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A thymine tetrad in d(TGGGGT) quadruplexes stabilized with Tl^+/Na^+ ions

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ABSTRACT

We report two new structures of the quadruplex $d(TGGGGT)_4$ obtained by single crystal X-ray diffraction. In one of them a thymine tetrad is found. Thus the yeast telomere sequences $d(TG_{1-3})$ might be able to form continuous quadruplex structures, involving both guanine and thymine tetrads. Our study also shows substantial differences in the arrangement of thymines when compared with previous studies. We find five different types of organization: (i) groove binding with hydrogen bonds to guanines from a neighbour quadruplex; (ii) partially ordered groove binding, without any hydrogen bond; (iii) stacked thymine triads, formed at the 3′ends of the quadruplexes; (iv) a thymine tetrad between two guanine tetrads. Thymines are stabilized in pairs by single hydrogen bonds. A central sodium ion interacts with two thymines and contributes to the tetrad structure. (v) Completely disordered thymines which do not show any clear location in the crystal. The tetrads are stabilized by either Na^+ or Tl^+ ions. We show that by using MAD methods, Tl^+ can be unambiguously located and distinguished from Na^+. We can thus determine the preference for either ion in each ionic site of the structure under the conditions used by us.

INTRODUCTION

The telomeric ends of chromosomes contain stretches of guanine-rich segments (1) that can form a variety of four-strand structures (2–4). There is strong evidence that these structures vary in their organization as a function of the sequence between consecutive tandems of guanines, but also depending on the ions present (5,6) and pH (7). The sequence we report here d(TGGGGT) is related to the telomeric repeat sequence found in Tetrahymena. A high resolution crystal structure of the latter sequence has been reported (2) showing that, in the presence of Na^+ and Ca^{2+} ions, the guanine quadruplexes are parallel-stranded. The four phosphodiester chains in the quadruplex show an identical 5′–3′ orientation. This structure will be named S3 in what follows. The same parallel conformation is found in solution in the presence of either Na^+ or K^+ ions (8). In a related study (9), Deng and co-workers have found that the RNA quadruplex $(UGGGGU)_4$ also forms parallel-stranded structures in the presence of Sr^{2+}. The main difference between both structures is the organization of the uracil/thymine groups, which in d(TGGGGT) are mostly stacked in external positions, whereas in UGGGGU they form either U4 tetrads, previously described by Cheong and Moore (10), or G4U4 octads.

In contrast with the invariable parallel structure found in d(TGGGGT), longer guanine rich oligonucleotides usually form antiparallel structures with thymine folds (3,11–13). However, divalent cations may induce a change into parallel structures (14). An interesting case is the human telomere sequence $d[AG_3(T_2AG_3)_3]$, which has been reported to be antiparallel by NMR (15), whereas a recent crystallographic study shows it to be parallel (4). The latter study developed an increased interest for the study of parallel-stranded guanine quadruplexes. Therefore, we decided to study the structure of d(TGGGGT) in the presence of Tl^+ ions, known to stabilize quadruplexes (16). Such ions are also helpful in crystallographic studies due to their high scattering power. MAD methods also allow their clear-cut localization. In fact our aim was to co-crystallize the oligonucleotide with anthracene drugs, but we were not successful. Other investigators have been recently able to obtain such co-crystals with a different drug, daunomycin (17). However, most thymines are very disordered and could not be visualized in the latter structure.

Our study shows substantial differences in the packing arrangement of thymines when compared with previous studies (2,9). We have obtained two different types of crystals (S1 and S2) which differ on the organization of thymine residues. In both cases thymines are found in the grooves of the quadruplexes and also form characteristic triads and tetrads different from those previously described (9).

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MATERIALS AND METHODS

Crystallization and data collection

The deoxyhexanucleotide d(TGGGGT) was synthesized by the phosphoramidite method and purified by gel filtration and reverse-phase HPLC. The ammonium salt of the hexamer was prepared by ion-exchange chromatography.

The purified oligonucleotide was dissolved at 5 mM concentration in 20 mM sodium cacodylate (pH 6.5) and quadruplex formation was induced by slow cooling from 70°C.

Crystals were grown at 4°C by vapour diffusion from hanging drops containing 20 mM sodium cacodylate (pH 6.5), 30 mM $MgCl_2$, 1 mM spermine tetrahydrochlorhide, 0.25 mM Thallium acetate, 20 µM lysine-anthraquinone, 0.5 mM d(TGGGGT). In S2 crystals, 2.5% (v/v) polyethylene glycol 400 was also present in the drops. We expected the drug to be co-crystallized with the oligonucleotide, but it was not incorporated into the crystal structure. Drops were prepared very carefully to avoid the disruption of the quadruplex; after 3 h, additives and drug were added; the precipitant was added the day after. The droplets were equilibrated against reservoirs containing 60 mM $MgCl_2$ and, either 10% MPD for S1 or 25% (v/v) polyethylene glycol 400 for S2. Crystals were non-colored rods and grew within 2 weeks to ~0.1 × 0.05 × 0.05 mm^3. Crystals were flash-cooled in a fiber loop at 100 K.

Data were collected at the BM14 beam line in the Grenoble synchrotron, using a CCD detector. Data collected at λ = 0.9170 Å were used to solve the S1 structure. MAD data were collected for both structures at wavelengths appropriate for thallium: λ_1 = 0.9800 Å (inflexion), λ_2 = 0.9772 Å (peak) and λ_3 = 0.9076 Å (remote), but the anomalous contribution was insufficient for the direct determination of the structures. Resolution and completeness were practically identical to those given in Table 1. However they could be used in the localization of Tl^+ as described below. The S2 structure was solved by using the data collected at λ_1 = 0.9800 Å. Processing was performed with the DENZO and SCALEPACK programs (18): crystals obtained under similar conditions, except for the precipitant, gave two different cells. Unit-cell parameters and data collection statistics are given in Table 1.

Structure determination and refinement

The quadruplexes in S1 crystals were localized by molecular replacement using the AMoRe program (19). Refinement was initially performed with CNS version 1.1 (20). The structure of Phillips and co-workers (2) was used as a model. Refinement data are shown in Table 1. Subsequent refinement was done with Shelx-97 (21) and CNS. Changes in the value of R-free were taken into account throughout the whole process. Water molecules were automatically generated by Shelx-97. Their positions were ascertained with the help of omit maps and intermolecular distances. No drug was visible in the electron density maps. A scheme of both structures is shown in Figure 1.

We did not find full occupancy of Tl^+ in any case (Table 2). One of the ions (Nr 1106) is described as Na^+, although the anomalous map showed the presence of Tl^+ at this site, with a low occupancy (~15%). Furthermore the ion-06(G) distances at this site correspond to Na^+ (Table 2). In general the degree of occupancy was determined by matching the electron density of the ionic sites. It should be noted that a Tl^+ occupancy of 10–15% is sufficient to detect this ion, given its strong scattering power. The presence of Tl^+ at ionic sites

Table 1. Crystal data and refinement of d(TGGGGT) structures

	Type S1	Type S2
Space group	P1	$P2_1$
Cell dimensions	a = 28.26,b = 35.41, c = 32.06 α = 83.73°,β = 61.78°, γ = 76.68°	a = 28.32,b = 56.34, c = 51.75 β = 99.17°
Asymmetric unit contents	(two quadruplexes)	(three quadruplexes)
	963 DNA atoms	1432 DNA atoms
	2 Na^+	6 Na^+
	5 Tl^+	6 Tl^+
	1 Mg^{2+}	–
	112 H_2O	87 H_2O
Wavelength (Å)	0.9170	0.9800
Resolution range (Å)	20–2.2	20–2.5
Number of unique reflections	5055	10915
Overall redundancy	3.16	5.43
Completeness (%) (overall/last shell)	93.5/83.3	98.3/97.3
R merge (Overall/last shell)	0.06/0.11	0.08/0.16
Work/test reflections	4218(78%)/503(9.3%)	7670(70.2%)/892(8.2%)
R-work/R-free (%)	18.5/24.2	22.2/31.3
R.m.s.d. (bonds/angles)	0.0039/0.66	0.0045/0.74

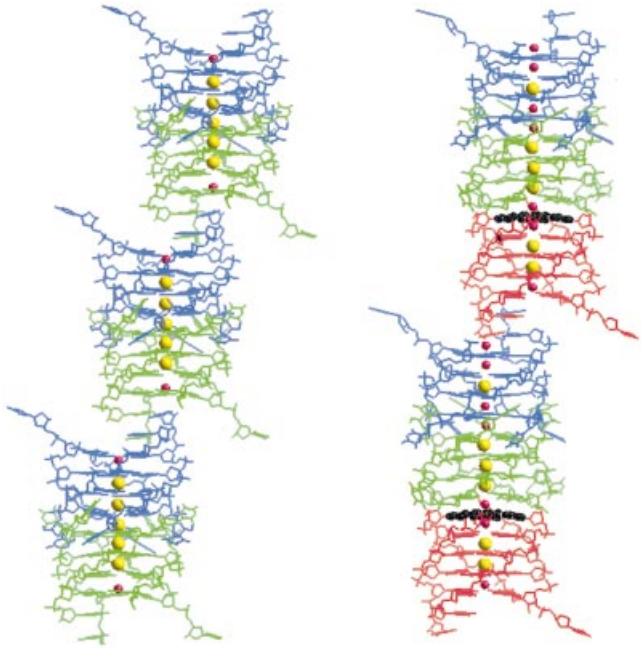

Figure 1. Quadruplex columns in d(TGGGGT) crystals; left, the S1 structure in space group P1; right, the S2 structure in space group $P2_1$. Each quadruplex type is shown in a different color. A thymine tetrad is shown in black, with atoms as small spheres. It belongs to the red quadruplex. Ions are shown as yellow (Tl^+) and pink (Na^+) balls. Parallel columns are packed on the plane of the paper and form layers, which are stabilized by thymine interactions between neighbor columns. Layers parallel to the plane of the paper show no apparent contacts among themselves.

Table 2. Average ion-guanine distances in S1 quadruplexes

Ion	Occupancy	Ion-O6(G) average distance in Å[a]
Tl 1101	0.60	2.85 (0.16)
Tl 1102	0.60	2.89 (0.15)
Tl 1103	0.70	3.00 (0.12)
Tl 1104	0.30	2.78 (0.10)
Tl 1105	0.30	2.83 (0.09)
Na 1106	0.85	2.48 (0.09)
Na 1107	1.00	2.51 (0.05)

[a]The averages correspond to eight ion-O6(G) distances in Tl^{+1} and to four distances in Na^{+1}.

was confirmed by the use of high-resolution anomalous diffraction data. The phases of the model were introduced in the anomalous data and Tl^+ ions appeared very clearly. An example is given in Figure 2. This approach is particularly useful, since Na^+ ions exhibit very little anomalous effect (22).

The quadruplexes in S2 crystals were located with EPMR (23). The S1 structure was used as a search model. Two quadruplexes could be immediately located, but the third one appears to be less ordered and could not be easily located. Refinement was carried out with CNS. Due to the lower quality and resolution of the data available, only the main features of this structure will be presented in this paper.

A general problem in refining this type of quadruplexes is the disorder found in some of the thymines, which occupy flipped-out positions. Some of them are not apparent in the electron density map. This feature does not depend on resolution, since in the S3 structure, determined at 0.95 Å resolution, several thymines could not be located.

RESULTS

Thallium ions as a crystallographic aid

Thallium ions have a high electron density and a strong phasing power for MAD experiments. For that reason we introduced Tl^+ in our crystallization trials. In DNA duplexes it is known that this ion binds with low occupancy to many sites (24). Our aim was to direct Tl^+ specifically to the ion sites placed between G-tetrads. Thus we used a low concentration of Tl^+ in order to avoid overall binding to phosphates and bases (24). The presence of chloride ions in the crystallization buffer is not sufficient to precipitate TlCl, since it has a solubility of ~3 g/l. It is known (5,16) that Tl^+ has a strong affinity for G-tetrads, much higher than Na^+, also present in the crystallization buffer. Therefore we expected to find a substantial amount of Tl^+ in the quadruplex crystals. Nevertheless Na^+ will compete with Tl^+ and we only found partial occupancies (Table 2).

Packing

We have obtained two types of crystals: S1 and S2. S1 has two quadruplexes in the asymmetric unit in the P1 space group, whereas S2 has three quadruplexes in the P21 space group. We will compare our results with the S3 structure described by Phillips and co-workers (2) for the same oligonucleotide.

Packing of quadruplexes in the S1 and S2 crystals is similar to that found in the S3 structure; they are organized in columns as shown in Figure 1. The columns form layers stabilized by thymine interactions with quadruplexes in neighbor columns. Divalent cations also contribute to stabilize this side-by-side organization. Both S3 and S1 crystallize in the P1 space group, but the S3 structure contains four quadruplexes in the asymmetric unit, whereas our S1 structure contains only two. In both cases, the quadruplexes are packed in a head-to-head fashion, with direct stacking between the G-tetrads at the 5′-ends of neighbor quadruplexes. The main difference between S1 and S3 resides in the organization of the thymines at the 5′-end of the quadruplexes. At the 3′-ends, two layers of thymine triads are found between the G-tetrads. The organization of thymines will be described in detail below.

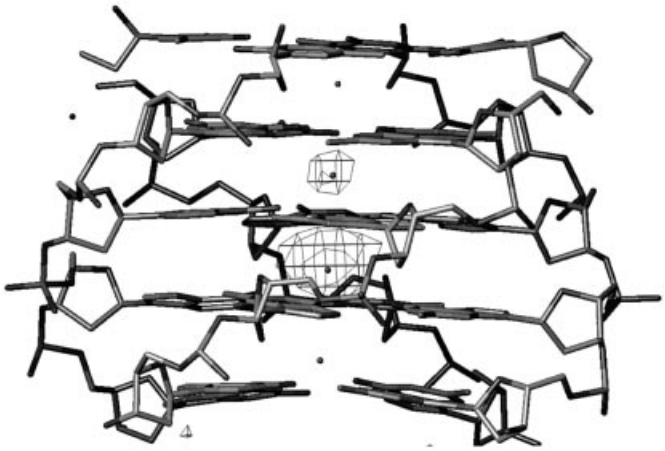

Figure 2. A view of one quadruplex in the S2 structure, with the anomalous fourier map superimposed. The thymine tetrad is at the top of the figure. Ions (Na^+, Tl^+) are shown as small spheres. The largest density peak is centered over TL1703 (occupancy 0.6) and the smaller peak over TL1704 (occupancy 0.4). Above and below are sodiums, and these do not show any density in the anomalous map.

The quadruplex columns in the S2 structure show remarkable differences with S1 and S3. Two of the quadruplexes (shown in green and blue in Fig. 1) are also packed head-to-head with the G-tetrads at the 5′-end stacked. At the 3′-end, one of the quadruplexes (red) also shows two floors of thymine triads upon interaction with the next quadruplex in the column (blue). The unique feature of S2 is that thymines at the 3′-end of one quadruplex (red) form a tetrad layer. The four thymines (black) are stacked between two G-tetrads, one of them from the 3′-end of the next quadruplex (green) in the column. The 5′–3′ orientation of the phosphodiester chains is the same in the red and green quadruplexes, whereas those in the blue quadruplexes run in opposite directions. Thus, in the asymmetric unit of S2, we find a unique stacking pattern with 12 G-tetrads, two T-triads and one T-tetrad. Such an organization of quadruplexes differs from those described in S3 and in UGGGGU(9).

Quadruplex structure

The quadruplex structure is practically identical to those previously described, as reported in the Introduction. The main difference lies in the use of Tl^+ ions; we report the first X-ray quadruplex structure stabilized with Tl^+ ions. The ions are found at the usual position, between two G-tetrad layers.

However, in no case we have found full occupancy by Tl^+, as shown in Table 2. This is expected, since the concentration of Na^+ is much higher than that of Tl^+. In fact, one of the sodium ions (Nr 1106) in a 3′-terminal G-tetrad of S1 (Fig. 1) has been refined as a Tl^+ ion with 15% occupancy. We have chosen to represent it as a Na^+ ion due to the low Tl^+ occupancy. Furthermore the ion-guanine distances correspond to those of Na^+.

All guanine sugar rings are found in the C2′-endo conformation, with the exception of those belonging to the first guanine tetrad in one of the two quadruplexes of S1, which are in the C3′-endo conformation. This is a clear difference between the two quadruplexes in the asymmetric unit, which is also found in S3. It appears that the stacking of the terminal G-tetrads of two quadruplexes requires such conformation in the sugar rings of one of the tetrads in order to avoid steric clashes (2).

Groove binding thymines

There are three different types of organization shown by thymines: groove binding, triads and a tetrad. We will analyze them separately, starting with the groove binding case.

The 5′ terminal thymines occupy an external position next to the region where two neighbor quadruplexes present a direct stacking between two G-tetrads, as shown in Figure 3. However, their organization is different for each of the two stacked quadruplexes. One set of thymines is rather disordered and only shows van der Waals interactions with the grooves of the next quadruplex. The other set of thymines penetrate more deeply into the groove and form hydrogen bonds between their O2 atoms and the N2 atoms of guanines in the first tetrad of the next quadruplex. The thymines are located in the grooves of the quadruplex between the first and second guanine tetrads and are further stabilized by van der Waals interactions. This organization is similar to the guanine/uridine octad described in UGGGGU, but in that case hydrogen bonds are instead formed with the second tetrad of the quadruplex (9).

As mentioned above, the sugar pucker of G2 is different in the two stacked quadruplexes. The quadruplex that starts with the thymines hydrogen bonded to guanines in the grooves has a C2′-endo conformation in both T1 and G2, whereas the next quadruplex has thymines with different puckers and G2 has a C3′-endo conformation. The latter thymines are less well ordered. In contrast, in the S3 structure, hydrated Ca^{2+} ions occupy this region. Thymines do not enter the grooves. They are rather disordered and occupy the solvent channels between quadruplexes. Some of them stack in pairs, as described in detail by Phillips and co-workers (2).

Thymine triads

Triads are formed by thymines at the 3′-ends of the quadruplexes, as shown in detail in Figure 3B and C. Those from the stacked quadruplexes form hydrogen-bonded pairs that involve their N3 and O4 atoms. A third thymine from a neighbor quadruplex interacts through a water molecule. In the S3 structure, some of the latter thymines are rather disordered and do not show this interaction. In all cases (S1, S2 and S3) the fourth 3′-terminal thymine in all quadruplexes is disordered and has not been located in the electron density maps.

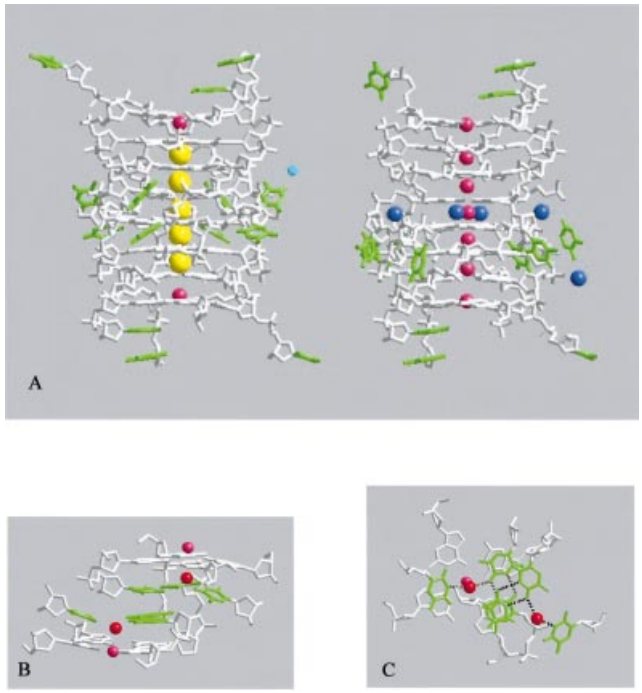

Figure 3. (**A**) Comparison of the S1 (left) and S3 (right) structures. Thymines are shown in green. In S3, hydrated Ca^{2+} ions (in blue) occupy the central region of contact between G-tetrads, as described by Phillips and co-workers (2). In the S1 structure this region is occupied by thymines, four of which are well ordered in the grooves of the next quadruplex. The other four thymines are also found in the grooves of the other quadruplex, but are less well ordered. Note that in the S3 structure, the thymines occupy external positions and are poorly ordered, some sugars are even missing in the coordinate file. Divalent cations contribute to stabilize neighbor quadruplex columns: Ca^{2+} in S3 and Mg^{2+} (cyan) in S1. Hydration waters are not included. Monovalent ions are shown as yellow (Tl^+) and pink (Na^+) balls. Water molecules are red. (**B** and **C**) The two thymine triads (green) intercalated between the 3′-terminal G-tetrads of two quadruplexes. Sodium ions are shown in pink and water molecules in red. In (C), shown in projection, the upper G-tetrad has been removed. Hydrogen bonds (black dashed lines) are formed between the N3 and O4 atoms of two thymine pairs. The third thymine interacts through a water molecule. The drawing corresponds to S1, similar triads are present in S2 and S3.

Thymine tetrad

The thymine tetrad observed in S2 is found at the 5′-end of the sequence. It is presented in Figure 4. Two of the thymines show more disorder. It appears that they are stabilized in pairs by single O4-N3 hydrogen bonds. A central Na^+ interacts with two thymines. The four thymines are intercalated as a rather planar structure between two guanine tetrads. A similar thymine tetrad has been described by NMR (25) in d(TGGTGGC). The authors claim that all thymines form O4-H3 hydrogen bonds, an interaction which is not found in the tetrad structure obtained by us (Fig. 4). Such interaction does not appear possible due to O2-methyl clashes. In fact the results reported by Patel and Hosur (25) are consistent with a structure such as the one found by us, shown in Figure 4, which has no O2-methyl clashes.

The organization of thymines is different from the uridine tetrads reported in a related RNA study (9). In the latter case, the uridines are at the other end of the sequence (3′-end) and are all hydrogen bonded in a similar way, but have a pyramidal

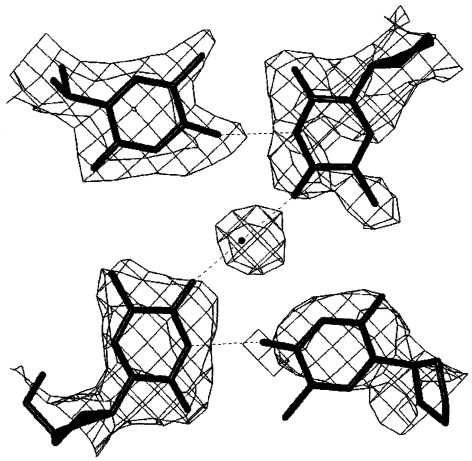

Figure 4. Electron density omit map of the thymine tetrad in S2. The map, calculated using all the resolution range data, was contoured at 0.8 σ. Two of the thymines show higher disorder as it is apparent from the figure. The central ball corresponds to Na^+. Hydrogen bonds and ionic interactions are shown as dashed lines.

conformation and a central Na^+ ion. They are stacked on a guanine tetrad on one side, whereas on the other side they are in contact with water molecules. The absence of the methyl group in uridine compared with thymine allows O4-N3 hydrogen bonding of all uridines in the tetrad, which does not appear to be possible in the S2 thymine tetrad due to O2-methyl clashes.

DISCUSSION

The results we have obtained show that the G quadruplexes have a uniform structure in the three crystal structures compared, although the interactions of each terminal G-tetrad are different. A peculiar feature is that the conformation of G2 is different in each pair of quadruplexes which is associated with the conformation of the thymine bases. Thymines do not intercalate into the quadruplexes, they always occupy external positions. In our crystallization trials an intercalating drug was present, but it appears that it cannot easily disrupt the quadruplex structure for intercalation between two internal G-tetrads. In fact, all drugs that have been co-crystallized are found stacked on a terminal floor of the quadruplex (17,26).

Although some of the thymines are disordered, other thymines show a clear pattern of interactions, different from those described by Phillips *et al.* (2) in S3. It appears that the thymines at both ends of the oligonucleotide may contribute to stabilize the parallel structure of the quadruplex, in contrast with the antiparallel structures found in other cases (6,14).

The structure of d(TGGGGT) is apparently similar to that described for UGGGGU (9), which is also parallel, but in the latter structure most sugar rings are in the C3′-endo conformation, as expected for an RNA chain.

The terminal thymines show a considerable variability in conformation, as shown in Figures 3 and 4. Although the two quadruplexes in the asymmetric unit are similar and appear to be related by a pseudo-dyad through the central Tl^+ ion (Fig. 3), the thymines in the central region are clearly different: one quadruplex has thymines hydrogen bonded inside the grooves, whereas those in the other quadruplex are external and less well ordered. In the S3 structure, also shown in Figure 3, the same region is occupied by Ca^{2+} ions and thymines occupy external positions and are poorly ordered.

The thymine tetrad found in S2 (Fig. 4) is clearly different from the uracil tetrad found in UGGGGU (9). In the latter case all bases show a hydrogen bond with two neighbor uracils, but this is not possible in the case of S2 due to the methyl groups in thymines. Furthermore, the thymine tetrad is found at the 5′-end of the sequence and is stacked between two guanine tetrads (Fig. 1), whereas in UGGGGU it is found at the 3′-end. Furthermore, in the latter case it deviates significantly from planarity and interacts only with one guanine tetrad, water molecules cover the other side.

The finding of an intercalated thymine tetrad, similar to that reported by Patel and Hosur (25), indicates that such tetrads might be present in telomere sequences such as TG_{1-3} found in yeast (27). In fact there are reports (28,29) that suggest that (TG)n sequences may also form quadruplex structures.

With regard to the ions, the distances to the O6 guanine atoms (Table 2) appear to be optimal for Tl^+ (5), but it is often replaced by Na^+. It should be noted that Tl^+ has an ionic radius similar to K^+, which is known to be optimal (5,30) to stabilize G4 quadruplexes. Na^+ prefers shorter distances, with an average close to 2.5 Å, as is the case in S1. Thus Na^+ ions tend to be found closer to the plane of the G-tetrad, rather than between two G-tetrads, although some Na^+ ions do not follow this trend, as is very clear in the S3 structure (Fig. 3) (2). These observations confirm that monovalent cations may substitute each other in oligonucleotide structures, as found in standard duplexes (31). However each ion has its preferred interatomic distances and may thus influence the overall structure and its stability (5). It has been shown that Tl^+ can substitute other monovalent cations by competing for binding regions within duplex DNA, of between 20 and 35% occupancy (24). We observed that the thalliums positioned between the G-tetrad planes, confirmed by its anomalous signal, also show a wide range of occupancies (Table 2), inferring a mixed population with other available counterions at these sites. In the quadruplex structures S1 and S2 we observe a single density peak between the G-tetrads implying that the folded quadruplex defines the size of the central cavity, that is filled by the availability of suitably sized counter ions.

In summary, our results show that the parallel G4-quadruplex has a very uniform structure, independent of the ions present, whereas the terminal thymines can adopt a variety of structures, as illustrated in the figures presented in this paper. As shown in Figure 2, anomalous scattering data allow a clear analysis of occupancy in each ion site.

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