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Sequence effects of single base loops in intramolecular quadruplex DNA

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Abstract **We have examined the properties of intramolecular G-quadruplexes in which the G3 tracts are separated by single base loops. The most stable complex contained 1′,2′-dideoxyribose in all three loops, while loops containing T and C were slightly less stable (by about 2 °C). Quadruplexes containing loops with single A residues were less stable by 8 °C for each T to A substitution. These folded sequences display similar CD spectra, which are consistent with the formation of parallel stranded complexes with double-chain reversal loops. These results demonstrate that loop sequence, and not just length, affects quadruplex stability.**

Keywords: DNA quadruplex; DNA structure

1. Introduction

DNA sequences that contain four or more G-tracts can fold to form intramolecular structures that consist of stacks of G-quartets [1–5]. The four G-tracts are separated by different length loops, which can be as short as a single nucleotide [6–8]. The complexes can adopt a range of structures in which the bases are either *anti* or *syn* and in which the strands run parallel or antiparallel [4,5]. The loops can be arranged in several different ways; double chain reversal (propeller) loops link two adjacent parallel strands by a connection between the top and bottom G-tetrads [9], while edgewise or diagonal loops link two antiparallel strands [10]. Some structures contain both edgewise and propeller loops [11–13]. G-rich sequences with the potential to adopt these structures are found in several gene promoters; most notably the c-*myc* [8,14,15], *bcl*2 [16,17] and c-*kit* [18,19] oncogenes and several of these contain single base loops. Although it has been shown that loop length affects quadruplex stability and structure [6,20–23] there have been few studies on the effect of loop sequence, though single base changes can have a significant effect (e.g. changing TTA in the human telomeric repeat to TTG [10] in the Tetrahymena repeat [24,25]).

One very stable intramolecular quadruplex contains four G_3 tracts that are linked by single T residues [7,26]. We have investigated the effect of loop sequence on its stability using sequences of the type d(TGGGNGGGNGGGNGGGT), where N is each base in turn (except G) together with 1′,2′-dideoxyribose, Φ.

2. Materials and methods

2.1. Oligonucleotides

All oligonucleotides were synthesized on an Applied Biosystems ABI 394 automated DNA/RNA synthesizer on the 0.2 or 1 μmole scale using the standard cycles of acid-catalysed detritylation, coupling, capping and iodine oxidation procedures. Phosphoramidite monomers and other reagents were purchased from Applied Biosystems or Link Technologies. The sequences of the oligonucleotides used in this work are shown in Table 1. All oligonucleotides were prepared with 5′-fluorescein and 3′-dabcyl (fluorescein C6 phosphoramidite and dabcyl cpg purchased from Link Technologies Ltd.) for use in the fluorescence melting experiments and the same sequences were used for the circular dichroism studies. The bases adjacent to the fluorophore and quencher were the same (T) for all the oligonucleotides to ensure that the terminal base did not affect quadruplex formation and stability. Inclusion of this base also hinders any fluorescence quenching between G and fluorescein.

2.2. Fluorescence melting

Fluorescence melting curves were determined in a Roche LightCycler as previously described [22,26,27] in a total reaction volume of 20 μL. Oligonucleotides (final concentration 0.25 μM) were prepared in 10 mM lithium phosphate pH 7.4, which was supplemented with various concentrations of potassium or sodium chloride. The LightCycler has one excitation source (488 nm) and the changes in fluorescence were measured at 520 nm. In order to avoid hysteresis between heating and cooling curves melting experiments were performed at a slow rate of temperature change (0.2 °C min^{-1}). This was achieved by changing the temperature in 1 °C steps and leaving the samples to equilibrate for 5 min at each temperature before recording the fluorescence. In a typical experiment the oligonucleotides were first denatured by heating to 95 °C for 5 min. They were then annealed by cooling to 30 °C at 0.2 °C min^{-1} and melted by heating to 95 °C at the same rate. The fluorescence was recorded during both the annealing and melting steps.

T_m values were obtained from the maxima of the first derivatives of the melting profiles using the LightCycler software or, together with ΔH, from van't Hoff analysis of the melting profiles [23,26–28]. The fraction folded was calculated as previously described [28] from the difference between the measured fluorescence and the upper and lower baselines. All reactions were performed at least twice and the T_m values differed by <0.5 °C with a 5% variation in ΔH. Since $\Delta G = 0$ at the T_m, ΔS was estimated as $\Delta H/T_m$. It should therefore be noted that ΔS is not determined independently of ΔH and T_m. This analysis assumes a simple two-state equilibrium between the folded and unfolded forms. The presence of polymorphic quadruplex structures will lead to shallower melting profiles and therefore smaller apparent values for ΔH.

2.3. Circular dichroism

CD spectra were measured on a Jasco J-720 spectropolarimeter as previously described [22]. Oligonucleotides solutions (5 μM) were prepared in 10 mM lithium phosphate pH 7.4, containing either 100 mM potassium chloride or 100 mM sodium chloride. The samples were

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Table 1
Sequences of the oligonucleotides used in this work

Name	Oligonucleotide sequence
TTT	d(Fam-TGGGTGGGTGGGTGGGT-dabcyl)
CCC	d(Fam-TGGGCGGGCGGGCGGGT-dabcyl)
AAA	d(Fam-TGGGAGGGAGGGAGGGT-dabcyl)
ΦΦΦ	d(Fam-TGGGΦGGGΦGGGΦGGGT-dabcyl)
TAT	d(Fam-TGGGTGGGAGGGTGGGT-dabcyl)
ATA	d(Fam-TGGGAGGGTGGGAGGGT-dabcyl)

Fam = fluorescein; Φ = 1′,2′-dideoxyribose.

heated to 95 °C and annealed by slowly cooling to 15 °C over a period of 12 hours. Spectra were recorded between 220 and 320 nm in 5 mm path length cuvettes. Spectra were averaged over 10 scans, which were recorded at 100 nm min^{-1} with a response time of 1 s and 1 nm bandwidth. A buffer baseline was subtracted from each spectrum and the spectra were normalized to have zero ellipticity at 320 nm.

Table 2
T_m values (°C) for the various oligonucleotides, determined in 10 mM lithium phosphate pH 7.4 containing different concentrations of KCl or NaCl

		TTT	AAA	CCC	ΦΦΦ	ATA	TAT
KCl	0	46.0	<30	44.4	49.5	36.9	37.9
	1	73.9	50.0	71.8	77.0	59.6	67.3
	5	85.6	64.4	83.2	88.1	72.1	79.3
	10	>90	70.0	>90	>90	78.0	84.2
	50	>90	82.2	>90	>90	>90	>90
NaCl	1	48.3	<30	47.3	53.2	<30	40.0
	10	55.4	<30	53.6	60.5	39.6	46.3
	50	63.7	<30	62.3	68.0	47.0	55.6
	100	68.3	43.7	67.1	72.5	52.2	60.5
	200	73.3	50.2	72.1	77.0	57.8	65.6

The oligonucleotide concentration was 0.25 μM and the samples were heated and cooled at 0.2 °C min^{-1}. All T_m values are ±0.5 °C.

3. Results

We have investigated the effect of sequence on the stability of quadruplexes that contain single nucleotide loops using oligonucleotides of the type (TGGGNGGGNGGGNGGGT), where N is each base in turn. The sequences of these oligonucleotides are shown in Table 1; they were each labelled at the 5′-end with fluorescein and with dabcyl at the 3′-end. Fluorescence melting curves were used to assess the thermal stability of these complexes. When the quadruplex is folded the fluorophore and quencher are in close proximity and the fluorescence is quenched [22,26–28]. When the structure unfolds the fluorophore and quencher are separated and there is an increase in fluorescence. In the presence of 100 mM KCl all these sequences formed structures that melted above 90 °C. However, by using only 1 mM KCl the melting temperatures decreased to a measurable range. Although this concentration is lower than that used in most quadruplex studies, higher potassium ion concentrations produced melting curves that were too stable to measure. The fluorescence melting profiles (in which the fluorophore and quencher are close together) and the similar patterns of stability in both 1mM KCl and 100 mM NaCl, strongly suggest that these sequences adopt an intramolecular quadruplex. Melting profiles for the four oligonucleotides that contain the same base in each loop (TTT, CCC, AAA or ΦΦΦ) are shown in Fig. 1 in the presence of 1 mM KCl or 100 mM NaCl and the melting temperatures determined at a range of ionic strengths are summarized in Table 2. At this rate of heating (0.2 °C min^{-1}) there was no hysteresis between the melting and annealing profiles and the T_m values were independent of oligonucleotide concentration between 0.1 and 10 μM.

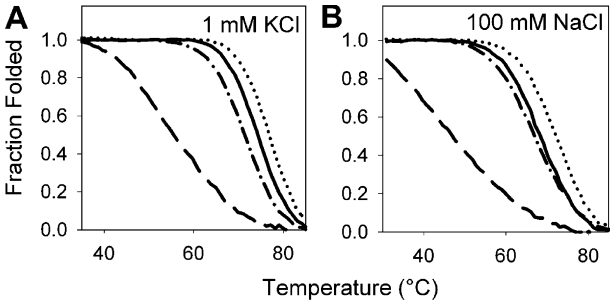

Fig. 1. Fraction of the different oligonucleotides folded at different temperatures (A) in 10 mM lithium phosphate pH 7.4 containing 1 mM KCl. (B) in 10 mM lithium phosphate pH 7.4 containing 100 mM NaCl. Solid line, TTT; dotted line, ΦΦΦ; dot and dash line, CCC; dashed line, AAA. These plots were derived from the melting curves by assuming that the complexes are folded at low temperature and single stranded at elevated temperatures [27].

It can be seen that loops with T, C and 1′,2′-dideoxyribose produce the most stable complexes (Φ > T > C), while the T_m of the complex with As in the loops is about 25 °C lower. The complexes are less stable in sodium containing buffers, but the rank order of stabilities is the same. The complex with A-containing loops has a similar stability to a complex containing propanediol linkers [22]. Thermodynamic parameters for the folding of each complex in the presence of potassium were estimated from van't Hoff analysis of the melting profiles and these are presented in Table 3. The values are consistent with those previously determined for intramolecular quadruplexes, which are typically between 65 and 100 kJ mol^{-1} per quartet [23,26]. These show that the complexes with the lower stability are characterized by a smaller enthalpy and higher entropy.

To further study the effect of loop sequence on stability we examined the properties of oligonucleotides with two Ts in the loops and one A (TAT), or one T in the loop and two As (ATA). The melting profiles of these sequences are shown in Fig. 2, alongside those for AAA and TTT and the thermodynamic parameters are presented in Table 3. Each T to A modification reduces the melting temperature by about 8 °C in both sodium and potassium-containing buffers, with a change in enthalpy of 46 kJ mol^{-1} per A substitution in 1 mM KCl and 33 kJ mol^{-1} in 100 mM NaCl.

CD spectral signatures are often used as indicators of the folding pattern of intramolecular quadruplexes; parallel structures (with *anti* glycosidic bonds) typically have positive maxima around 260 nm, while antiparallel structures (containing both *anti* and *syn* bonds) show maxima around 295 nm [29–32]. CD spectra of these sequences in the presence of 100 mM KCl are shown in Fig. 3 and show that the complexes produce very similar CD spectra with clear single maxima at 265 nm. These CD spectra were identical in sodium and potassium-containing buffers and strongly suggest that all these complexes adopt the same topology, which is likely to be the