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Exceptionally slow kinetics of the intramolecular quadruplex formed by the *Oxytricha* telomeric repeat

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We examined the stability and kinetics of folding of the *Oxytricha* telomeric repeat sequence $(G_4T_4)_4$. Fluorescence melting experiments show that this intramolecular quadruplex, which is more stable in potassium- than sodium-containing buffers, shows considerable hysteresis between the melting and annealing profiles, even when heated at a rate of 0.05 °C min^{-1}. Quantitative analysis of this hysteresis, together with temperature-jump relaxation experiments show that the dissociation is exceptionally slow with a half-life of about 10 years at 37 °C in the presence of 50 mM K^+. The association reaction has a half-life of a few seconds at 37 °C, but becomes slower at elevated temperatures consistent with the suggestion that association occurs by a nucleation-zipper mechanism.

Introduction

G-Rich DNA sequences can assemble into four-stranded structures that are generated by stacks of quartets of G-residues.[1–4] These structures are stabilized by monovalent cations (K^+ > Na^+) which fit within the central core of guanine carbonyls and can lie between or within the plane of each quartet.[5,6] Intermolecular complexes are generated by the association of four separate DNA molecules[7] or by dimerization of molecules that each contain two G-tracts,[8] while intramolecular quadruplexes are formed by folding a single strand of DNA.[9–12] There has been considerable interest in these structures as they may be induced in telomeric repeats, such as $(GGGTTA)_n$ in humans, $(GGGGTT)_n$ in *Tetrahymena* and $(GGGGTTTT)_n$ in *Oxytricha.*

Intramolecular G-quadruplexes can adopt several conformations, which differ in the orientation of the strands.[1] For the human sequence a parallel arrangement has been suggested from a crystal structure generated in the presence of potassium,[11] while NMR studies showed an antiparallel arrangement in the presence of sodium.[10] NMR studies have also shown that the dimeric two-repeat human telomeric sequence d(TAGGGTTAGGGT) can form both parallel and antiparallel structures in the presence of potassium,[13] while CD has been used to suggest that the human sequence forms a parallel structure in the presence of potassium.[14] In contrast, only an antiparallel arrangement has been suggested for the *Oxytricha* repeat.[12,15,16] The dimeric *Oxytricha* telomeric repeat $d(G_4T_4G_4)$ also forms an antiparallel structure which has been observed in both NMR[17] and crystallographic[18] studies and this antiparallel structure is maintained in the presence of potassium and sodium.[19] The CD spectrum of this sequence is also typical of an antiparallel arrangement, with a positive peak around 295 nm.[20] In addition, studies with oligonucleotides of the type $(G_3T_n)_4$ have shown that sequences with longer T_n tend to form antiparallel structures.[21] It therefore seems reasonable to suggest that the *Oxytricha* sequence folds to form an antiparallel quadruplex structure.

Quadruplexes are very stable and some G-rich sequences preferentially fold into this structure, even in the presence of an excess of the complementary C-rich DNA strand.[22,23] They also display extremely slow association and dissociation rates.[24–26] NMR studies have shown that the guanine imino protons exchange on a time scale of days to weeks, even when dissolved in D_2O,[27] in contrast to DNA duplexes (for which these protons have half-lives which are measured in milliseconds). The formation of intermolecular quadruplexes is also very slow, though since it is a fourth order reaction it is highly concentration dependent. For example, the intermolecular complex formed by $T_2G_4T_2$ showed a fourth order association rate constant of 6×10^4 M^{-3} s^{-1} at 37 °C, which extrapolates to a half life of 10^6 years at a concentration of 1 μM, but only 10 h at 1 mM. The dissociation rate for this complex was 1.3×10^{-7} s^{-1} (*i.e.* a half life of 60 d) at 37 °C.[26] We have also reported similar slow dissociation rates for a series of short intermolecular quadruplexes.[28] We would expect intramolecular complexes to form more rapidly than their intermolecular counterparts, though there is less information on their rates of association and dissociation. In one study, the unfolding of the *Oxytricha* telomeric sequence was determined by measuring the rate of duplex formation on adding an excess of the complementary C-rich strand and showed half lives of about 4 and 18 h at 37 °C in the presence of 50 mM Na^+ and K^+, respectively.[29] A similar study using a PNA trap with the human telomeric repeat showed dissociation half lives of about 30 min and over 40 h at 20 °C in the presence of 100 mM Na^+ and K^+, respectively,[30] though a similar study using complementary DNA strands suggested folding and unfolding half-lives of a few minutes.[31] Another study on the quadruplex formed by the thrombin-binding aptamer showed rates of quadruplex opening of 62×10^{-5} s^{-1} and 3200×10^{-5} s^{-1} at 10 °C in the presence of K^+ and Na^+ respectively.[23]

These studies with different quadruplexes emphasise their slow kinetics of folding and unfolding. We have discovered that the *Oxytricha* telomeric repeat sequence $(G_4T_4)_4$ displays exceptionally slow kinetics and have used fluorescence melting and temperature jump kinetics to explore its rates of folding and unfolding.

Results

Fluorescence melting

We have previously used fluorescently labelled oligonucleotides to study the stability of intramolecular quadruplexes, especially those related to the human telomeric repeat (GGGTTA).[22,32–35] In these studies, fluorescein and methyl red are attached at opposite ends of the sequence; these are in close proximity when the oligonucleotide folds into an intramolecular quadruplex and the fluorescence is quenched. When the quadruplex melts, the fluorophore and quencher are separated and there is a large increase in fluorescence. In our previous studies we have

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employed a heating and cooling rate of 0.1 °C s^{-1} and, under these conditions, the human telomeric repeat shows little or no hysteresis in sodium containing buffers, though the melting and annealing curves differ by a few degrees in the presence of low concentrations of potassium.[32,33] These differences in melting and annealing profiles, which are exaggerated for some related sequences,[32,33] indicate that the thermal melting curves are not at thermodynamic equilibrium and that at least one of the steps is slow. An extreme example of this phenomenon is the formation of intermolecular quadruplexes, generated by the association of four DNA strands, for which the dissociation reaction is essentially irreversible at sub-micromolar oligonucleotide concentrations.[26,28] The results of similar melting experiments with the *Oxytricha* sequence (performed at a heating and cooling rate of 0.1 °C s^{-1}) are shown in Fig. 1. Panel A shows the results in the presence of 50 mM Na^+, for which there is considerable hysteresis between the melting (T_m = 49 °C) and annealing (T_m = 39 °C) curves. However, a biphasic melting profile is apparent when this complex is melted a second time; the T_m of the first component is similar to that of the first melt, but about 25% of the fluorescence change occurs with a T_m of about 70 °C. This is exaggerated at lower concentrations of Na^+ (not shown). The T_m values for heating and cooling are almost coincident in the presence of 100 mM Na^+ (inset to panel A). However, at this higher salt concentration about 20% of the melting (but not the annealing) transition occurs at about 70 °C. The reannealed oligonucleotide has a higher fluorescence than the original complex, suggesting that it has not properly reassociated. When this complex is melted again it displays a biphasic profile in which the second transition has a similar T_m to the minor transition in the first melt, but is a greater proportion of the reaction. Further annealing and melting of this complex produces identical fluorescence profiles, demonstrating that the differences are not caused by degradation of the oligonucleotide.

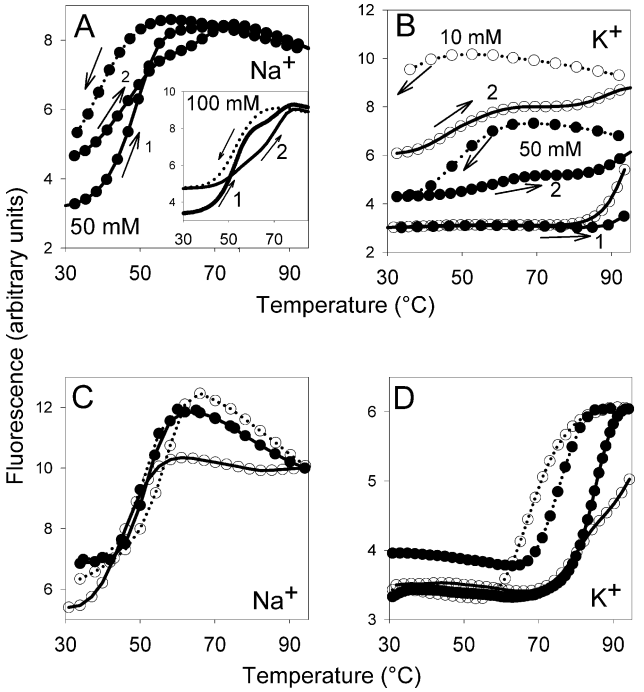

Fig. 1 Fluorescence melting and annealing curves for the *Oxytricha* telomeric sequence determined in different buffers and at different rates of heating and cooling. (A) 10 mM sodium phosphate pH 7.4 containing 10 mM NaCl determined at a heating rate of 0.1 °C s^{-1}. The first and second melting curves (1 and 2) are shown with solid lines, while the annealing curves are joined with dotted lines. For the sake of clarity only about 10% of the data points have been included. The inset shows the profiles in the presence of 10 mM sodium phosphate pH 7.4 containing 90 mM NaCl. (B) 10 mM potassium phosphate pH 7.4 (open circles) and 10 mM potassium phosphate containing 40 mM KCl (filled circles), determined at a heating rate of 0.1 °C s^{-1}. The first and second melting curves (1 and 2) are shown with solid lines, while the annealing curves are shown with dotted lines. (C) 10 mM sodium phosphate pH 7.4 containing 40 mM NaCl, determined at a heating rate of 0.5 °C min^{-1} (open circles) and 0.05 °C min^{-1} (filled circles). Melting curves are shown with solid lines, while annealing curves are indicated with dotted lines. (D) 10 mM potassium phosphate pH 7.4 containing 40 mM KCl, determined at a heating rate of 0.5 °C min^{-1} (open circles) and 0.05 °C min^{-1} (filled circles). Melting curves are shown with solid lines, while annealing curves are indicated by dotted lines.

Panel B of Fig. 1 shows corresponding melting and annealing profiles in the presence of 10 mM (open circles) and 50 mM K^+ (filled circles). In both cases the first melting transition is too high to measure, though when these are subsequently re-annealed the T_m of the transition is too low to determine in 10 mM K^+, and about 49 °C in 50 mM K^+. Re-melting of these complexes produces biphasic profiles. These melting curves demonstrate that melting and annealing of the *Oxytricha* telomeric repeat is a slow process with considerable hysteresis at a heating rate of 0.1 °C s^{-1}, and that more than one complex is generated at this rate of annealing. These experiments were therefore repeated at much slower rates of heating and annealing (0.5 °C min^{-1} and 0.05 °C min^{-1}) and the results are shown in panels C and D in the presence of 50 mM Na^+ and K^+ respectively. In Na^+ (panel C) hysteresis is still evident when heating at 0.5 °C min^{-1} (open circles) with T_m values of 57.1 °C (melting) and 45.7 °C (annealing), though there is no hysteresis at 0.05 °C min^{-1} (T_m = 51 °C). In the presence of potassium (panel D) the apparent T_ms for annealing and melting are 67.0 and 80.5, respectively, when heated at 0.5 °C min^{-1} (open circles), though it can be seen that the melting transition is biphasic. Under these conditions there is still considerable hysteresis even at the slowest rates of temperature change (0.05 °C min^{-1}) with T_ms for melting and annealing of 75.0 and 84.5, respectively. These experiments demonstrate that the rate of folding and/or unfolding of this intramolecular quadruplex is extremely slow.

Representative melting and annealing curves at the slowest rate of temperature change (0.05 °C min^{-1}) are shown in Fig. 2 in the presence of different concentrations of Na^+ and K^+ and the apparent T_m values are summarised in Table 1. It can be seen that although hysteresis is still evident at the lowest Na^+ concentration (20 mM), this is hardly evident at 50 mM and above. In contrast there is substantial hysteresis in the presence of K^+ which persists beyond 50 mM, and biphasic melting curves are evident at the lowest K^+ concentrations (10 and 20 mM).

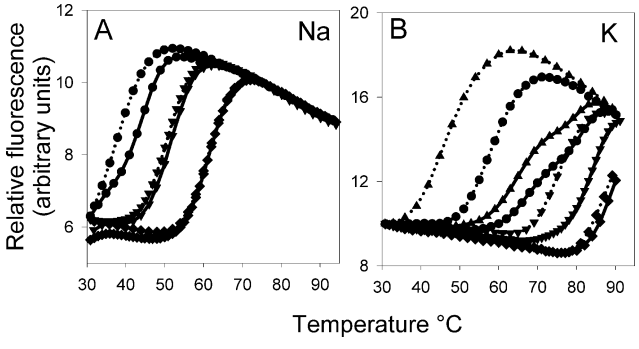

Fig. 2 Fluorescence melting and annealing curves for the *Oxytricha* telomeric sequence determined at a heating rate of 0.05 °C min^{-1}. (A) 10 mM sodium phosphate pH 7.4 containing 10 mM NaCl (circles), 40 mM (triangles) and 90 mM (diamonds). Melting curves are shown with solid lines, while annealing curves are indicated by dotted lines; at the higher ionic conditions these two curves coincide. (B) 10 mM potassium phosphate pH 7.4 containing 0 mM (triangles), 10 mM (circles), 40 mM (inverted triangles) and 90 mM KCl (diamonds). Melting curves are shown by solid lines, while annealing curves are indicated by dotted lines.

The hysteresis arises because the melting profiles are not in thermodynamic equilibrium and demonstrate that the *Oxytricha* sequence has very slow kinetics of folding and unfolding, and that in some instances the properties of the complex depend on

Table 1 Apparent annealing and melting temperatures (°C) for the *Oxytricha* telomeric repeat in the presence of different concentration of Na^+ and K^+, at different rates of temperature change

	Na^+				K^+			
	0.5 °C min^{-1}		0.05 °C min^{-1}		0.5 °C min^{-1}		0.05 °C min^{-1}	
Concentration/mM	Melt	Anneal	Melt	Anneal	Melt	Anneal	Melt	Anneal
10	<30	<30	<30	<30	37.0/75.0[a]	<30	65.0/80.0[a]	45.5
20	36.0/54.0[a]	<30	45.0	36.0	78.0	45.0	67.5/81.5[a]	57.5
30	37.5/55.5[a]	37.5	47.5	42.5	55.0	78.3	82.5	65.0
50	57.0	45.5	51.5	50.0	80.5[a]	67.0	84.5	75.0
75	59.0	53.0	55.5	55.5	>90	78.5	86.0	81.5
100	61.5	58.0	59.5	59.5	>90	81.0	>90	86.0

[a] Indicates the presence of a biphasic melting profile.

the method by which it was prepared (fast or slow annealing). Association and dissociation rate constants can be obtained from these data by comparing the melting and annealing curves, as described in the Experimental section, and we have derived these for the ionic conditions which generate monophasic melting transitions. Arrhenius plots derived from this analysis are shown in Fig. 3 and the thermodynamic parameters obtained are summarized in Table 2. It is immediately clear that, not only are the kinetic parameters extremely slow, but the association reaction appears to have a negative activation energy (*i.e.* the reaction becomes faster at lower temperatures). This has been observed previously in other studies with quadruplexes and triplexes, and is explained by suggesting that association occurs *via* a nucleation zipper mechanism in which a transient intermediate is stabilised at lower temperatures. The association parameters are similar in the presence of K^+ and Na^+, though

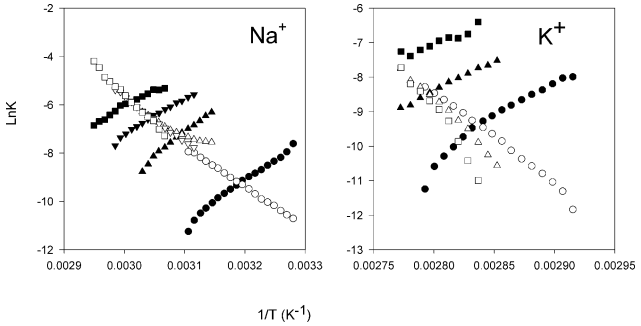

Fig. 3 Arrhenius plots for the association and dissociation of the *Oxytricha* telomeric sequence determined in the presence of different concentrations of sodium or potassium. The parameters were estimated from analysis of the hysteresis between the melting and annealing profiles. In both panels open symbols show the dissociation rate constants (k_{-1}) while filled symbols correspond to the association rate (k_1). The left hand panel shows the reaction rates in the presence of Na^+: circles, 20 mM; triangles, 50 mM; inverted triangles, 75 mM; squares 100 mM. The right hand panel shows the reaction rates in the presence of potassium: circles, 50 mM; triangles 75 mM; squares 100 mM.

the activation energies in potassium are greater by between 10–30 kJ mol^{-1}. Overall, the association is faster in the presence of potassium (with half-lives of a few seconds at 37 °C) than Na^+. The rate of dissociation is very slow in the presence of both ions, but it is especially slow with potassium, for which the $t_{1/2}$ in 50 mM of this cation is about 10 years at 37 °C. The activation energies in potassium are about twice those measured in Na^+, and these increase with increasing ionic strength, as do the pre-exponential factors. We can calculate precise melting temperatures from these kinetic parameters, as this will be the temperature at which k_{-1} equals k_1; these values are tabulated in the final column of Table 2. From these data we can calculate that the dissociation half-lives at the T_m are 1.5 h and 30 min in 50 and 100 mM K^+, respectively. This explains why there is still hysteresis between the melting and annealing profiles when the temperature is changed at a rate of 1 °C every 20 min (0.05 °C min^{-1}).

We confirmed these exceptionally slow reaction kinetics by performing temperature-jump relaxation kinetics. In this technique, the temperature of the sample is rapidly increased and the rate of change of the fluorescence is measured as the reaction relaxes to a new equilibrium. We have previously used this technique for examining the stability of intermolecular triplexes.[33] Representative relaxation profiles for this reaction are shown in Fig. 4, in the presence of Na^+ and K^+, and are compared with those for the human telomeric repeat sequence (which will be reported in detail elsewhere). The kinetics of both sequences are slower in the presence of K^+ than Na^+. However, the kinetic profiles for the *Oxytricha* sequence are much slower than for the human sequence; Fig. 4 shows that, in the presence of 50 mM K^+, the *Oxytricha* repeat is about 200 times slower at a temperature which is 20 °C higher. The rate at which the reaction relaxes to a new equilibrium is equal to the sum of the forward and reverse rate constants ($k_1 + k_{-1}$) and since these are independent of concentration for a unimolecular reaction it is not possible to determine each one independently. Fig. 5 shows the effect of temperature on this composite relaxation rate (open circles), and these are compared with the values of

Table 2 Thermodynamic parameters for association (k_1) and dissociation (k_{-1}) of the *Oxytricha* telomeric repeat sequence. E_a is the activation energy, while A is the pre-exponential factor in the equation $k = Ae^{(-Ea/RT)}$. The calculated half-life for each reaction at 37 °C is shown, along with the calculated T_m (*i.e.* the temperature at which $k_{-1} = k_1$)

	k_1			k_{-1}			
	E_a/kJ mol^{-1}	ln(A)/s^{-1}	$t^{37}_{1/2}$/s	E_a/kJ mol^{-1}	ln(A)/s^{-1}	$t^{37}_{1/2}$/s	T_m (°C)
50 mM K^+	−200 ± 12	−78 ± 4	0.9	228 ± 5	69 ± 2	3.7×10^8	77.0
75 mM K^+	−146 ± 4	−57 ± 2	1.8	284 ± 9	87 ± 3	8.1×10^9	86.3
100 mM K^+	−109 ± 2	−44 ± 5	2.9	405 ± 26	128 ± 9	5.4×10^{12}	86.6
20 mM Na^+	−205 ± 19	−88 ± 7	3770	123 ± 5	38 ± 2	12680	40.3
50 mM Na^+	−170 ± 6	−71 ± 2	65	94 ± 6	28 ± 2	3900	47.9
75 mM Na^+	−126 ± 4	−53 ± 2	31	155 ± 2	51 ± 1	13700	52.1
100 mM Na^+	−112 ± 5	−47 ± 2	15	208 ± 4	69 ± 2	51000	59.0

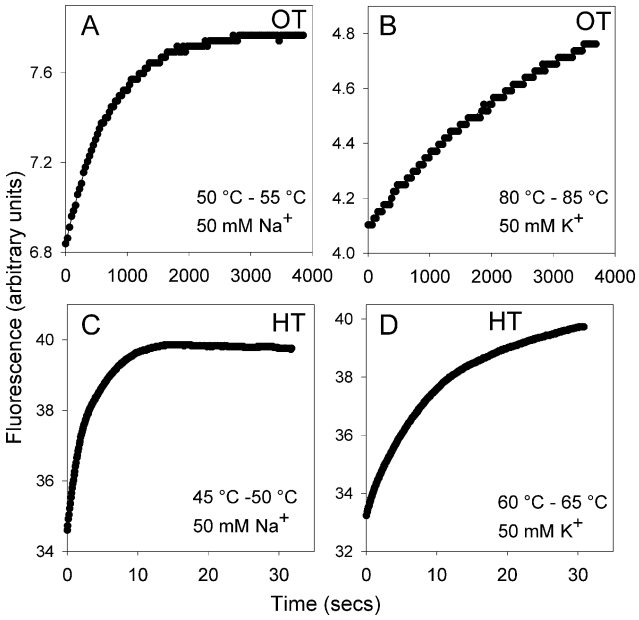

Fig. 4 Temperature-jump fluorescence profiles for the *Oxytricha* (A and B) and human (C and D) telomeric repeat sequences. (A) and (C) were performed in 50 mM sodium phosphate pH 7.4, while (B) and (D) were performed in 50 mM potassium phosphate pH 7.4. The temperature changes were (A) 50–55 °C, (B) 80–85 °C, (C) 45–50 °C, (D) 60–65 °C.

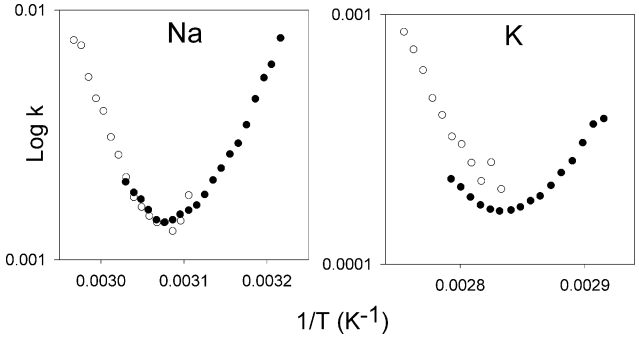

Fig. 5 Arrhenius plots showing the variation in the relaxation rates ($k_1 + k_{-1}$) with temperature in the presence of 50 mM sodium or potassium phosphate. Open symbols were derived from the temperature-jump experiments while filled symbols were derived from the analysis of the hysteresis in the melting and annealing profiles.

$k_1 + k_{-1}$ determined from the hysteresis. There is good agreement between these two techniques, confirming the exceptionally slow reaction kinetics. These Arrhenius plots are clearly non-linear as would be expected, since temperature has opposite effects on the association and dissociation rates, and a simple analysis predicts that the minimum in this profile will approximate to the T_m of the reaction.

Discussion

The results presented in this study demonstrate that the rates of folding and unfolding of the intramolecular quadruplex formed by the *Oxytricha* telomeric repeat are very slow and are much slower than the human telomeric repeat. The rate of association is between 10–60 times faster in K^+ than Na^+ (see Table 2), but the greatest difference between these two ions is in the dissociation rate constants. The equilibrium constants for the complex in 100 mM Na^+ (k_1/k_{-1}) is about 3400 at 37 °C, which is higher than that reported for the human telomeric sequence at this temperature, as expected since the *Oxytricha* sequence has a higher T_m. The stability in the presence of K^+ is greater by several orders of magnitude, as exemplified by the higher T_m and slower rates of dissociation. These results suggest that, although there are differences in the rates of association of quadruplexes under different conditions, the dissociation rate constant is the most important factor that affects the relative stabilities.

The association rate constants reported in this work show that the reaction is faster at lower temperatures. This has been reported in several other studies[37–39] and is consistent with a nucleation-zipper mechanism for quadruplex formation. However, since the rate limiting step in the reaction may change with temperature, these Arrhenius plots should be extrapolated to 37 °C with caution. It is also possible that addition of the fluorescent groups will affect the kinetics of folding, though our previous studies suggest that these effects will be relatively small and cannot be responsible for the extremely high stability of this potassium complex.

Why does this intramolecular quadruplex display much slower reaction kinetics than those of other published sequences? We can envisage several reasons. Firstly, it is possible that it adopts a different structure, though several studies have shown that the sodium form of this sequence adopts an antiparallel structure with a crossed central loop,[12,15,16] similar to that adopted by the human telomeric sequence under similar conditions, and as proposed for quadruplexes with longer loops.[21] We can be less sure of the structure of the potassium form, and examination of its CD spectrum[20] reveals subtle differences from the sodium form. Secondly, the slower kinetics may be caused by the long loops, though unusual kinetics were not reported for the quadruplex formed by $(G_3T_4)_4$,[21] and we also find much less hysteresis between annealing and melting curves with this oligonucleotide (Rachwal, Brown and Fox, unpublished observations). Thirdly the slow rates could be a feature of stacks of four, rather than three G-quartets. This would be consistent with studies on intermolecular quadruplexes formed with G_4-containing short strands,[26,28] which also display exceptionally slow dissociation rate constants, though these form parallel rather than antiparallel structures. However, we do not find similar hysteresis with the sequence $(G_4T_3)_4$. The slow kinetics therefore appear to result from a combination of long loop length and longer G-stack. The longer loop may give rise to a more flexible structure, allowing the stacked quartets to adopt a configuration that is similar to the very stable intermolecular complex. Given the extremely slow dissociation kinetics it is clear that, if this structure forms *in vivo*, it will require protein factors to unfold it.

Experimental

Synthetic oligonucleotides

All oligonucleotides were purchased from Oswel DNA service, Southampton UK. The human and *Oxytricha* telomeric repeats consisted of the sequences Q–A$(GGGTTA)_3$GGGT–F and Q–T$(GGGGTTTT)_3$GGGGT–F respectively, where F is fluorescein (FAM-dR) and Q is methyl red-dR.

Fluorescence melting studies

The thermal melting temperature of the quadruplexes was determined using the fluorescence melting technique that we have previously developed[32] and have used for assessing the stability of various other quadruplexes.[22,32–35] Oligonucleotides were labelled at the 5′-end with a quencher (methyl red) and at the 3′-end with a fluorophore (fluorescein). These are in close proximity when the oligonucleotide folds to form an intramolecular quadruplex and as a consequence the fluorescence is quenched. When this melts these groups become separated and there is a large increase in fluorescence. These experiments were performed in the Roche LightCycler, which has an excitation source at 488 nm and the changes in fluorescence emission were measured at 520 nm.

Quadruplexes were prepared in either 10 mM potassium or sodium phosphate buffers, pH 7.4 to which different concentrations of NaCl or KCl were added. Melting experiments were performed in a total volume of 20 μL and contained 0.25 μM

quadruplex-forming oligonucleotide. For experiments at the fastest rates of temperature change the complexes were first denatured by heating to 95 °C at a rate of 0.1 °C min^{-1} and left to equilibrate for 10 min. They were then cooled to 30 °C at a rate of 0.1 °C s^{-1}. Recordings were taken during both the melting and annealing reactions to check for hysteresis. Slower rates of heating and cooling (0.5 and 0.05 °C min^{-1}) were achieved by changing the temperature in 1 °C steps, leaving the samples to equilibrate for 2 or 20 min before each fluorescence reading. Recordings were taken during both the heating and cooling steps. T_m values were determined from the first derivatives of the melting profiles using the Roche LightCycler software. Each value was recorded in triplicate and these usually differed by less than 0.5 °C.

Obtaining kinetic parameters from hysteresis in melting and annealing curves.

The hysteresis that is observed between the melting and annealing profiles is caused by the slow association and/or dissociation reactions, as a result of which the reaction is not at thermodynamic equilibrium. Individual association and dissociation rate constants can be derived from this hysteresis as previously described.[37–39] Briefly, if a_c and a_h are the fractions of the folded quadruplex at any temperature in the cooling and heating profiles, respectively, and t and T are the time and temperature, then $d(a_c)/dT = d(a_c)/dt \times (dT/dt)^{-1}$ and $d(a_h)/dT = d(a_h)/dt \times (dT/dt)^{-1}$.

If k_1 and k_{-1} are the association and dissociation rate constants for folding of the quadruplex then $d(a_c)/dt = k_1(1 - a_c) - k_{-1}\ a_c$ and $d(a_h)/dt = k_1(1 - a_h) - k_{-1}\ a_h$. By measuring $d(a_c)/dT$, $d(a_h)/dT$, a_c and a_h, the individual rate constants can be estimated at each temperature.

Temperature jump kinetics

The kinetics of quadruplex dissociation were determined by measuring the rate of change of fluorescence after rapidly increasing the temperature, in a manner similar to that of temperature-jump relaxation kinetics.[36] The complexes were equilibrated in the LightCycler at a temperature around the T_m. The temperature was then rapidly increased by 5 °C at the fastest rate on the LightCycler (20 °C s^{-1}) and the time dependent change in fluorescence was recorded. This temperature increase promotes dissociation of some of the quadruplex, moving along the dissociation melting curve. Although the theoretical dead-time under these conditions is only 0.25 s all fluorescence changes that occurred in the first 2 s were ignored during equilibration to the new temperature. Successive temperature-jumps were then recorded on the same sample by further increasing the temperature by 5 °C. Initial experiments with the *Oxytricha* telomeric repeat sequence showed that the time-dependent change in fluorescence was extremely slow, requiring up to 1 h for completion. In order to prevent photobleaching over this prolonged period, the samples were degassed before use and readings were only taken every 30 s. To exclude any possible evaporation these samples were maintained under mineral oil. Each experiment was repeated at least 3 times. The time dependent changes in fluorescence were fitted by an exponential function $F_t = F_f{*}(1 - e^{-kt}) + F_0$ where F_t is the fluorescence at time t, F_f is final fluorescence and F_0 is the initial fluorescence. The relaxation rate constant (k) obtained from this analysis is equal to the sum of k_{-1} and k_1. Arrhenius plots of $\ln(k)$ against $1/T$ were constructed using these data.

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