes by self-folding, forming two Watson–Crick G–C pairs and one mismatch pair (13–16). Long hairpins have long
lifetimes and inhibit duplex reannealing (17, 18). A controversy remains on how different are these structures from classical B-DNA. Besides forming ‘slipped duplexes’ (19), CGG repeats have been reported to form quadruplexes (20–23). However, a recent study demonstrated that CGG repeats are reluctant to form tetraplexes under physiological conditions and this structure is unlikely to be involved in the disease (24): these sequences preferentially fold into antiparallel homoduplexes or hairpins in a length-dependent manner.

Concerning the structure of the bimolecular (CTG–CAG)$_n$ duplex, several results revealed a ‘polyhairpin concept’. Studies on (CAG–CTG)$_{30,50}$ showed that these duplexes formed alternative DNA duplex structures named SDNA and SiDNA (9, 19, 25–27). This conclusion was reached using atomic force microscopy, electron microscopy, native gel electrophoresis, Mung Bean (MB) and T7 endonuclease cleavage assays. SDNA is a well-described bimolecular polyhairpin structure composed of multiple short (CTG)$_n$ and (CAG)$_n$ ($n = 1–10$) slipped-out structures with a hairpin-like single-stranded character. SDNA is formed in the absence of replication when the two complementary strands have the same length. Instead, SiDNA is formed during replication between two complementary strands with a different number of repetitions and results in repeat expansion or deletion. SiDNA is composed of one major slipped-out structure containing 20–30 repeats of the longest (CTG)$_n$ or (CAG)$_n$ strand.

To date, only the short hairpin structures (containing <10 repeats) involved in the formation of SDNA have been clearly established (15, 28) revealing an intramolecular stem with several repetitions of a T·T or A·A mismatch sandwiched between 2 G–C base pairs. In contrast, the nature of the structures observed for longer repeats involved in the formation of SiDNA is still unclear. Even if several studies have previously demonstrated that long trinucleotide repeats adopt more compact structures than the short ones, suggesting the hypothesis of a multi-folded structure (16, 29), but little is known about their exact conformation. The elucidation of this point is the purpose of our study; i.e. the analysis of the conformational properties of individual (CTG)$_n$ or (CAG)$_n$ strands, which may constitute the single slipped-out structures of particular DNA regions.

Initial thermodynamic studies showed the stabilities of CAG and CTG hairpins to be nearly identical under physiological salt concentrations in vitro (17). In contrast, Völker et al. (30) showed that, within a conformationally confined system, (CAG)$_n$ and (CTG)$_n$ form stable, ordered structures with the former triplet less stable than the latter, as also recently confirmed by another group (31). In this study, we analysed the folding of (CTG)$_n$ and (CAG)$_n$ individual sequences by using biophysical [UV-absorbance, circular dichroism (CD) and microcalorimetry] and biochemical techniques (native gel electrophoresis and enzymatic footprinting). In agreement with the previous results, we found that the folding of these sequences is intramolecular rather than bimolecular and that these structures are stable in physiological conditions. The study of the thermodynamic data obtained by thermal denaturation and microcalorimetry led us to propose an intramolecular ‘bis-hairpin’ like model for (CTG)$_n$ and (CAG)$_n$ individual strands ($n = 12–25$), with longer strands possibly giving rise to the formation of multi-branched hairpins. These structures are actually distinct from the multiple hairpins found in SDNA and SiDNA, in which several short hairpins protrude from a DNA duplex at different positions. Our model suggests that each slipped-out single-stranded region found in SDNA and SiDNA can actually fold into structures more complex than previously thought, as soon as 10–12 repeats are present in a single protruding loop. Similar results were also found for (CAG)$_n$ repeats.

**MATERIALS AND METHODS**

**Oligodeoxynucleotides**

Oligodeoxynucleotide probes were synthesized by Eurogentec (Belgium) on the 0.2 or 1 μmol scale. As all oligomers studied here correspond to DNA, the ‘d’ prefix was omitted from most sequences. Purity was checked by gel electrophoresis. All concentrations were expressed in strand molarity using a nearest-neighbour approximation for the absorption coefficients of the unfolded species (32).

**Thermal difference spectra for CTG and CAG trinucleotide repeats**

The thermal difference spectrum (TDS) of a nucleic acid is obtained by simply recording the UV-absorbance spectra of the unfolded and the folded states at temperatures, respectively, above and below its melting temperature ($T_m$). The difference between these two spectra is defined as the TDS. The TDS has a specific shape that is unique for most structures (33, 34) (J.L. Mergny et al., manuscript in preparation); thus, providing a simple, inexpensive and rapid method to gain structural insight into nucleic acid structures, both DNA and RNA, ranging from short oligomers to polynucleotides. Spectra were recorded between 220 and 335 nm with a Kontron Uvikon 940 UV/Vis spectrophotometer using quartz cuvettes with an optical pathlength of 0.2 or 1 cm. The differential spectrum of a CTG repeat structure gives a maximum differential absorbance at an unusually high wavelength (∼277 nm) (34).

**UV-melting experiments**

The thermal stability of the different trinucleotide repeat structures was estimated by heating/cooling experiments, recording the UV-absorbance at several wavelengths as a function of temperature using a Kontron Uvikon 940 spectrophotometer thermostated with an external ThermoNeslab RTE111 or ThermoHaake Phoenix C25P1 waterbath. The temperature of the bath was typically increased or decreased at a rate of 0.2°C/min, using 0.2 or 1 cm pathlength quartz cuvettes. All experiments were carried out in 10 mM sodium cacodylate buffer (pH 7.0) containing 30–500 mM KCl. Taking into consideration the TDSs and the high strand concentrations used for some UV-melting experiments, we chose to record the denaturation process at 290 nm (using cuvettes of 0.2 cm optical pathlength for the highest concentrations) in order to obtain an absorbance between 0.1 and 1.5. On the contrary, for the thermodynamic analysis (see below), lower strand concentrations were used and the denaturation process was followed at 275 nm where the signal is maximal. However, the observed melting temperatures were in excellent agreement (usually within 0.5°C, data not shown) with the ones determined at other wavelengths (e.g. 260 nm). All melting profiles were...
perfectly reversible at the chosen temperature gradient, demonstrating that these curves correspond to true equilibrium curves (35).

Thermodynamic analysis

For all parameters listed below, the assumed direction of the thermal process is the single-strand-to-hairpin transition. The thermal reversibility and the known molecularity of the process allow one to calculate the value of the equilibrium constant ($K_e$) assuming a simple two-state transition model. One must first convert absorbance measurements into folded fraction by manually selecting two baselines corresponding to the completely folded and unfolded form. An uncertainty may therefore arise because of the subjectivity in baseline determination. Starting from the classical Gibbs enthalpy equation,

$$\Delta G^o = \Delta H^o - T\Delta S^o$$

one can write, for a reversible reaction [in which $\Delta G^o = -RT \ln(K_e)$], the following van’t Hoff equation:

$$\ln(K_e) = -\frac{\Delta H_{VH}^{o}}{R}(1/T) + \frac{\Delta S_{VH}^{o}}{R},$$

where $T$ is the temperature in Kelvin, while $\Delta H_{VH}^{o}$ and $\Delta S_{VH}^{o}$ are, respectively, the standard enthalpy and entropy change of the reaction. In other words, provided that $\Delta H_{VH}^{o}$ and $\Delta S_{VH}^{o}$ are temperature-independent (see below), the so-called van’t Hoff representation or Arrhenius plot [ln($K_e$) versus 1/T] should give a straight line, with a slope of $-\Delta H_{VH}^{o}/R$ and a y-axis intercept of $\Delta S_{VH}^{o}/R$. The $\Delta H_{VH}^{o}$ of this reversible reaction is called the van’t Hoff enthalpy and is defined by

$$\Delta H_{VH}^{o} = \frac{R \ln(K_e)}{d(T^{-1})}.$$

In the case of temperature-dependent enthalpies and entropies, one will obtain a significant deviation from linearity. Nevertheless, the above equation is still valid, but the slope at each point may be different, leading to temperature-dependent $\Delta H_{VH}^{o}$ and $\Delta S_{VH}^{o}$ values. van’t Hoff enthalpies and entropies are said to be ‘model-dependent’: they rely on a two-state equilibrium hypothesis. They are, therefore, less robust than the ‘model-independent’ thermodynamic values provided by calorimetry (see below).

Determination of $\Delta C_{p}^{o}$

The linear fit of an Arrhenius plot assumes that $\Delta H^o$ is temperature-independent, which in turn means that $\Delta C_{p}^{o} = 0$. $\Delta C_{p}^{o}$ is the heat capacity change at constant pressure which is occurring during the thermal process (considered in the single-strand-to-hairpin direction). $\Delta H_{VH}^{o}$ and $\Delta S_{VH}^{o}$ are linked to $\Delta C_{p}^{o}$ by the following relations:

$$\frac{d(\Delta H_{VH}^{o})}{d(T)} = \Delta C_{p}^{o},$$

$$\frac{d(\Delta S_{VH}^{o})}{d(ln(T))} = \Delta C_{p}^{o}.$$
DSC and UV-melting analysis were in good agreement. The systematic small ($\pm 2^\circ$C) difference in favour of the DSC $T_m$ is the result of the calorimetric definition of the melting temperature, which corresponds to the temperature of maximum heat release/uptake rather than to the temperature of half association/dissociation. The real $T_m$ (half association/dissociation temperature) is therefore $\pm 2^\circ$C below the DSC $T_m$, referred to in the text as ‘$T_m^{cal}$’. The calorimetric enthalpy ($\Delta H^{cal}_{cal}$) and entropy ($\Delta S^{cal}_{cal}$) for the transition process were determined in a model-independent way from the DSC curve. Comparison of the $\Delta H^{cal}_{cal}$ with the van’t Hoff value obtained by the UV-thermal curve ($\Delta H^{cal}_{th}$) allowed us to confirm or inform the correctness of the previously assumed two-state model used to describe the entire thermal process ($\kappa = \Delta H^{cal}_{th}/\Delta H^{cal}_{cal} = 1$). Additional fitting of the experimental DSC curve with a more general model equation (provided by the CpCalc software) and its resolution by deconvolution analysis allowed us to obtain the value of $\Delta S^{cal}_{cal}$ for the whole process, as well as the thermal profile of the ‘daughter’ subcurves corresponding to the intermediate transitions. Deconvolution of the DSC curves represents one of the major advantages of the calorimetric analysis over the spectroscopic one. In fact, while by UV analysis small differences in melting temperature between independent subunits may be masked by an even smaller conformational change in the overall structure (giving rise to an apparent two-state transition profile of the UV-thermal curve), deconvolution of the calorimetric curve into its components can allow for unravelling intermediate states.

**RESULTS**

**Confirmation of the folded form and intramolecular folding of CTG repeats**

Several groups have previously demonstrated that repeats adopt an intramolecular duplex structure [for a recent example see (31)]. For this reason, we will very briefly describe the experiments that were carried out to confirm this model in our system. As shown in Supplementary Figures S1A and S1B, DNA composed of pure (CTG)$_n$ repeats ($n = 4$–10) exhibit fast, strand concentration-independent, mobility on polyacrylamide gels (29,43–45) in good agreement with an intramolecular structure. This increased mobility is almost completely lost under denaturing conditions (Supplementary Figures S1C and S1D). CD spectra of CTG oligomers are similar to the CD spectral signature of B-DNA with a negative peak $\sim 255$ nm and a positive peak $\sim 285$ nm (Supplementary Figure S2) (31).

**Thermal absorbance difference spectra**

Another method, we recently proposed, to study the conformation of a nucleic acid is to record the ‘thermal absorbance difference spectrum’ between its high and low temperature UV-absorbance spectrum (33,34). The normalized TDS has a shape which is specific for each nucleic acid conformation studied so far, ranging from duplexes to quadruplexes: as shown in Figure 2A, the shapes of TDS for (CTG)$_n$ ($n = 8$–20) were all very close (red curves) suggesting that these oligomers adopt a similar folded conformation. Furthermore, these spectra with two maxima $\sim 275$ and $235$ nm are highly reminiscent of pure GC-rich B-DNA TDS [blue curve and (34)]. A similar analysis was performed for CAG repeats (Figure 2B); although the TDS were not superimposable (compare panels A and B), these spectra were also reminiscent of GC-rich B-DNA TDS, but completely different from the ones of other structures such as quadruplexes (33). It is interesting to note that the CTG spectra are very close to the 100% GC spectra (47) (Figure 2A, in blue; these duplexes correspond to sequences where only GC base pairs are formed), whereas CAG repeats resemble mixed duplexes (Figure 2B, in black; these duplexes involve 67% GC/33% TA base pairs).

**Analysis of the melting profiles**

Careful analysis of the UV-melting profiles revealed several observations. First, the melting temperature of (CTG)$_n$ and (CAG)$_n$ was weakly dependent on oligonucleotide length (example provided in Supplementary Figure S3; see also Figure 1B). For the CTG oligomers tested here, the $T_m$s varied between 54 ($n = 6$) and 58.7°C ($n = 25$) (Table 1), while for (CAG)$_n$ the $T_m$s varied between 51.6 ($n = 6$) and 54°C ($n = 25$) (Supplementary Table S1).
Second, the Arrhenius representation of the melting curves also revealed a more complex behaviour (35). Determination of the folded fraction for the two samples analysed in Supplementary Figure S3 led to the Arrhenius plots are shown in Figure 3. A significant and reproducible deviation from linearity is seen in both cases. As described previously (Materials and Methods), it is possible to fit these curves to obtain the $D_{C_{ph}}/C_{14}$ values (Table 1). Assuming a reaction in the single-strand-to-hairpin direction, these values were found to be always negative, in agreement with the studies on different DNA structures but with surprisingly high absolute values for long sequences [e.g. (CTG)$_{20}$, (CTG)$_{25}$ and (CAG)$_{25}$]. Additionally, even if some discrepancies may be found, the $D_{C_{ph}}$ values deduced from van’t Hoff plots ($D_{C_{ph}}$) were generally in good agreement with those deduced from calorimetry ($D_{C_{ph}}$; Tables 1 and S1).

Third, the thermal stability of these trinucleotide repeats was analysed as a function of KCl concentration. As expected, the $T_m$ values for the (CTG)$_8$ oligomer were found to be indeed dependent on KCl concentration (Supplementary Figure S4): a 10-fold increase in potassium concentration led to a 7°C increase in $T_m$ (from 50°C at 10 mM KCl to 57°C at 100 mM KCl). This ionic strength dependence is somewhat lower than previously reported (16) and lower than expected for regular double-stranded hairpins.

Calorimetric analysis of (CTG)$_n$

The calorimetric values of the enthalpy ($\Delta H_{cal}$) and entropy ($\Delta S_{cal}$) of renaturation for the CTG oligomers are reported in Table 1 and an example of a DSC denaturation run of (CTG)$_{25}$ is provided in Figure 4A. As in the UV-thermal analysis,
the $T_{\text{max}}^\text{cal}$ determined by DSC was almost sequence-length independent (Figure 4B). Next, the van’t Hoff enthalpy determined by the analysis of the UV-thermal transition curve ($\Delta H_{\text{VH}}^\circ$) was compared to the calorimetric value obtained by DSC ($\Delta H_{\text{cal}}^\circ$) (Figure 4C and D). For relatively short sequences ($n = 4–12$) the ratio $x = \Delta H_{\text{VH}}^\circ / \Delta H_{\text{cal}}^\circ$ was close to 1 (1.08 ± 0.13; Table 1). The similarity of the two values validated the hypothesis of the two-state model used in the van’t Hoff analysis to describe the thermal transition of the intramolecular trinucleotide repeat. However, in the case of longer sequences, the $\Delta H_{\text{VH}}^\circ / \Delta H_{\text{cal}}^\circ$ ratio dramatically dropped to values ~0.4 (Figure 4D). The $\Delta H_{\text{VH}}^\circ$ failed to become more negative with increased repeat number, whereas $\Delta H_{\text{cal}}^\circ$ continued to increase (in absolute terms) explaining why the agreement was lost (Figure 4C and Table 1). The difference was highly significant as the errors bars (Figure 4C) on $\Delta H_{\text{VH}}^\circ$ and $\Delta H_{\text{cal}}^\circ$ were 6% or lower.

**Calorimetric analysis of (CAG)$_n$**

The thermal behaviour observed for the CTG repeats was compared with that obtained from CAG repeats of identical length under the same experimental conditions. An example of a DSC denaturation run for a (CAG)$_n$ oligo is provided in Figure 5A. Again, we observed that, for all sequences tested, the $T_{\text{max}}$ and the $T_{\text{m}}$ values found by DSC and UV-melting analysis were in good agreement and almost independent on sequence-length (Figure 5B). As shown in Figure 5C and D, the values of $\Delta H_{\text{VH}}^\circ$ and $\Delta H_{\text{cal}}^\circ$ for the (CAG)$_n$ thermal denaturations were compared and plotted against the number of triplet repeats, leading to the same conclusions drawn for the (CTG)$_n$ analogs (see also Supplementary Table S1).

**Enzymatic probing of long CTG repeats**

Nuclease sensitivity studies were performed with S1 and MB nucleases that are two single-strand specific endonucleases. The bases contained in the single-stranded regions of the structures adopted by long CTG repeats are preferentially cleaved with respect to the ones contained in the double-stranded regions. The cleavage sites for the two nucleases were determined by comparison with size markers (Materials and Methods). The enhanced MB digestion of (CTG)$_{15}$ occurred at three main regions (Figure 6A): (i) two sites at the 3’ end, G13 and G14; (ii) five sequence-centred sites around the (CTG)$_7$ (T4) marker, i.e. C7, T7, G7, C8 and T8 resulting from a TGCT-loop; and (iii) two sites around the 5’ end region identified as G1 and C2 representing a CTG-loop. S1 digestion of (CTG)$_{15}$ presents only two sites around the same 5’ end region cleaved by the MB nuclease: G1 and C2 (Figure 6B). Similarly, two loops were characterized for (CAG)$_{15}$: one central loop composed of two repeats

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**Table 1. Thermodynamic parameters for (CTG)$_n$ repeats**

| $n$  | $T_{\text{VH}}^\circ$ (°C)$^a$ | $T_{\text{cal}}^\text{max}$ (°C)$^b$ | $\Delta H_{\text{VH}}^\circ$ (kcal/mol)$^d$ | $\Delta H_{\text{cal}}^\circ$ (kcal/mol)$^b$ | $x = \Delta H_{\text{VH}}^\circ / \Delta H_{\text{cal}}^\circ$ | $\Delta S_{\text{VH}}^\circ$ (cal/mol/K)$^e$ | $\Delta S_{\text{cal}}^\circ$ (cal/mol/K)$^f$ | $\Delta C_{\text{VH}}^\circ$ (cal/mol/K)$^g$ | $\Delta C_{\text{cal}}^\circ$ (cal/mol/K)$^h$ |
|-----|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| 6   | 54            | 55.1           | −33.0          | −22.6          | 1.28           | −101           | −69            | −450           | nd             |
| 8   | 56.8          | 58.1           | −44.0          | −40.5          | 1.09           | −133           | −122           | −1030          | −380           |
| 10  | 57            | 60.1           | −51.0          | −45.9          | 1.11           | −155           | −138           | −710           | −720           |
| 11  | 57.1          | 59.4           | −53.9          | −51.0          | 1.06           | −165           | −153           | −1360          | −1040          |
| 12  | 57.8          | 60.0           | −56.6          | −62.8          | 0.90           | −171           | −188           | −1410          | −1210          |
| 13  | 57.7          | 60.1           | −52.7          | −62.6          | 0.77           | −159           | −188           | −1700          | nd             |
| 14  | 57.6          | 60.8           | −52.6          | −78.7          | 0.67           | −159           | −235           | −1510          | nd             |
| 15  | 57.7          | 59.6           | −51.9          | −82.1          | 0.63           | −156           | −293           | −1500          | nd             |
| 16  | 57.7          | 61.4           | −51.5          | −95.2          | 0.54           | −156           | −296           | −1500          | nd             |
| 20  | 57            | 59.3           | −56.5          | −125.7         | 0.45           | −171           | −385           | −2060          | −1260          |
| 25  | 58.7          | 61.3           | −61.7          | −147.2         | 0.42           | −186           | −440           | −2120          | −1880          |

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For all parameters listed, the assumed direction is the single-strand-to-hairpin transition. nd, not determined.

$^a$Number of (CTG) repeats.

$^b$Melting temperature deduced from the UV-melting curve.

$^c$Melting temperature deduced from the DSC profile.

$^d$Thermodynamic parameters deduced from the UV-melting curves, using a non-linear fit of the Arrhenius plots, where the $\Delta H_{\text{VH}}^\circ$ is close to the $\Delta H^\circ$ determined at the $T_{\text{cal}}^\text{max}$.

$^e$Thermodynamic parameters deduced from the DSC profiles. (Error bars for $\Delta T^\circ$ values are shown in Figure 4C; highest relative error of 6.4%).

$^f$Thermodynamic parameters deduced from the DSC profiles. (Error bars for $\Delta T^\circ$ values are shown in Figure 4C; highest relative error of 5.1%).

$^g$Heat capacity change deduced from general model fitting of the DSC profiles.

$^h$Heat capacity change deduced from non-linear fitting of the Arrhenius plots.

$^i$Heat capacity change deduced from general model fitting of the DSC profiles.
(CAGCAG) and one CAG loop at the 5' end (Supplementary Figure S5).

The enhanced digestion of (CTG)₁₅ occurred at three main regions (Figure 7A): (i) two sites at the 3' end: G14 and G15; (ii) two sequence-centred sites around the (CTG)₈ (T₃) marker: T₈ and G₈ resulting from a CTG-loop; and (iii) two sites around the 5' end region identified as G₁ and C₂, indicating a CTG-loop. These two CTG-loops are conserved in (CTG)₂₀ (Figure 6A and B) with the following digestion sites: (i) two sequence-centred sites around the (CTG)₁₀ (T₂) marker: T₁₀ and G₁₀ and (ii) two sites around the 5' end region identified as G₁ and C₂.

Finally, (CTG)₂₅ presents the same kind of loops as (CTG)₁₅ (Supplementary Figure S6): (i) four sequence-centred sites around the (CTG)₁₂ (T₁) marker: T₁₂, G₁₂, C₁₃ and T₁₃ resulting from a 4 nt loop; and (ii) two sites around the 5' end region identified as G₁ and C₂, representing a CTG-loop. Thus, our data show that all these sequences present one centred loop of 3 or 4 nt and one loop at the 5' end containing 3 nt (Figures 6C and 7B). In theory, this terminal loop could also correspond to DNA end fraying, but in this case we would have obtained four cleavage sites: C₁, T₁, G₁ and C₂ at the 5' end region instead of the only two sites G₁ and C₂.

Furthermore, two NMR studies on short CTG repeat sequences have demonstrated that the T–T mismatches, all over the stem of the hairpin, are very well stacked between the two adjacent CpG base pairs and are bound to each other by two hydrogen bonds (35). The DNA end fraying hypothesis is therefore unlikely, as will be confirmed in the following part.

**CTG)₁₅ variant sequences**

In order to confirm these results and to ensure that the proximal cuts of the 5' end nucleotides do not represent DNA end fraying, the C and G bases of the single-strand regions of (CTG)₁₅ previously defined by footprinting experiments...
were replaced by T bases. The $\Delta T_{\text{cal}}$ and renaturation enthalpy of the modified oligonucleotide sequences were then measured by DSC and compared to the ones of the unmodified analog (Table 2). Replacement of C2 and G2 (as well as of G7 and C8) by two T bases did not influence the renaturation enthalpy. In fact, despite a little decrease in the $\Delta T_{\text{cal}}$ ($-2.5^\circ C$) of the modified sequences, their enthalpy remained around $-80$ kcal/mol as for the unmodified (CTG)$_{15}$. This suggests that these four bases, i.e. C2, G2, G7 and C8, do not belong to the stem region of the hairpin but to unpaired regions of the structure. The small difference in $\Delta T_{\text{cal}}$ could be the result of little variations in the ionic conditions between the modified and the unmodified samples or errors in data acquisition/baseline determination. More importantly, this $\Delta T_{\text{cal}}$ is defined as the maximum of the DSC curve (Materials and Methods) and does not necessarily correspond to the real $T_m$ (half association/dissociation temperature): it is therefore not the best parameter to assess the stability of a structure. At this purpose, the model-independent $\Delta H^\circ_{\text{cal}}$ of the renaturation process is a more suitable parameter, directly related to the number of base pairs involved in the molecule. Thus, the constancy of this value for T-replacement of C2, G2, G7 and C8, undoubtedly indicates that these four bases do not belong to the stem region of the hairpin but to unpaired regions of the structure. In contrast, we obtained a dramatic decrease, in absolute value, of the renaturation enthalpy (from $-80$ till $-54.8$, $-51.8$ and $-51.4$ kcal/mol) by replacing C3, C7 and G8 by a T base, thus showing that these three bases belong instead to the stem region of the structure.

**DISCUSSION**

The goal of this study was to analyse the assumption of a quasi ‘normal’ hairpin duplex for trinucleotide repeats, namely the CTG and CAG repeat motifs. Little is known about the exact conformation of long trinucleotide repeats. Several studies have previously demonstrated that long trinucleotide repeats adopt more compact structures than the short ones, suggesting the hypothesis of a multi-folded structure rather
than a single-hairpin (16,29). Few NMR or crystallographic studies have actually analysed 'pure' CNG repeats, as they are usually embedded into different sequence motifs [e.g. GCGGTTTGCGG in (48) and TGGCGGC in (49); for a review see (50)]. Disease-related CNG repeats exhibit a propensity for folding at chain lengths as short as 12 residues (14). CTG-containing sequences have been studied with a variety of other techniques, including PAGE, KMnO₄ modification, P1 nuclease digestion, UV-absorbance and molecular dynamic simulations [reviewed in (51)]. To date, the hairpin structure of sequences shorter than 10 repeats has been clearly established (15,28), but the nature of the compact structures observed for longer repeats is still unclear.

In this paper, CD, electrophoresis, UV and DSC data revealed sharp structural transitions, in agreement with the formation of a rather simple canonical B-DNA hairpin, with a stem length growing with the repeat number. However, independent results indicate that the energetics and/or structure of these intramolecular trinucleotide repeats were significantly altered when compared with the canonical B-DNA, especially for (relatively) long (CTG)ₙ sequences. Qualitatively similar results were obtained for (CAG)ₙ repeats, suggesting that this behaviour may be a general phenomenon for (CNG)ₙ oligonucleotides.

First, the thermal stability of this structure (its Tₘ) was very weakly dependent on the oligonucleotide length (Figures 4B and 5B and Supplementary Figure S3). The weak sequence-length dependence of the Tₘ of these repeats was previously observed for (CTG)₁₀ and (CTG)₂₅ (16); (CTG)₁₀ and (CTG)₂₅ (17); (CTG)₁₀ and (CTG)₁₅ (31) as well, but has never been systematically studied [with a notable exception for (CUG)ₙ RNAs; n = 5–69 (52)]. In our experience, the thermal stability of DNA hairpins increases rapidly with the stem length, in complete opposition to what is observed here. This behaviour...
is therefore highly irregular for a simple hairpin system. Second, the analysis of the melting curves did not lead to simple Arrhenius plots, as significant and reproducible curvatures were observed. We actually had to manually select an unlikely lower baseline in order to ‘linearize’ the Arrhenius representations (Supplementary Figure S7). At least three non-exclusive reasons may be proposed to explain this non-linear behaviour: (i) a non-two-state transition (partially melted structures are significantly populated), (ii) two (or more) intermingled transitions occur or (iii) a single two-state transition occurs with a highly negative $\Delta C_{\text{VH}}/C_14$ (i.e. a temperature-dependent $\Delta H_{\text{VH}}/C_14$). This last possibility which was, until recently (53,54), overlooked for nucleic acids transitions, should be seriously considered here. The comparison of the model-dependent and model-independent $\Delta H_{\text{VH}}/C_14$ values provides interesting clues.

**Length-dependent discrepancy between van’t Hoff and calorimetry enthalpy**

Several groups have reported important discrepancies between the van’t Hoff enthalpy ($\Delta H_{\text{VH}}$) and the calorimetry enthalpy ($\Delta H_{\text{cal}}$) for various nucleic acids structures (55), although not for trinucleotides. For (CTG)$_n$ and (CAG)$_n$ repeats, we observed that the melting temperatures found by DSC and van’t Hoff analysis of the UV-melting curves were always in reasonable agreement; however, the model-dependent and the model-independent enthalpies were not always identical (Figures 4C and 5C and Tables 1 and S1). For relatively short (CTG)$_n$ or (CAG)$_n$ sequences ($n = 4–12$) the $\Delta H_{\text{VH}}/\Delta H_{\text{cal}}$ ratio was close to 1, validating the hypothesis of the two-state model used in the van’t Hoff analysis. In the
case of longer sequences, the $\Delta H_{\text{cal}}/\Delta H_{\text{cal}}^\text{VH}$ ratio dramatically dropped (Figures 4D and 5D) to values $\sim 0.4$ (for CTG repeats) or 0.5 (for CAG repeats). This length-dependent difference between the two enthalpy values suggests the hypothesis of a non-two-state transition model for longer sequences. That is, above a critical threshold number of trinucleotide repetitions ($n = 10–12$), a change in the structural organization of the oligonucleotides occurs, going from a simple hairpin–stem model to a structure of higher complexity, composed of different independent units. Therefore,

(i) Below 12 repeats, no significant difference between the two $\Delta H$ values was observed, leading to their similar increase with sequence-length. For these relatively short sequences the two-state transition model was confirmed by deconvolution of the DSC profiles (Figure 8A) into a single transition, indicating their folding into a simple hairpin structure.

(ii) Beyond 12 repeats, instead, an increasing discrepancy between the $\Delta H$ values appeared, with the model-independent $\Delta H_{\text{cal}}$ still increasing in a length-dependent manner, whereas the two-state model-dependent $\Delta H_{\text{VH}}$ remained quasi-constant. In contrast with a previous report (31), no plateau was observed in $\Delta H_{\text{cal}}$ for sequences longer than 15 trinucleotide repeats (Figures 4C and 5C). For long oligomers, the two-state hypothesis was no longer valid, suggesting the formation of a more complex structure with at least two independent units. This observation was confirmed by deconvolution of the DSC profiles into a three-state equilibrium with two intermediate transitions (Figure 8B).

In other words, for short sequences, folding into a single-hairpin structure is relatively simple, leading to a good concordance between $\Delta H_{\text{cal}}$ and $\Delta H_{\text{VH}}$. For long sequences (at least 12), rather than adopting a regular long stem, the oligonucleotide seems to maintain a relatively short and constant length of the stem region while the remaining part fold into several (at least two) independent folding units, of not necessarily the same length, which melt in an independent fashion. In that case, a multi-branched hairpin should have a relatively apparent constant van’t Hoff enthalpy, as seen by UV-melting analysis, but an increased calorimetric enthalpy, as observed by DSC. This different folding trend for longer sequences may be explained in terms of a favourable enthalpy–entropy compensation. In fact, although a long single-hairpin structure should indeed have a more favourable renaturation enthalpy (owing to maximization of the number of base pairs), it has not necessarily the most favourable free energy. On the contrary, branched hairpin structures may offer additional degrees of freedom, possibly lowering the unfavourable entropic term and thus explaining their preferential formation for longer sequences.

**Nuclease assays**

The enzymatic assays on long sequences (Figures 6 and 7 and Supplementary Figures S5 and S6), additionally confirmed by the DSC analysis of the (CTG)$_n$-modified sequences (Table 2), suggested that a simple hairpin was not necessarily the chosen structure, as other bases close to the 5’ end of the sequence were cleaved by single-strand specific nucleases.
3 nt CTG-loop hairpin. It is interesting to note that, in our study, this tendency is reversed for longer sequences: (CTG)_15 and (CTG)_25 present a centred 4 nt TGCT-loop, while (CTG)_16 and (CTG)_20 present a centred 3 nt CTG-loop. Thus, this inversion confirms the presence of a second loop composed of an odd number of CTG repeats at the 5′ end of the sequence.

CONCLUSIONS

Above a critical threshold number of trinucleotide repetitions (n = 12–15), a change in the structural organization of the oligonucleotides occurs going from a simple hairpin–stem model to a structure of higher complexity composed of different independent units such as a bis- (or multi-branched) hairpin-like structure. In contrast with a recent report (31), our calorimetric data suggest that the enthalpic stability of this structure is not compromised as the length of the hairpin overcomes 15 repeats. The possible intramolecular bis-hairpin model could be generalized as an intramolecular ‘multi-branched’ hairpin model for pathological sequences as long as 3000 repetitions. The 3 or 4 nt bulges disseminated all over the repeated sequence could be involved in the instability process during DNA replication through the formation of slipped-out DNA structures, but may also represent an important feature to discriminate trinucleotide repeats against canonical B-DNA for targeting by small repeat-specific bulge-ligands. This instability has been ascribed to the energy of the structure but in our case it could be explained in terms of number of hairpin loops that introduce an additional steric parameter to the energetic one. These results have encouraged us to carry on further investigations for a better understanding of the thermodynamics of these repetitions, and stimulating additional studies directed to the structural elucidation of long trinucleotide repeat models. We will now pursue similar studies on other DNA and RNA trinucleotides [(CGG)_n, (CCG)_n and (CUG)_n (58,59)] to see whether these observations may be extended to other CNG repeats.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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