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The thrombin binding aptamer GGTTGGTGTGGTTGG forms a bimolecular guanine tetraplex

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Abstract

In the literature, the thrombin binding aptamer GGTTGGTGTGGTTGG is generally taken as a prototype of an intramolecular guanine tetraplex of DNA. Our results, however, show that this notion is not true in aqueous solutions. This conclusion is based on a dependence of the CD spectra on aptamer concentration, migration of the aptamer in polyacrylamide gels, and the Ferguson analysis of the gel migration data. The presented data document that the aptamer forms a bimolecular tetraplex. We furthermore show that only an extension of the aptamer by a sequence containing further guanines, or an elongation of loop regions, causes that its tetraplex folding is intramolecular.
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Keywords: DNA tetraplex; Thrombin binding aptamer; CD spectroscopy; Gel electrophoresis

Aptamers are nucleic acid molecules that specifically bind to various biologically important molecules. As such, they represent a potential tool for therapeutic purposes. Various aptamers were synthesized and their structural properties were studied to optimize binding properties to the target molecules. One of the most intensely studied aptamers is the DNA 15-mer GGTTGGTGTGGTTGG that specifically binds to thrombin and inhibits thrombin activity in the cascade of reactions resulting in blood clotting [1–3]. With the molecule of the thrombin aptamer, one three-dimensional structure was proposed and studied in detail [4–16]. It was suggested that the aptamer folded into a compact intramolecular tetraplex with an antiparallel orientation of strands in the chair-like conformation [4–13].

In the present work, we have studied the thrombin aptamer and related DNA molecules by a combination of CD spectroscopy, PAGE, and UV absorption spectroscopy. It was surprising in the light of the literature interpretations that the magnitude of the tetraplex CD signal depended on the aptamer concentration. This challenged the idea of an intramolecular aptamer tetraplex structure. And indeed, PAGE showed that the tetraplex was bimolecular. This conclusion was unambiguously confirmed by the Ferguson analysis of the aptamer gel migration. In parallel, we have studied the thrombin aptamer variants with slightly different primary structures to find that lengthening of the sequences that generate the loops between the guanine tetrads, or addition of a 5′ end tail containing additional G's, leads to formation of an intramolecular tetraplex.

Materials and methods

The oligonucleotides were purchased from VBC Genomics Bioscience Research (Vienna, Austria). The lyophilized oligonucleotides were dissolved in 1 mM sodium phosphate and 0.3 mM EDTA, pH 7, to give a stock solution concentration of ~100 OD/ml. The accurate sample concentrations (related to nucleosides) were determined from their absorption measured at 90 °C in the above buffer using molar extinction coefficients calculated according to [17]. The UV absorption spectra were measured on a UNICAM 5625 UV/VIS spectrometer. Before starting the experiments, all the oligonucleotide samples were denatured (10′ at 90 °C) in the above low salt solution to remove aggregates. The sample was then left to cool to room temperature. Buffers and salts were added to denatured samples

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directly in the CD cells. The salt and DNA concentrations were then corrected for the increase of the sample volume. Formation of tetraplexes was followed at 0 °C, unless stated otherwise.

The CD spectra were measured using a Jobin-Yvon Mark VI dichrograph in 0.1–2 cm pathlength Hellma cells, placed in a thermostatted holder. DNA concentration was taken to give absorption around 0.8, which corresponds to the best signal to noise ratio. Unless stated otherwise, the DNA concentration was ~0.8 mM (in nucleosides—0.1 cm cell). Circular dichroism was expressed as the difference in the molar absorption of the right-handed and left-handed circularly polarized light, $\Delta\varepsilon$, in units of M^{-1} cm^{-1}. To compare the oligonucleotides of different lengths, the molarity (M) was related to the whole DNA molecules.

The thermodynamic parameter ΔH of the individual tetraplexes was determined from the melting curves generated by monitoring the 295 nm CD band as a function of temperature according to [18]. The molecularity of the tetraplex, occurring in the equation for calculation of the Van't Hoff enthalpy change, was taken 2 or 1 depending on the gel electrophoresis results.

Non-denaturing polyacrylamide gel electrophoreses were performed in a thermostatted submersible apparatus (SE-600, Hoefer Scientific, San Francisco). The gels (12%, 14%, 16%, 18%, and 20%, 29:1 monomer/bis ratio), 14 × 16 × 0.1 cm in size, were run for 16 h at 60 V (~5 V/cm) and 2 °C. Two micrograms of DNA was loaded on gels. The gels were stained with Stains-All (Sigma). Densitometry was performed using a Personal Densitometer SI, Model 375-A (Molecular Dynamics, Sunnyvale, CA).

Results

The GGTTGGTGTGGTTGG aptamer generates a guanine tetraplex even at very low ionic strength, i.e., in 1 mM sodium phosphate (Fig. 1). Its CD spectrum has a positive maximum at 295 nm and a negative maximum at 265 nm, which is characteristic of an antiparallel tetraplex arrangement. This conclusion is in line with the literature [10,19–21]. The same type of CD spectrum is displayed by the aptamer in the presence of KCl (Fig. 1), only the CD amplitudes are larger, which may reflect an increased tetraplex population. Also, ethanol induces the same type of CD spectrum, which is in line with our recent finding [22] that ethanol stabilizes DNA guanine tetraplexes. In contrast, the CD amplitudes are smaller in the presence of NaCl but the type of the CD spectrum is the same again. Thus, the aptamer is very stable in the antiparallel guanine tetraplex under various solvent conditions. The UV absorption spectra exhibit hyperchromicity at 295 nm, which is also characteristic [23] of guanine DNA tetraplexes. The differential absorption spectra (the spectrum at the actual temperature minus the spectrum measured at 81 °C) are shown in inset A of Fig. 1. Isosbestic points in the UV absorption spectra reflect a two-state thermal melting of the aptamer, which indicates the presence of a single tetraplex arrangement before melting. The same conclusion follows from the presence of isoelliptic points in the temperature dependence of the CD spectra (Fig. 2).

At low salt (1 mM Na phosphate), the aptamer melts at room temperature (Fig. 2 and inset). The presence of 0.12 M KCl or of 50% ethanol at low salt shifts the melting temperature upward by 30 °C and 25 °C, respectively (Fig. 2, inset, and Table 1). In contrast, NaCl does not stabilize the tetraplex. Similarly, ΔH significantly increases in the presence of KCl (Table 1) [10,15,20,23] as well as in ethanol, but it even decreases after addition of NaCl (Table 1).

It was surprising that the CD amplitudes (Fig. 1B) as well as the melting temperatures (not shown) depended

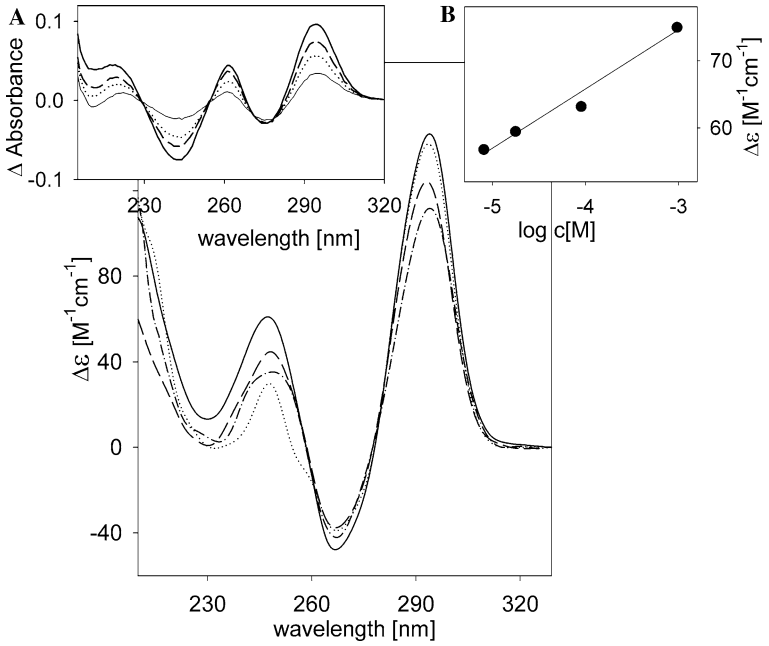

Fig. 1. CD spectra of the thrombin binding aptamer measured in (dashes) 1 mM Na phosphate + 0.3 mM EDTA, pH 7, (dots) ethanol, added to the previous sample up to a 50 % concentration, (dash-dot) 10 mM Na phosphate + 0.125 M NaCl, pH 7, and (solid line) 10 mM K phosphate + 0.1 M KCl, pH 7. DNA concentration was 0.8 mM (in nucleosides), temperature 0 °C. (A) Differential absorption spectra measured in the above 0.115 M K^+ at (solid line) 11 °C, (dashes) 37 °C, (dots) 45 °C, and (thin full line) 52 °C. The spectrum measured at 81 °C was subtracted. (B) The value of the positive amplitude at 295 nm as a function of the logarithm of the aptamer concentration measured in 10 mM Tris–HCl, pH 7.1, at 15 °C.

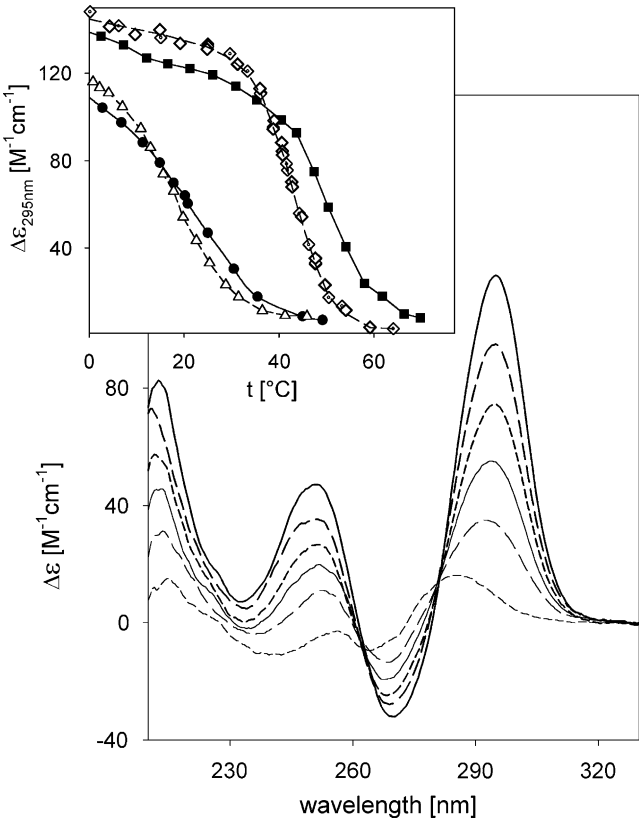

Fig. 2. CD spectra of the aptamer measured in 1 mM Na phosphate + 0.3 mM EDTA, pH 7, at (from the solid to the thin short dashed line) 0, 10.8, 15.6, 19.8, 25.4, and 45.9 °C. (Inset) Melting curves of the aptamer in (full squares) 10 mM K phosphate + 0.1 M KCl, pH 7, (rhombs and dotted rhombs) 50 % ethanol, measured in 1 cm and 2 cm cells, respectively, (open triangles) 1 mM Na phosphate + 0.3 mM EDTA, pH 7, and (full circles) 10 mM Na phosphate + 0.125 M NaCl, pH 7. Melting is monitored by $\Delta\varepsilon$ changes at 295 nm.

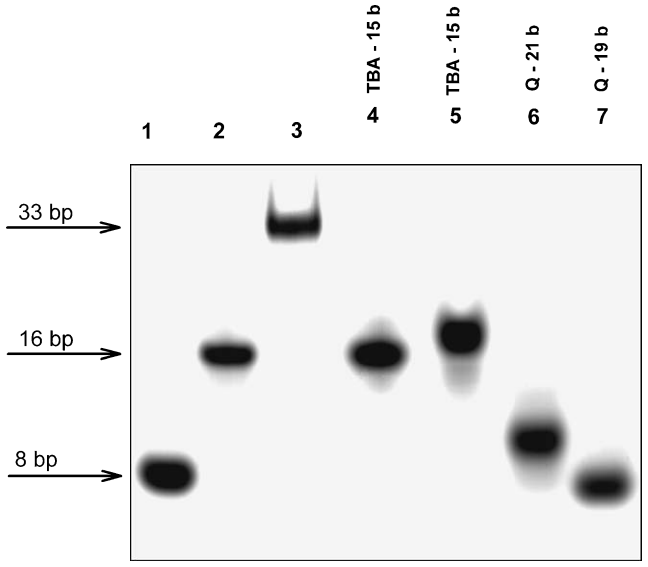

Fig. 3. Native polyacrylamide gel electrophoresis run in 10 mM K phosphate + 0.1 M KCl, pH 6.1, at 2 °C. Lanes 1–3, markers for 8 (C_4G_4*C_4G_4), 16 ($G_3TG_4AG_3TG_3$*$C_3AC_3TC_4AC_3$), and 33 ($TG_4AG_3TG_4AG_3TG_4A_2G_2TG_4A$*$TC_4AC_2T_2C_4AC_3TC_4AC_3TC_4A$) base-pair DNA duplexes, respectively; lanes 4 and 5, thrombin binding aptamer loaded at 1 mM and 0.06 mM nucleoside concentration, respectively; lane 6, the 21-mer $GTAG_2TG_2T_2G_2TGTG_2T_2G_2$, and lane 7, the 19-mer $G_2T_3G_2T_2GT_2G_2T_3G_2$. The samples were transformed into 10 mM K phosphate + 0.1 M KCl, pH 6.1, at room temperature and then denatured (5′/90 °C) and left to cool slowly before loading on the gel.

on aptamer concentration. This indicates that the tetraplex is intermolecular. As this conclusion on intermolecular thrombin aptamer conformation contrasts with claims in the literature e.g., [4–13], we performed parallel PAGE studies of the aptamer and related DNA molecules. The electrophoresis in Fig. 3 shows that the aptamer comigrates with the duplex of the fragment with a comparable length. Thus, the aptamer tetraplex is an associate of two molecules. The conditions of the electrophoresis (110 mM K^+ and pH 6.1) correspond to those of NMR measurements [4]. The same result was obtained at neutral pH, in NaCl or at low salt. We compared aptamer migration with the migration of DNA fragments that had very similar primary structures to exclude the possibility that the base content or the secondary structure is what slows down the migration of the aptamer (Fig. 3). An appropriate molecule was an analogue of antiasthmatic oligonucleotides [24], the GTAGGTGGTTGGTGTGGTTGG 21-mer, which shared the 15 nucleotides with the aptamer and was extended by the GTAGGT hexamer on the 5′ end. Furthermore, we analyzed a 19-mer fragment GGTTTGGTTGTTG GTTTGG, where an additional T was present at the place of each T in the original 15-mer aptamer. Both the 21-mer and the 19-mer migrated as monomers (Fig. 3). Fig. 4 shows that both formed tetraplexes under the conditions of electrophoresis. They were slightly less thermostable than the thrombin aptamer tetraplex (Table 1) and, also, their ΔH values were lower (Table 1). Thus, the 21-mer and the 19-mer folded into intramolecular guanine tetraplexes while, in contrast, the tetraplex of the aptamer 15-mer was bimolecular. The denaturation curve of the

Table 1
T_m and ΔH of the studied tetraplexes

	Conditions	T_m [°C]	$-\Delta H$ [kJmol^{-1}]	Molecularity
Thrombin aptamer	1 mM Na phosphate	~18	167	2
	0.12 M K^+	47.7	212	2
	0.14 M Na^+	21.3	131	2
	50% Ethanol	42.7	173	(1)
$GTAG_2TG_2T_2G_2TGTG_2T_2G_2$	0.12 M K^+	39.6	141	1
$G_2T_3G_2T_2GT_2G_2T_3G_2$	0.12 M K^+	41.7	141	1

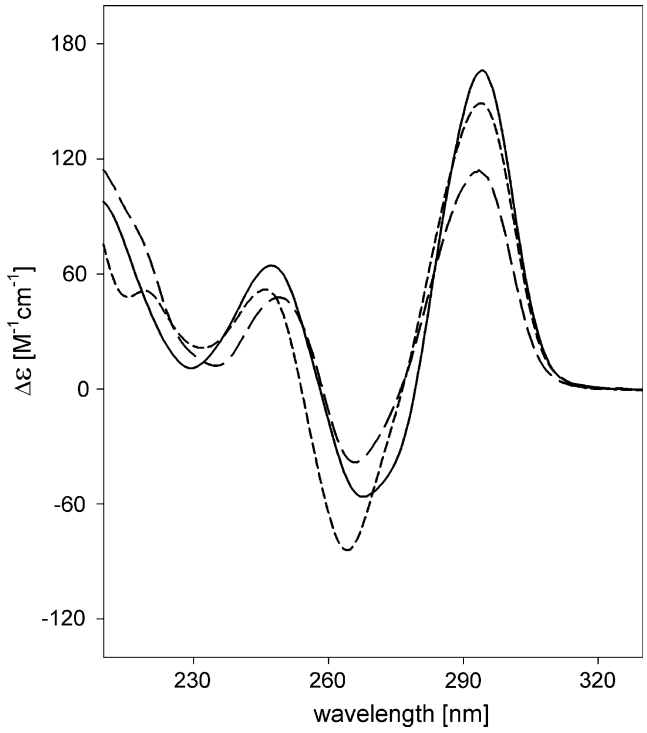

Fig. 4. CD spectra of tetraplexes of (solid line) the thrombin binding aptamer, (short dashes) the 21-mer $GTAG_2TG_2T_2G_2TGTG_2T_2G_2$, and (long dashes) the 19-mer $G_2T_3G_2T_2$ $GT_2G_2T_3G_2$ measured in 0.1 M KCl and 10 mM K phosphate, pH 7, at 0 °C.

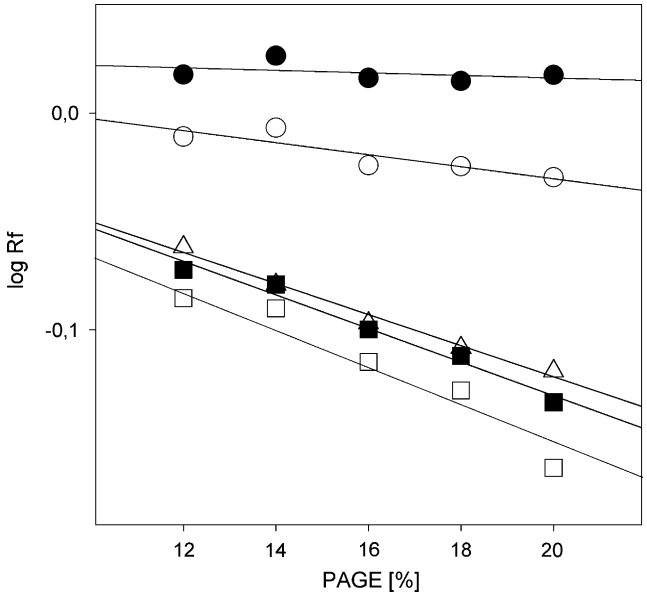

Fig. 5. Ferguson plots of the thrombin aptamer tetraplex loaded on the gel at (open triangle) 1 mM and (open square) 0.05 mM nucleoside concentration, of (full circle) the 19-mer $G_2T_3G_2T_2GT_2G_2T_3G_2$, and of (open circle) 21-mer $GTAG_2TG_2T_2G_2TGTG_2T_2G_2$ tetraplexes, and of (full square) the $G_3TG_4AG_3TG_3{*}C_3AC_3TC_4AC_3$ duplex. The concentration of the latter oligonucleotides was 0.4 mM in nucleosides. The logarithm of relative mobility (with respect to that of $C_4G_4{*}C_4G_4$ duplex) is plotted against the concentration of acrylamide in the gel. The gels were run at 2 °C in 0.1 M KCl and 10 mM K phosphate, pH 6.1.

aptamer tetraplex stabilized by ethanol was the same in the 1 cm and 2 cm pathlength cells (Fig. 2, inset). In the 1 mm pathlength cells, the denaturation temperature was curiously still a bit lower (not shown).

We performed the Ferguson analysis of migration data of the studied oligonucleotides (Fig. 5). Electrophoretic migration of the aptamer 15-mer was compared with those of the 19-mer, 21-mer, and a normal 16 bp long heteroduplex. The aptamer was loaded on the gel at two concentrations differing about 20 times. In both cases, the slope of the Ferguson plots corresponded to that of the heteroduplex. This confirmed a bimolecular nature of the tetraplex formed by the 15-mer aptamer GGTTGGTGTGGTTGG in aqueous solution.

Discussion

The 15-mer aptamer GGTTGGTGTGGTTGG was originally selected from a large pool of DNA fragments as the molecule that selectively bound thrombin [25]. Subsequently, it was subject to numerous detailed NMR [4–6,8,10–13], X-ray diffraction [7,9], and other studies [1,16,20,22] to identify the origin of the thrombin binding selectivity [1–3,26]. It was clear very soon that a guanine tetraplex stood behind the selective binding. However, guanine tetraplexes are of many kinds [27] differing by the number of molecules in the tetraplex (intramolecular, bimolecular, tetramolecular, and octamolecular), mutual orientation of strands (parallel, antiparallel), position of the loops in the tetraplex, and syn-anti glycosidic orientation of the dG residues. In this direction, the crystal study showed that the tetraplex of GGTTGGTGTGGTTGG was antiparallel and intramolecular. The solution structures, however, often do not correspond to structures observed in crystals [28,29]. The NMR results [4] are consistent with both intramolecular and bimolecular tetraplex of the aptamer. The authors preferred the intramolecular interpretation because a mixture of two aptamer analogues, where G was substituted by I at various places, indicated no dimerization. Then, papers accumulated [5–16] considering only the intramolecular model. Our studies, in line with these studies, confirm that the tetraplex is antiparallel because CD spectroscopy is reliable regarding discrimination between parallel and antiparallel guanine tetraplexes [30]. However, in this paper we present conclusive evidence that the tetraplex is bimolecular and not monomolecular in aqueous solution.

Our results cannot be directly compared with the results of crystal studies because the crystallization agents and/or the crystal packing stabilize different DNA conformations in the crystal than those adopted by DNA in aqueous solution. This holds not only with DNA duplexes [28,29] but also with tetraplexes [31]. A comparison of our results with NMR studies is more straightforward though the conditions of NMR, CD, and PAGE experiments are mostly by far not the same. First of all, NMR usually works at much higher DNA concentrations. However,

high DNA concentrations as well as high salt concentrations promote molecular associations, so that the low DNA concentrations used in the present study would rather stabilize intramolecular folding. In spite of this, the conclusion did not change under various salt conditions and nor even at very low salt concentrations—the tetraplex was invariably bimolecular. The only exception might be the aptamer tetraplex stabilized by ethanol. Unfortunately, we cannot run electrophoresis in the presence of ethanol to get an unambiguous answer regarding the tetraplex molecularity in this case. Hence, we must consider the possibility that the thrombin aptamer is a single molecule tetraplex in the presence of ethanol. This would be in line with the finding that alcohol stabilizes the structures observed in crystals [28,29].

The tetraplex of GGTTGGTGTGGTTGG was long considered to be a prototype of the intramolecular guanine tetraplex. Here, we show that this notion is not correct in aqueous solution. In order to get the intramolecular tetraplex, one has either to add a tail containing other guanines to the 15-mer or to lengthen the loops.

Acknowledgments

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