A new class of DNA quadruplexes formed by oligodeoxyribonucleotides containing a 3′-3′ or 5′-5′ inversion of polarity site†

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Received (in Cambridge, UK) 31st March 2005, Accepted 31st May 2005
First published as an Advance Article on the web 22nd June 2005
DOI: 10.1039/b504455c

Unprecedented DNA quadruplex structures containing a 3′-3′ or 5′-5′ inversion of polarity site in the G-tract are presented; the quadruplexes are characterized by different elements of symmetry and glycosidic angle conformations.

The biological relevance of DNA G-quadruplex structures mainly lies in two features: their probable presence in many important regions of genomes and their fundamental role as a scaffold in several aptamers provided with useful biological properties.[1] In fact, the interest in G-quadruplexes is witnessed by hundreds of reports dealing with various aspects of these DNA secondary structures, such as the chemical nature and the topology of the backbone and the loops (where present), the conformation of the glycosidic linkage, as well as the influence of structural modifications on the stability of the complexes.[1] Several studies dealing with modified sugar-phosphate backbone containing G-quadruplexes have also been described.[2,3] However, to the best of our knowledge, no reports describing G-quadruplexes containing a 3′-3′ or 5′-5′ inversion of polarity included in the guanine tract have been reported yet, even though these backbone modifications are well-known to increase nuclease resistance in therapeutic oligodeoxyribonucleotides (ODNs) such as antisense ODNs[4] and aptamers.[5] In this frame, we wish to report here the NMR and CD studies of the two quadruplexes named **Q33** and **Q55**, formed by the ODNs $^{5'}$TGG$^{3'}$-$^{3'}$GGT$^{5'}$ and $^{3'}$TGG$^{5'}$-$^{5'}$GGT$^{3'}$, respectively, in which four strands are characterized by the presence of a 3′-3′ or 5′-5′ inversion of polarity site within the G-stretches.

ODNs $^{5'}$TGG$^{3'}$-$^{3'}$GGT$^{5'}$ and $^{3'}$TGG$^{5'}$-$^{5'}$GGT$^{3'}$ were synthesized by standard methods for the 3′-5′ tracts, and using 5′-phosphoramidites for the 5′-3′ tracts. For the former, a CPG resin linked to a thymine residue through the 5′OH function was also employed. The NMR samples of **Q33** and **Q55** were prepared at a concentration of 2.0 mM (0.6 ml, 90% H_2O/10% D_2O) and they were studied in two different buffers: 10 mM potassium phosphate, 70 mM KCl, 0.2 mM EDTA (pH 7.0) and 10 mM sodium phosphate, 70 mM NaCl, 0.2 mM EDTA (pH 7.0). The 1D proton spectra of both samples in the two buffers are almost superimposable.

Apart from some weak resonances due to very minor conformations also present in solution (whose relative intensities turned out to be insensitive to temperature changes), the one-dimensional proton spectrum of **Q33** (see ESI†) consists of well defined signals, suggesting that, in the conditions used here, the modified ODN mainly forms a single well-defined hydrogen-bonded conformation. This was confidently identified as a quadruplex structure as reported below. Particularly, two sharp resonances in the region corresponding to imino protons involved in Hoogsteen hydrogen bonds are present, along with one methyl and three base proton signals at higher fields. A comparison between the ^{1}H-NMR spectra of **Q33** and that of the unmodified [d(TGGGGT)]$_4$,[6] led us to conclude that the species under investigation is a quadruplex structure as well. Furthermore, the halved number of resonances in the spectrum of **Q33** clearly indicates it to possess further elements of symmetry, in addition to the fourfold symmetry.

The NOE pattern of **Q33** turned out to be similar to that observed for other parallel quadruplex structures.[6,7] As a matter of fact, an unbroken path of NOE connectivities between H8/H6 protons and the deoxyribose protons of the adjacent nucleoside at the 5′ side is clearly observable, which is typical of right-handed helix structures.[6,7] Furthermore, the lack of strong NOEs between any G H8 and H1′ of the same residue, in comparison to those observed between each G H8 and its deoxyribose H2′/H2″, suggests that all G residues are in the *anti* glycosidic conformation.[6,7]

It is interesting to note that, since all residues assume an *anti* conformation, all bases possess the same orientation with respect of the strand subunit orientation. Thus, the two tetrads comprising the inversion of polarity site will be characterized by an opposite (clockwise and anticlockwise) disposal, leading to a less efficient (head-to-head) stacking between a couple of Gs in each strand (Fig. 1, model **II**).

As for **Q55**, its ^{1}H-NMR spectrum (see ESI†) clearly indicates the presence of a unique species in solution, most likely a quadruplex structure, as suggested by a set of four resonances in the region of imino protons involved in Hoogsteen hydrogen bonds. Surprisingly, the number of signals in the spectrum of **Q55** matches that of [d(TGGGGT)]$_4$, rather than that of **Q33**. This datum points to a different degree of symmetry for the two novel complexes.

Useful information to elucidate the structure of **Q55** arose from ^{31}P-NMR spectroscopy. First, the one-dimensional proton-decoupled phosphorus spectrum displays five signals. After assigning the ^{1}H resonances within each deoxyribose by a 2D TOCSY experiment, the 2D proton-detected heteronuclear ^{1}H–^{31}P COSY allowed us to correlate each phosphorus resonance to the

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† Electronic supplementary information (ESI) available: expanded regions of 1D ^{1}H-NMR spectra of **Q33** and **Q55**. CD spectra and CD melting experiments of **Q33**, **Q55** and [d(TGGGGT)]$_4$. See http://dx.doi.org/10.1039/b504455c

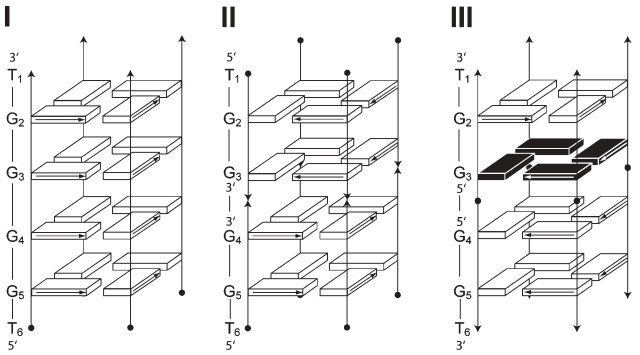

Fig. 1 Schematic illustration of the structures of $[d(TGGGGT)]_4$ (**I**), **Q33** (**II**) and **Q55** (**III**). Black arrowheads and circles indicate 3′ and 5′ edges of each subunit/strand, respectively. The *anti* and *syn* guanines are depicted as white and black solids, respectively. Arrows on solids indicate the direction of the proton donors and acceptors in Hoogsteen hydrogen bonds.

pertinent protons. Following this procedure, we unambiguously assigned the protons of six deoxyribose rings and five phosphorus. These data led us to hypothesize that, differently from that observed for **Q33**, no further elements of symmetry are present, whereas the four strands are equivalent to each other (fourfold symmetry). Confirmative evidences were gained from an extensive analysis of NOESY spectra of **Q55**, as described below.

Interestingly, only one G nucleotide shows an intense H8–H1′ NOE (Fig. 2), diagnostic of a *syn* glycosidic conformation. In agreement with literature data,[8] the H8 resonance of this *syn* G residue is upfield shifted with respect to those of the *anti* ones. This nucleotide shows a single sequential connectivity to another G residue which, in turn, is sequentially connected to a T residue. Furthermore, sequential connectivities between H8/H6 protons and the deoxyribose protons of the adjacent nucleoside at the 5′ side could be clearly observed for the remaining portion of the strand, comprised of two more G and a T residues.

The whole of the above data could be plainly interpreted assuming that the *syn* G residue is adjacent to the inversion of

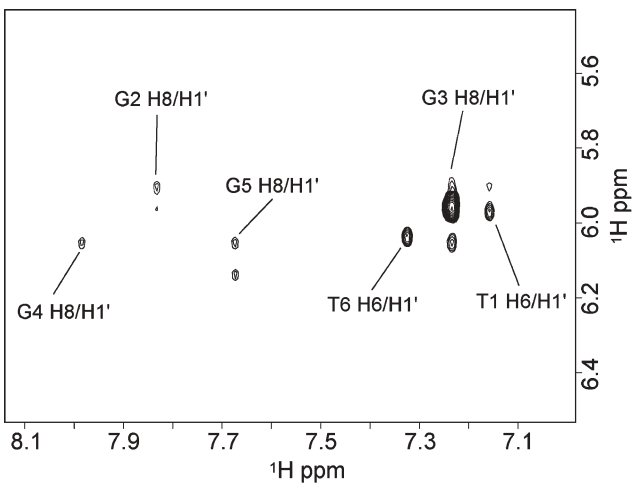

Fig. 2 2D NOESY (700 MHz, 100 ms mixing time, $T = 5$ °C) contour plots correlating base and sugar H1′ protons in **Q55**.

polarity site (Fig. 1, model **III**), and that the path of NOE connectivities is broken at this level. On the other hand, the existence of NOE sequential connectivities along the two subunits of the strand suggests that the backbone of **Q55** adopts a right-handed helix conformation.[6,7]

In the NOESY spectrum of **Q55** recorded in H_2O, we observed sequential imino–imino NOEs arising from intra-strand contacts, along with the connectivities between imino protons of G2 and G5 nucleotides with the adjacent methyls of T1 and T6 residues, respectively. Furthermore, NOE connectivities G3-H8/G3-NH, G3-NH_2 and G4-H8/G4-NH, G4-NH_2, also present in the spectrum, were ascribed to contacts between protons belonging to distinct, although equivalent, bases within each tetrad, thus further confirming the C_4 symmetry of the complex.

CD spectra for **Q33** and **Q55** and their natural counterpart $[d(TGGGGT)]_4$ (Fig. 1, model **I**) were acquired at 20 °C and are reported as supporting information.† All the measurements were performed at a concentration of 1×10^{-4} M, with both buffers used for NMR experiments. The CD spectrum of $[d(TGGGGT)]_4$ is characterized by negative and positive bands at 243 and 263 nm, respectively. The CD spectrum of **Q33**, instead, is characterized by a profile never observed before for quadruplex structures, having two positive bands at 253 and 294 and two negative bands at 232 and 270 nm, respectively. On the other hand, **Q55** is characterized by a CD profile very similar to those of folded antiparallel quadruplexes involving deoxyguanosine alternating between *syn* and *anti* conformations about the glycosidic bond[9] (two maximum bands at 250 and 295 nm and a minimum band at 270 nm). CD profiles of **Q33** and **Q55** do not match literature data regarding parallel and antiparallel quadruplexes so far reported. This is not quite surprising, considering the unprecedented structural features present in the novel complexes. Nevertheless, in order to elucidate the relationship between structure and CD profile, further studies on similar molecules are in order.

In order to estimate their thermal stability, **Q33** and **Q55** were subjected to melting and annealing CD experiments in comparison with $[d(TGGGGT)]_4$, under the same experimental conditions. All the measurements were performed at a concentration of 1×10^{-4} M, using both Na^+ and K^+ buffers. Taking into account that the rates of quadruplex formation/dissociation are very slow, we collected the data at 10 °C h^{-1}. **Q55** and $[d(TGGGGT)]_4$ were still structured even at 95 °C using a K^+ buffer, while we were able to obtain good melting profiles using a Na^+ buffer for all three samples (see ESI†). In spite of the very slow scan rate used, an extreme hysteresis phenomenon was observed (no sigmoidal annealing profile was obtained), thus indicating that the systems were not at equilibrium. Therefore, from the melting curves, the apparent melting temperatures (T_m) of 65 °C, 52 °C and 90 °C could be measured for $[d(TGGGGT)]_4$, **Q33** and **Q55**, respectively. A considerable increase (25 °C) in the apparent melting temperature could be determined for **Q55** when compared with the natural counterpart, while **Q33** was the least stable complex.

The above finding concerning the relative stabilities of the three complexes is intriguing and certainly deserves further studies in depth. At the moment, we can only hypothesize that, as far as **Q33** and **Q55** are concerned, the origin of the different behavior is to be sought for in the higher flexibility of the inversion of polarity site in **Q55**, where one of the two tetrads comprising the inversion of polarity site is allowed to arrange all residues in the *syn* glycosidic

conformation. Thus, the two subunits of each strand having opposite polarity, the two tetrads have the same orientation, similar to a canonical parallel quadruplex[6,7] (Fig. 1, models **I** and **III**), allowing a better stacking between the residues of the two G-quartets. The energy gain deriving from the more efficient stacking in the central, more structured part of the quadruplex exceeds the loss of stability due to the adoption of the less favorable *syn* glycosidic conformation for the G3 residues and the less efficient stacking between the G2 and G3 tetrads at the edge of the complex. More importantly, the higher structural flexibility, along with the unprecedented arrangement of the two tetrads in the middle of **Q55**, might also favor a more efficient complexion with Na^+, thus accounting for the increased T_m value of $[d(TGGGGT)]_4$. Molecular mechanics and dynamics calculations are currently in progress to verify these hypotheses.

In conclusion, the results described here could shed more light on the understanding of the variables involved in the formation and the stability of quadruplex structures. Furthermore, the new features found in the two complexes **Q33** and **Q55** might guide the design of novel aptameric and catalytic nucleic acids provided with novel structural motifs for ligand-binding pockets and/or diverse molecular recognition capabilities from those possessed by native RNA/DNA sequences.[10]

This work is supported by Italian M. I. U. R. (PRIN 2003 and 2004) and Regione Campania (L.41, L.5). The authors are grateful to "Centro Ricerche Interdipartimentale di Analisi Strumentale", C. R. I. A. S., for supplying NMR facilities.

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