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Bioorganic & Medicinal Chemistry Letters 15 (2005) 2047–2050
Bioorganic & Medicinal Chemistry Letters

Inosine substitutions demonstrate that intramolecular DNA quadruplexes adopt different conformations in the presence of sodium and potassium

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Received 14 January 2005; revised 2 February 2005; accepted 17 February 2005
Available online 19 March 2005

Abstract—We have examined the stability of fluorescently-labelled oligonucleotides that are based on the human telomeric repeat [$(GGGTTA)_3GGG$], in which one of the guanines in turn is substituted with inosine. We show that the relative stability of the substitutions is different in the presence of sodium and potassium. The data for potassium suggest a parallel arrangement of the strands, while the sodium form is mixed parallel and antiparallel.

Guanine-rich DNA sequences can form four-stranded structures that contain stacks of G-quartets[1–3] (Fig. 1a). These complexes can be generated by the intermolecular association of two or four separate DNA strands[4–7] or the intramolecular folding of a single DNA strand that contains at least four G-tracts.[8–18] The formation of these structures requires the presence of monovalent cations (especially potassium)[19,20] while they are destabilised by lithium.[21] The cations fit within the central core of guanine carbonyls and can lie between or within the plane of each quartet.

G-rich sequences with the ability to form quadruplex structures are found in telomeric regions of DNA, which contain highly repeated sequences such as $(GGGTTA)_n$ in humans and most other higher eukaryotes.[22–24] Several high resolution X-ray[5,15,16] and NMR[10–14] structures have been determined for DNA quadruplexes and these reveal that these are polymorphic. The strands are all parallel in intermolecular quadruplexes, while the arrangement in intramolecular complexes can be parallel, antiparallel or a combination of both.[2] In the usual representation of the intramolecular quadruplex (shown in Fig. 1b) the strands are antiparallel, with the TTA loops crossed at the top of the stack. This structure is consistent with the NMR data on a complex in the presence of sodium.[10] However, a crystal structure of the potassium complex reveals that this sequence can adopt a fully parallel structure in which the loops run between the bottom of one stack and the top of the next[15] (Fig. 1d, lower). More recent NMR studies have confirmed the formation of the parallel form or have suggested that these structures can co-exist.[13,14,17,18]

In this study we have examined the properties of an intramolecular G-quadruplex, based on the human telomeric repeat sequence $(GGGTTA)_3GGG$, by measuring the stability of oligonucleotides that contain single inosine substitutions at different locations. The stability of these intramolecular complexes was determined using a fluorescence melting technique employing oligonucleotides that contain a fluorescent group (fluorescein) at one end and a quencher (methyl red) at the other end, as previously described.[25,26] On forming an intramolecular complex these groups are in close proximity and the fluorescence is quenched. When the structure melts these groups become separated and there is a large increase in the fluorescence signal (Fig. 1b). We have previously used this technique to study the stability of G-rich intramolecular complexes.[26,27] Although these studies demonstrate that the oligonucleotides fold into stable structures, they do not give any information about the folding pattern. We have addressed this problem by examining the properties of oligonucleotides in which one of the guanines in turn is replaced with inosine. Inosine lacks the 2-amino group of guanine, thereby

Keywords: G-quartet; Quadruplex; Fluorescence; Molecular beacon; DNA melting; Inosine.
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doi:10.1016/j.bmcl.2005.02.050

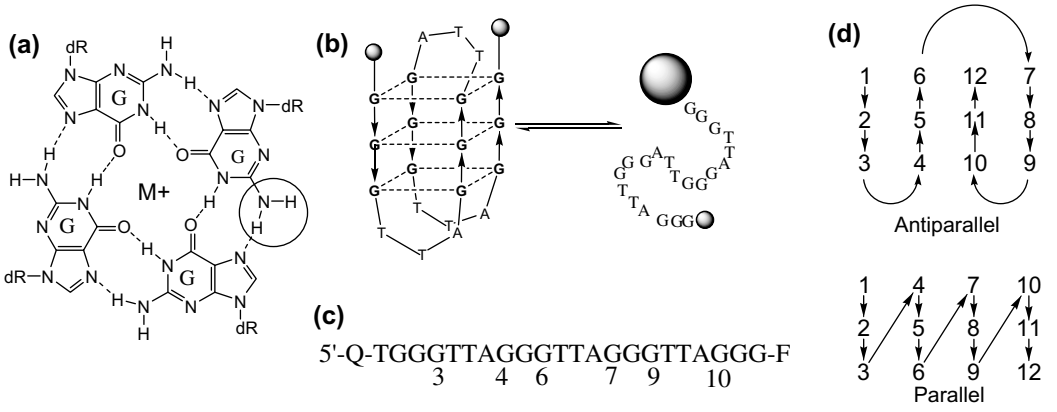

Figure 1. (a) Chemical structure of the G-quartet. The 2-amino group of one of the guanines is circled, indicating the group, that is lost when this base is substituted with inosine. (b) Schematic representation of the fluorescence melting technique. The fluorophore (fluorescein) and quencher (methyl red) are close together in the folded complex (shown as an antiparallel crossed structure); these are separated when the quadruplex melts, and there is a large increase in fluorescence. (c) Sequences of the DNA fragments used in this work. The positions of the guanines that are substituted with inosine are indicated by the numbers. Q is the quencher (methyl red) while F is the fluorophore (fluorescein). (d) Schematic representation of the antiparallel and parallel intramolecular quadruplexes indicating which numbered guanines are present in each G-quartet. The upper diagram corresponds to the antiparallel form shown in (b) with the crossed central loop, generating mixed parallel and antiparallel strands.

removing one hydrogen bond from the quartet (Fig. 1a), without affecting the central core of keto oxygens which interact with the monovalent cations.[28] In the parallel structure the upper quartet is composed of guanines 1, 4, 7 and 10, while in the antiparallel structure this contains guanines 1, 6, 7 and 12 (as illustrated in Fig. 1d). The lower quartet consists of guanines 3, 6, 9 and 12 in the parallel form but 3, 4, 9 and 10 in the antiparallel form. We therefore examined the thermal stability of oligonucleotides with single inosine substitutions at positions 3, 4, 6, 7, 9 and 10 (termed I3, I4, I6, I7, I9 and I10, respectively). These melting experiments were performed in the Roche LightCycler as previously described[25] using an oligonucleotide concentration of 0.25 μM.

Representative melting profiles are shown in Figure 2 in the presence of potassium or sodium and the T_m values derived from these are shown in Table 1. It can be seen that all these oligonucleotides produce clear melting profiles, which suggest the formation of the expected G-quadruplex structures. The T_m values are 10–15 K lower than those for the unsubstituted oligonucleotide.[26] Examination of these data shows that, in the presence of potassium, the most stable complexes are formed with oligonucleotides I3, I6 and I9, while I4, I7 and I10 melt a few degrees lower. Since positions 3, 6 and 9 are part of the same quartet in the parallel structure (as too are the positions 4, 7 and 10), but are part of different quartets in the antiparallel structure, the similar behaviour of I3, I6 and I9 (and I4, I7, I10) is consistent with the formation of the parallel form, as illustrated in the lower structure of Figure 1d.

A different pattern is seen in the presence of sodium, in which I3, I6 and I7 are the most stable while I4, I9 and I10 have lower stability. These data therefore suggest that the guanines at positions 3, 6 and 7 are part of the same quartet, as too are the guanines at positions 4, 9 and 10. This could be achieved if the first two guanine tracts are arranged parallel to each other, with the TTA loop running from the bottom of one stack to the top of the next, while the third and fourth G-tracts are arranged in an alternating antiparallel configuration with the loops positioned at the ends of the structure.

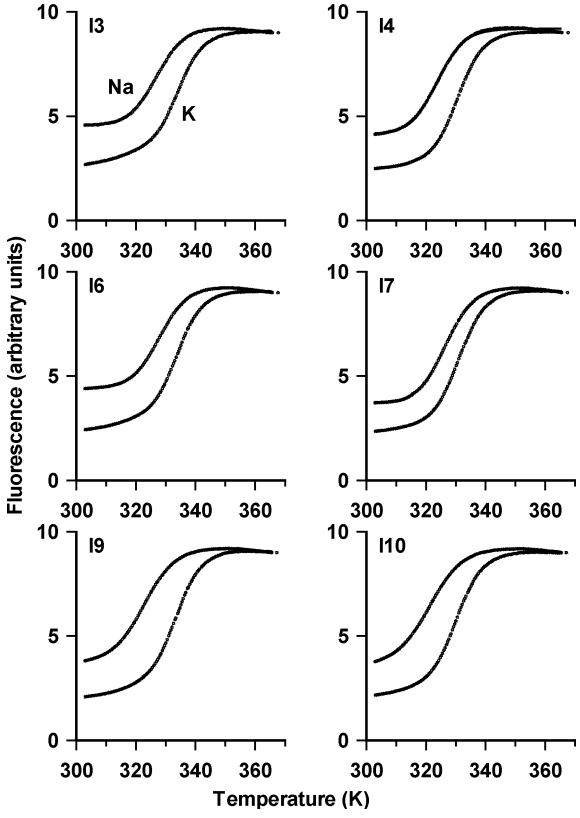

Figure 2. Representative fluorescence melting curves for the six oligonucleotides that contain single inosine substitutions. In each case the left curve corresponds to 100 mM sodium phosphate pH 7.4, while the right curve is for 50 mM potassium phosphate pH 7.4. The samples were heated at 0.1 °C/s. The *y*-axis shows the fluorescence intensity (arbitrary units), while the *x*-axis shows the temperature (K).

Table 1. T_m values (K) for melting of the intermolecular quadruplexes, determined in 50 mM potassium phosphate pH 7.4 or 100 mM sodium phosphate pH 7.4

	50 mM K^+		100 mM Na^+	
	T_m (K)	ΔH (kJ/mol)	T_m (K)	ΔH (kJ/mol)
Unmodified GGGTTA*	346.3	261	335.9	215
Single substitutions				
I3	336.3	230	328.0	178
I4	331.9	219	325.8	169
I6	335.1	231	329.4	178
I7	333.2	218	327.6	174
I9	334.8	218	324.0	153
I10	332.3	210	322.4	148
Double substitutions				
I3,9	326.0	163	316.7	129
I3,10	324.0	162	314.9	115
I6,7	324.0	180	321.8	150
I4,9	322.9	170	310.6	96
I4,10	320.1	161	310.4	83

ΔH values were calculated from van't Hoff analysis of the melting profiles, assuming simple two-state equilibrium. All reactions were performed at least twice and the calculated T_m values usually differed by <0.5 K with a 5% variation in ΔH.
* Data taken from Ref. 26.

This folding pattern, with three of the strands running parallel to each other with one in the opposite direction, has not previously been suggested for the human telomeric repeat, for which NMR studies suggest that the sodium form adopts the structure illustrated in the upper part of Figure 1d. However, this is similar to the structure proposed for the *Tetrahymena* telomeric repeat.[11,12] Although this folding pattern may not be correct, the different patterns of relative stability in the presence of sodium and potassium emphasise that different structures are formed in the presence of these two ions.

These experiments were performed in the presence of 50 mM potassium or 100 mM sodium (higher concentrations of sodium were used as this ion generates less stable structures) at a relatively fast rate of heating (0.1 °C/s). Under these conditions no hysteresis was observed between the melting and annealing curves in the presence of sodium, while there was a small (approximately 2 K) difference in the presence of potassium. Experiments performed at lower concentrations of these monovalent ions (not shown) produced lower T_m values as expected. The concentration dependence of these T_ms and the estimated ΔH values was similar to that previously determined for the unmodified quadruplex, generating values for n (the number of specifically bound K^+) of between 1.9 and 2.6.[27,29] These results suggest that, as expected, the inosine modification has not affected the interaction with the cations. No hysteresis was observed at low sodium concentrations, though the melting and annealing curves differed by 5–10 K when the experiments were performed in the presence of low potassium concentrations (10 mM). This is similar to that observed for the unmodified quadruplex,[26] and confirms that the modifications have not dramatically affected the properties of the folded quadruplexes.

We further examined the effects of these modifications by studying oligonucleotides containing two inosine substitutions: I3,9; I3,10; I4,9; I4,10 and I6,7. The results of these experiments in the presence of potassium and sodium are shown in Figure 3 and the T_m values are summarised in Table 1. It can be seen that this second substitution produces a further decrease in T_m of about 10 K. In the presence of potassium the most stable complex is formed with I3,9 while the least stable is I4,10; the T_m values of the other three complexes are very similar and have an intermediate stability. There is a 6 K difference between the most and least stable. A different pattern is evident in the presence of sodium, and again confirms that the identity of the cation affects the structure. In this case the most stable complex is generated by I6,7 (this can be clearly seen in Fig. 3 for which the difference between the potassium and sodium curves is much less than for the other oligonucleotides), I3,9 and I3,10 have equivalent stabilities; I4,9 and I4,10 are also equivalent and have the lowest stability.

We are not able to interpret these results in terms of one or other folded structure, since it is not clear whether two inosines within the same quartet will have a greater or lesser effect on the T_m than if they are at opposite ends of the folded structure. However, these data are consistent with the values obtained with singly substituted

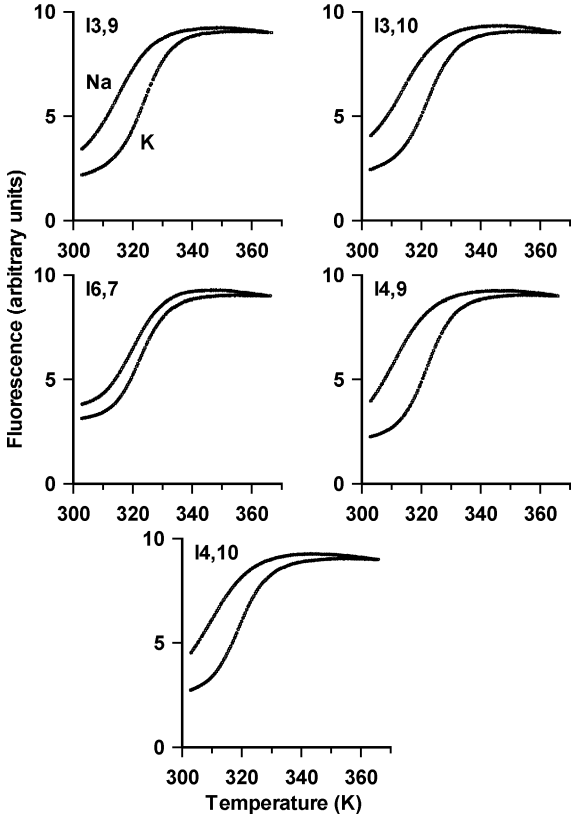

Figure 3. Representative fluorescence melting curves for the oligonucleotides that each contain two inosine substitutions. In each case the left curve corresponds to 100 mM sodium phosphate pH 7.4, while the right curve is for 50 mM potassium phosphate pH 7.4. The samples were heated at 0.1 °C/s. The *y*-axis shows the fluorescence intensity (arbitrary units), while the *x*-axis shows the temperature (K).

oligonucleotides. In each case the most stable complexes are produced when both inosines are from the most stable single substitutions (i.e., 3 and 9 for potassium and 6 and 7 for sodium), while the least stable are produced when both inosines are from the least stable position (i.e., 4 and 10 for potassium and 4 and 9 or 4 and 10 for sodium). The complexes with intermediate stability (I3,10; I6,7; I4,9 in potassium and I3,9 and I3,10 in sodium) correspond to substitutions in which one inosine is from the most stable and one from the less stable series.

ΔH values were estimated for each of the complexes by van't Hoff analysis of the melting curves and the values obtained are summarised in Table 1. It can be seen that a single inosine substitution changes ΔH by between 30 and 50 kJ/mol in the presence of potassium and between 37 and 67 kJ/mol in sodium. Similarly two substitutions affect ΔH by between 80 and 100 kJ/mol in potassium and 65 and 130 kJ/mol in sodium. These values do not show any correlation with the predicted parallel or antiparallel structures. In general inosine substitutions have a slightly greater effect in the presence of sodium, though the average change per substitution is about 45 kJ/mol.

These results demonstrate that the relative stabilities of these inosine-substituted quadruplex-forming oligonucleotides are different in sodium and potassium-containing buffers. This confirms that the quadruplex structure is altered by the identity of the monovalent cation. The results are consistent with a structure in which all four strands are parallel in the presence of potassium (Fig. 1d, lower part), while the sodium structure is mixed parallel and antiparallel.

Acknowledgements

This work was supported by a grant from the European Union.

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