Kinetics of G-Quartet-Mediated Tetramer Formation

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ABSTRACT: The phosphorothioate and phosphodiester oligodeoxynucleotides d(TTGGGGTT) form parallel-stranded tetramer structures stabilized by guanosine quartets. The phosphorothioate tetramer has been shown to inhibit human immunodeficiency virus (HIV) *in vitro*. The kinetics of association and dissociation of both tetramers have been determined as a function of temperature using size exclusion chromatography to measure the ratio of single strand to tetramer. In phosphate buffered saline (pH 7.2) at 37 °C, the fourth-order association rate of the phosphorothioate tetramer was 6.1 (±0.5) × 10^4 M^{-3} s^{-1}; the dissociation rate was 8.2 (±0.2) × 10^{-6} min^{-1}, resulting in a $t_{1/2}$ of about 60 days. The association rate of the phosphodiester was about one order of magnitude faster and the dissociation rate about one order of magnitude slower than that of the phosphorothioate tetramer. The association reaction had a negative energy of activation for both compounds. Despite thermodynamic instability of the tetramer at low concentrations, the extremely slow dissociation rate may allow use of the phosphorothioate tetramer for AIDS chemotherapy.

The guanosine base tetrad, or guanosine quartet (G-quartet), is an important structural motif observed in DNA or RNA oligonucleotides containing G-rich sequences. Interest in nucleic acid structures stabilized by G-quartets has increased since the proposal that telomeric DNA sequences adopt intramolecular G-quartet structures (Williamson et al., 1989). Subsequently, many studies of oligonucleotides that adopt G-quartet structures have been reported (Guschlbauer et al., 1990; Williamson, 1994; Pilch et al., 1995). The complexes formed by four strands were originally characterized by Sen and Gilbert (1988, 1990). These tetramers are parallel, right-handed helices with all nucleotides in the *anti* conformation (Cheong & Moore, 1992; Lu et al., 1992; Sarma et al., 1992; Wang & Patel, 1992, 1993; Aboul-ela et al., 1994; Laughlan et al., 1994).

The phosphorothioate oligonucleotide d(TTGGGGTT) is a specific inhibitor of transmission of human immunodeficiency virus (HIV) infection *in vitro*. The oligonucleotide forms a parallel-stranded tetramer stabilized by G-quartets; the tetramer structure and certain phosphorothioate linkages are necessary for inhibition of viral infection (Wyatt et al., 1994). The tetramer binds to the V3 loop domain of gp120 to inhibit both cell-to-cell and virus-to-cell spread of infection (Buckheit et al., 1994). The concentration of oligonucleotide required to inhibit 50% of virus-induced cytopathic effect (IC_{50}) is 0.3 μM (±0.05 μM) (Wyatt et al., 1994). As the tetrameric complex is required for antiviral activity, the thermodynamic and kinetic stability of the complex is important. In order to have clinical value, the active tetramer must remain intact at relatively low concentration *in vivo*. The present study extends the initial structural characterization of the tetramer complex and addresses the association and dissociation kinetics of the tetramer.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis and Purification. Phosphorothioate and phosphodiester oligonucleotides were synthesized using phosphoramidite chemistry (Sinha et al., 1984; Iyer et al., 1990). The phosphorothioate d(TTGGGGTT) was purified using reversed-phase HPLC column with a gradient of methanol in water. For some experiments, the tetrameric form was isolated using size exclusion chromatography on a Pharmacia Superdex 75 HiLoad 26/60 column. The running buffer was 10 mM sodium phosphate (pH 7.2) at a flow rate of 2 mL min^{-1}. The phosphodiester oligonucleotide was purified by precipitating twice from 0.5 M sodium acetate using two volumes of ethanol. The phosphorothioate oligonucleotide was desalted using a Pharmacia HR 16/30 column packed with Pharmacia Superdex 30 run in water with a flow rate of 0.5 mL min^{-1}.

Size Exclusion Chromatography. Analytical assay of the ratio of monomeric and tetrameric forms of the oligonucleotides was performed with a Pharmacia Superdex 75 HR 10/30 size-exclusion column using a buffer of 25 mM sodium phosphate (pH 7.2), and 0.1 mM EDTA at a flow rate of 0.5 mL min^{-1}. Detection was at 260 nm.

Dissociation Rate Analysis. First order dissociation rates were determined at several temperatures and several potassium and sodium concentrations. A stock of oligonucleotide at 2 mM strand concentration in 40 mM sodium phosphate (pH 7.2) and 100 mM KCl was diluted to 10 μM strand concentration into buffer. The stock solution was at least 96% tetramer as monitored by size exclusion chromatography. For analysis of the temperature dependence of the dissociation rate, oligonucleotide was diluted into phosphate-buffered saline at pH 7.2 (PBS: 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4). The sample was incubated at the indicated temperature and analyzed by size exclusion chromatography over time. The fraction tetramer was calculated after correction of peak areas for difference in extinction coefficient. The dissociation rate was calculated from a plot of ln (fraction tetramer) vs time. Where given,

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standard error is derived from two or three independent experiments.

Association Rate Analysis. Association was monitored at oligonucleotide concentrations, solution conditions, and temperatures indicated. A desalted sample of oligonucleotide in water at 1 mM strand concentration was diluted to the appropriate concentration. The sample was incubated in a boiling water bath for 10 min to dissociate any tetramer present. Association was monitored by size exclusion chromatography as a function of time. The fraction single strand was calculated after correction of peak areas for difference in extinction coefficient.

The fraction single strand was plotted as a function of time for each oligonucleotide concentration and the curves were fit to the following equation (Levine, 1978):

$$S^{(1-n)} = 1 + [C_T]^{n-1}(n-1)(kt)$$

where S is the fraction single strand at time t, C_T is the total strand concentration, n is the order of the reaction with respect to oligonucleotide concentration, and k is the rate constant for disappearance of single strands. The order and the rate constant were simultaneously varied to minimize the square of the difference between the measured and calculated fraction single strand at all times and concentrations for experiments at 22 °C. Where given, standard error is from two or three independent experiments.

Thermal Melting Analysis. Absorbance vs temperature curves were measured at 260 nm using a Gilford 260 spectrophotometer. A decade divider circuit added to the spectrophotometer allowed collection of data at heating rates as slow as 0.025 °C/min. Oligonucleotide was diluted into PBS from a stock at 2 mM strand concentration in 40 mM sodium phosphate (pH 7.2) and 100 mM KCl immediately before use. The oligonucleotide concentration in the melting experiments was approximately 20 μM; the actual concentration for each experiment was determined using the absorbance at 85 °C and the extinction coefficient calculated as described below. The melting temperature (T_{max}) is the temperature at which the derivative of the absorbance vs temperature curve was maximum. Reported values are averages of two or three experiments.

Circular Dichroism Spectra (CD). CD spectra were recorded in a Jasco J-600 spectropolarimeter. Solutions of 20 μM strand concentration were prepared from stocks at 2 mM strand in 50 mM sodium phosphate (pH 7.2) and 100 mM KCl immediately before use. Spectra were recorded at room temperature. Ellipticities were converted to $\Delta\epsilon$ and are reported per mole of residue.

RESULTS

Structure of the Tetrameric Complex. Previously, we provided evidence that the phosphorothioate oligonucleotide d(TTGGGGTT) formed a four-stranded complex (Wyatt et al., 1994). Gel electrophoresis experiments similar to those performed by others (Sen & Gilbert, 1990; Kim et al., 1991) suggested that the phosphorothioate d(TTGGGGTT) tetramer was parallel-stranded as are ribo- and deoxyoligonucleotides d(TGGGGT) (Aboul-ela et al., 1992; Cheong & Moore, 1992).

Further evidence for the parallel-stranded nature of the complex formed by the phosphorothioate d(TTGGGGTT) is provided by circular dichroism. The CD spectra of the phosphorothioate and phosphodiester analogs of d(TTGGGGTT) are qualitatively similar and are shown in Figure 1. The spectra are characteristic of spectra of parallel-stranded guanosine quartet structures reported by others (Balagurumoorthy et al., 1992; Hardin et al., 1992; Jin et al., 1992; Lu et al., 1992, 1993; Sarma et al., 1992) with a positive band at 260 nm and a negative band at 245 nm. Antiparallel guanosine quartet structures have a different CD profile with a positive band at 295 nm and a larger negative band at 265 nm (Balagurumoorthy et al., 1992; Lu et al., 1993).

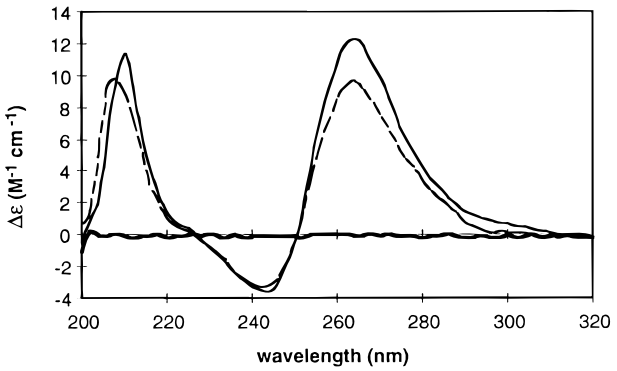

FIGURE 1: CD spectra of the phosphorothioate (—) and phosphodiester (- - -) d(TTGGGGTT) tetramers. The spectra of buffer alone is also shown ("bold line"). The spectra are characteristic of parallel-stranded G-quartet structures.

Kinetics of Tetramerization. Size exclusion chromatography was used to monitor the dissociation and association of the tetrameric complexes (Wyatt et al., 1994). The monomeric and tetrameric forms of the oligonucleotides were well resolved. When the phosphorothioate tetramer fraction was isolated using a preparative scale column and reinjected, no single strand was observed. Conversely, after heat denaturation and injection, only the single-stranded form was observed (data not shown). Thus, the conditions of the separation did not alter the ratio of single strand to tetramer.

The extinction coefficient for the monomeric form was calculated using the method described by Puglisi and Tinoco (1990). The extinction coefficient for the tetramer was determined by measuring the difference in absorbance at 260 nm of a sample of purified tetramer before and after denaturation. There was approximately a 6% difference in absorbance at 260 nm between the two forms. At 280 nm, the difference was about 9%. The extinction coefficient at 260 nm of d(TTGGGGTT) is 76.82×10^3 M^{-1} cm^{-1}; the extinction coefficient for the tetramer is 72.32×10^3 M^{-1} cm^{-1}.

Dissociation Rates. The dissociation rate of the phosphorothioate d(TTGGGGTT) quartet was determined in PBS at several temperatures. The concentration of oligonucleotide in each sample was 10 μM. Under these conditions, equilibrium lies far toward the single strand. The plot of ln (fraction tetramer) vs time at 37 °C is shown in Figure 2a. The plots are linear, implying that the dissociation was first order under these conditions. The dissociation rate constants in PBS at several temperatures are shown in Table 1. The dissociation rate of the tetramer was slow with a $t_{1/2}$ of approximately 60 days at 37 °C. The rate increased at higher temperature and the activation energy (E_a) obtained from a plot of ln of the dissociation rate constants as a function of temperature (Figure 2b) was 58 kcal mol^{-1}.

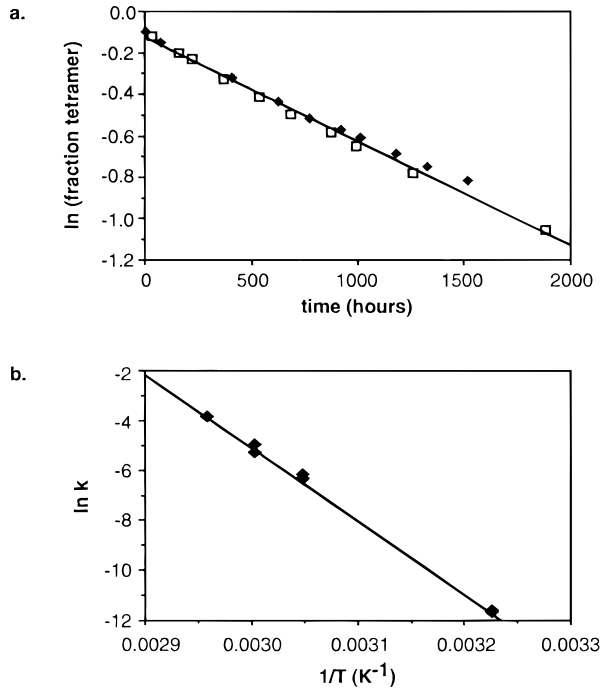

FIGURE 2: (a) Dissociation of the phosphorothioate tetramer in PBS at 37 °C. Fraction tetramer was measured as a function of time using size exclusion chromatography to resolve the single strand from tetramer. From the plot of ln (fraction tetramer) vs time, apparent first order rate for dissociation was determined (Table 1). Different symbols represent data from two independent experiments. (b) Arrhenius plot of the dissociation rates (min^{-1}) of the phosphorothioate tetramer in PBS. The energy of activation is 58 kcal mol^{-1}.

Table 1: Dissociation Rate Constants for Phosphorothioate and Phosphodiester d(TTGGGGTT) Tetramers as a Function of Temperature in PBS[a]

	phosphorothioate		phosphodiester	
temp (°C)	k (min^{-1})	$t_{1/2}$	k (min^{-1})	$t_{1/2}$
37	$8.2\ (\pm0.2) \times 10^{-6}$	59 days		
55	$1.7\ (\pm0.2) \times 10^{-3}$	6.8 h	1.6×10^{-4}	72 h
60	$5.5\ (\pm0.8) \times 10^{-3}$	2.1 h	6.0×10^{-4}	19 h
65	2.0×10^{-2}	35 min	2.5×10^{-3}	4.6 h

[a] Where given, the standard error is from two or three independent experiments.

Association Rates. The rates of association of the phosphorothioate and phosphodiester tetramers depended strongly on oligonucleotide strand concentration. At 100 μM strand concentration at room temperature, almost no association was observed over a 2 day period. In contrast, the association reaction at 1 mM strand was too rapid to measure accurately using the size exclusion chromatography method. Because of the strong dependence of reaction rate on oligonucleotide concentration, the concentration range accessible to determination of fraction single strand vs time using size exclusion chromatography was limited.

To calculate the order of the association reaction, experimental curves were obtained at several concentrations and fit to a single order and a single rate constant. This global analysis was necessary because if data were obtained only at a single concentration, small deviations could result in erroneous determination of the order of the reaction (Johnson, 1995). Figure 3a is a plot of fraction single strand vs time after incubation of the phosphorothioate oligonucleotide in PBS at 22 °C at several concentrations. Figure 3b plots data

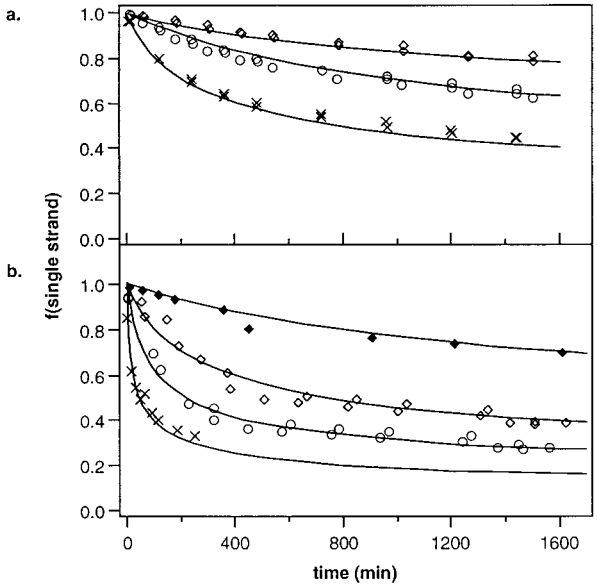

FIGURE 3: (a) Association of the phosphorothioate tetramer in PBS at 22 °C. Fraction single strand was measured as a function of time for strand concentrations of 200 μM (◇), 300 μM (○), and 500 μM (×). These data were simultaneously fit to determine order and rate constant. The curves were fit well by orders from 3 to 4.5. Solid curves were calculated using the fourth-order rate constant $3.8\ (\pm0.5) \times 10^5\ M^{-3}\ s^{-1}$. The fourth-order rate constants at other temperatures are listed in Table 2. (b) Association of the phosphorothioate tetramer in 100 mM KCl and 50 mM sodium phosphate (pH 7.2) at 22 °C. Fraction single strand was measured as a function of time for strand concentrations of 100 μM (◆), 200 μM (○), 300 μM (◇), and 500 μM (×). The curves were fit best by an order of 4.0. Solid curves were calculated using the fourth-order rate constant $6.5\ (\pm0.5) \times 10^6\ M^{-3}\ s^{-1}$.

Table 2: Association Rate Constants for Phosphorothioate and Phosphodiester d(TTGGGGTT) Tetramers as a Function of Temperature in PBS[a]

	k ($M^{-3}\ s^{-1}$)	
temp (°C)	phosphorothioate	phosphodiester
5	$3.0\ (\pm0.5) \times 10^7$	
22	$3.8\ (\pm0.5) \times 10^5$	$1.4\ (\pm0.5) \times 10^6$
37	$6.1\ (\pm0.5) \times 10^4$	$1.0\ (\pm0.5) \times 10^5$

[a] Where given, the standard error is from two or three independent experiments.

from a similar experiment in 100 mM KCl and 50 mM sodium phosphate (pH 7.2). The data shown in Figure 3a were fit well by reaction orders from 3 to 4.5. The data shown in Figure 3b were more conclusive and were best fit by a reaction order of 4.0. The fourth-order rate constant at 22 °C in PBS was $3.8\ (\pm0.5) \times 10^5\ M^{-3}\ s^{-1}$. In 100 mM K^+, tetramer formation was faster with a rate constant of $6.5\ (\pm0.5) \times 10^6\ M^{-3}\ s^{-1}$.

Association rates were also determined at 4 and 37 °C in PBS (Table 2). At these temperatures, fewer concentrations were studied so the fourth-order dependence on oligonucleotide strand observed at 22 °C was assumed. The association rate increased as the temperature decreased. An Arrhenius plot yielded a negative activation energy for tetramer formation of -33 kcal mol^{-1}. An elementary kinetic step cannot have a negative energy of activation (E_a), so this negative E_a must represent a composite of rate constants (Cantor & Schimmel, 1980).

Mechanism for Tetramer Association. We propose the mechanism for tetramer formation shown in Figure 4a.

Single strands (S) and dimers (D) are in rapid preequilibrium that favors single strand, and tetramer (Q) formation from dimer is rate limiting. The rate is

$$\frac{dQ}{dt} = k_2[D]^2 - k_{-2}[Q]$$

where k_2 and k_{-2} are the elementary rate constants for formation and dissociation of tetramer from dimer.

The rate of tetramer formation is

$$r_f = k_2[D]^2 = k_2(K_{eq})^2[S]^4$$

where K_{eq} is the equilibrium constant for the fast pre-equilibrium between single strand and dimer:

$$K_{eq} = [D]/[S]^2$$

The observed fourth-order association rate constant (k_{on}) is a composite of the elementary constants:

$$k_{on} = (K_{eq})^2 k_2$$

The observed dissociation rate constant is

$$k_{off} = k_{-2}$$

This mechanism is supported by the observed fourth-order dependence of the association rate constant on the concentration of single-stranded oligonucleotide.

The mechanism is also consistent with the observed negative energy of activation, which implies a rapid pre-equilibrium step. Short nucleic acid duplexes composed primarily of A-U base pairs have negative energies of activation (Craig et al., 1971; Porschke & Eigen, 1971; Breslauer & Bina-Stein, 1977; Drobnies, 1979). The negative E_a is explained by a mechanism in which formation of the *n*th base pair (where *n* is the nucleation length) is rate limiting. This rate-limiting step is preceded by a rapid pre-equilibrium between single strand and a rather unstable duplex of $n - 1$ base pairs (Riesner & Romer, 1973).

The proposed mechanism requires a stable dimer intermediate, yet a dimer of two strands was not observed in any association or dissociation experiments using either the phosphorothioate or phosphodiester d(TTGGGGTT). Complexes of this size were resolved from single strand and tetramer by size exclusion (data not shown). Therefore, the dimer intermediate may not be present at detectable levels. Kinetic simulations were performed using numerical integration to investigate this possibility. The equilibrium constant (K_{eq}) was varied to obtain agreement between simulated and observed data. The rate constant k_2 was calculated from k_{on} for the phosphorothioate tetramer in PBS at 22 °C and K_{eq}. The rate constant k_{-2} at 22 °C was calculated from the temperature dependence of the measured dissociation rates of the phosphorothioate tetramer in PBS. Values used for the simulation were $K_{eq} = 0.5\ M^{-1}$, $k_2 = 1.53 \times 10^6\ M^{-1}\ s^{-1}$, and $k_{-2} = 1.15 \times 10^{-9}\ s^{-1}$. The simulated fraction single strand was in good agreement with experimental data (Figure 4b). During the simulation, the concentration of dimer did not reach 1% of the total strand concentration (Figure 4c). An infinite number of combinations of K_{eq} and k_2 were consistent with the experimental data, so we were unable to independently determine values of K_{eq} and k_2.

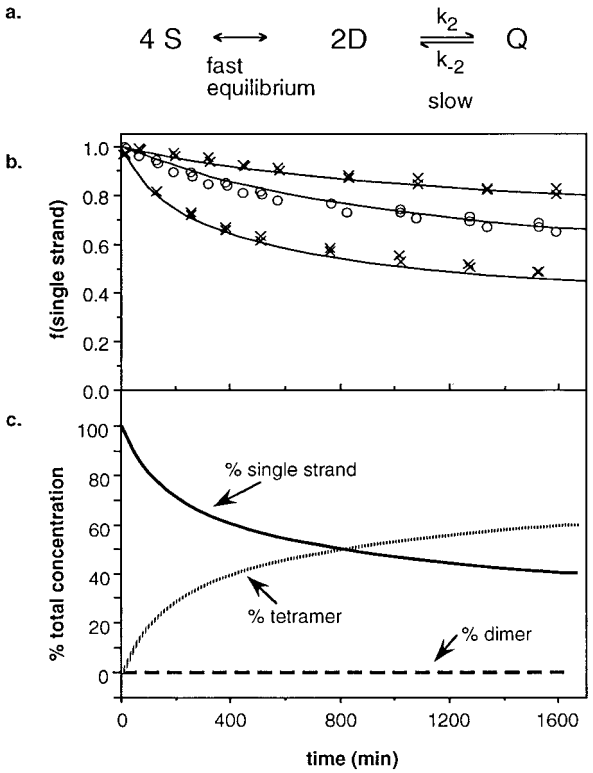

FIGURE 4: Proposed mechanism for formation of tetramer from single strand. (a) Four single strands (S) are in fast pre-equilibrium with two dimers (D). In the rate-limiting step, two dimers form the tetramer (Q). Kinetic simulations of tetramer formation at 22 °C in PBS were performed assuming this mechanism. Values used for the mechanistic rate constants are given in the text. (b) Calculated curves (—) are compared to experimental data at 22 °C in PBS for concentrations of 200 μM (◇), 300 μM (○), and 500 μM (×). (c) The percent of the total oligonucleotide concentration in single strands (—), tetramer (•••) and dimer (- - -) are plotted for the association reaction at 500 μM strand in PBS at 22 °C. The percent oligonucleotide in dimer complex never reached detectable levels.

Values for K_{eq} less than 10 M^{-1} and values of k_2 faster than $3.8 \times 10^3\ M^{-1}\ s^{-1}$ resulted in concentration of dimer less than 2% of the total strand concentration, well below the limit of detection of the assay.

Thermal Melting Experiments. The slow kinetics of tetramer formation and dissociation make equilibrium measurements impractical. As shown in Figure 5 the apparent melting temperature of the phosphorothioate tetramer changed with heating rate. The sample was diluted to 20 μM strand concentration in PBS immediately before the experiment. At a heating rate of 1 deg min^{-1}, a typical rate for a thermal denaturation experiment, the apparent melting temperature (T_{max}) was 73.4 (±0.5) °C. At the slowest heating rate used, 0.025 deg min^{-1} (1 °C every 40 min), T_{max} was 61.0 (±0.2) °C.

The melting temperature vs heating rate curve was simulated using dissociation rates calculated at each temperature from the slope and intercept of the Arrhenius plot (Figure 2b). For calculations, the sample was assumed to be 95% tetramer at 23 °C; this was confirmed by size exclusion chromatography. From the dissociation rates and the heating rate, the fraction single strand at each temperature above 23 °C was determined. Reassociation was ignored in the simulation because, at this oligonucleotide concentration, the association half-time at 23 °C is about 16 years and is slower at higher temperatures. The T_{max} values from

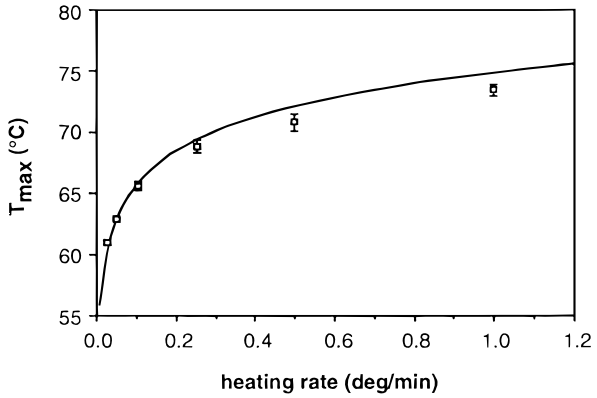

FIGURE 5: Apparent melting temperature (T_{max}) of the phosphorothioate tetramer changed with heating rate. The observed T_{max} at each temperature is indicated (□). The error bars are the standard deviation of two or three experiments. The calculated curve (—) was determined using the slope and intercept of the Arrhenius plot for the dissociation rate as described in the text.

the simulation are plotted with the experimentally determined T_{max} values in Figure 5. The Arrhenius data nearly quantitatively predicted the dependence of the T_{max} on heating rate and provided confirmation of the temperature dependence of the dissociation rates shown in Figure 2b.

Calculated Thermodynamic Parameters. As thermodynamic parameters could not be obtained from equilibrium melting experiments, parameters for formation of the phosphorothioate d(TTGGGGTT) tetramer in PBS (pH 7.2) were calculated from the rate constants and the activation enthalpy. The $\Delta G°$(37 °C) is −16 kcal mol^{-1}, $\Delta H°$ is −91 kcal mol^{-1}, and $\Delta S°$ is −240 cal mol^{-1} K^{-1}. The tetramer was kinetically stable at 37 °C in PBS (pH 7.2) with a dissociation $t_{1/2}$ of approximately 60 days. However, the tetramer was not thermodynamically stable at room temperature under conditions used in the thermal denaturation experiments described above. At 1 mM strand concentration, the equilibrium melting temperature is 49 °C. At 10 μM strand, the equilibrium melting temperature is 20 °C.

Kinetics of Phosphodiester d(TTGGGGTT) Tetramer Formation and Dissociation. The rate of dissociation of the phosphodiester d(TTGGGGTT) tetramer was determined at 55, 60, and 65 °C in PBS for comparison to the phosphorothioate (Table 1). The rates are roughly an order of magnitude slower than the dissociation rates of the phosphorothioate under the same conditions.

The association rates for the phosphodiester d(TTGGGGTT) were determined at two temperatures (Table 2). The phosphodiester oligonucleotide associated to form the tetramer more rapidly and the tetramer dissociated more slowly than the tetramer formed by the phosphorothioate oligonucleotide. Thus, the equilibrium constant for formation of the phosphodiester tetramer was considerably more favorable than that for the phosphorothioate tetramer.

Dependence of Rates on Monovalent Cation Concentration. The dissociation rate of the phosphorothioate tetramer was strongly dependent on the concentrations of both sodium and potassium. At 65 °C, the dissociation rate increased as the concentration of sodium in the buffer was increased, and dissociation rate slowed as the concentration of potassium was increased (Figure 1, Supporting Information). We previously observed that tetramer prepared in buffer containing no added potassium dissociated more rapidly at 37 °C than tetramer prepared in a mixture of sodium and potassium (Wyatt et al., 1994). This is consistent with increased stability of G-quartet structures formed in potassium relative to sodium that has been observed by others (Williamson et al., 1989; Hardin et al., 1992; Lu et al., 1992). The observed salt dependence at 65 °C is consistent with exchange of counter-ion within the G-quartet structure without dissociation of the tetramer.

The association rate of the phosphorothioate tetramer was increased when either sodium or potassium ion concentrations were increased. Fourth-order association rate constants were determined at 5 °C, in 50 mM sodium phosphate (pH 7.2) as a function of potassium and at 4 mM KCl and 50 mM sodium phosphate (pH 7.2) as a function of sodium. The extra monovalent ion concentration was varied from 20 to 500 mM. A plot of ln k vs ln (monovalent ion) showed a fourth-order dependence on potassium and a fifth-order dependence on sodium concentration (Figure 2, Supporting Information).

DISCUSSION

The phosphorothioate and phosphodiester oligonucleotides d(TTGGGGTT) adopt four-stranded structures presumably stabilized by G-quartets. Circular dichroism spectra (Figure 1) of the two oligonucleotides were characteristic of parallel-stranded tetrameric structures (Balagurumoorthy et al., 1992; Lu et al., 1993). Small, but significant, differences in the spectra of the phosphorothioate and phosphodiester oligonucleotides are presumably due to subtle structural differences between the two tetramers.

The slow kinetics of formation and dissociation of G-quartet structures have been discussed by Williamson (1994). In NMR studies of the parallel-stranded tetramer formed by the oligoribonucleotide d(TGGGGT) (Cheong & Moore, 1992) and of deoxyoligonucleotides that adopt inter- and intramolecular G-quartet structures (Smith & Feigon, 1992; Wang & Patel, 1992; Gupta et al., 1993), the normally labile imino protons do not exchange for days. Slow kinetics of dissociation of phosphodiester d(TTGGGGTT), under ionic conditions different from those used in this study, were reported previously (Shida et al., 1991). Thus, G-quartet structures exhibit kinetic stability unique for nucleic acid structures.

Previous studies have indicated that G-quartet structure formation is slow. Several groups have studied the kinetics of folding of oligonucleotides containing dimeric G-quartet structures (Sen & Gilbert, 1990; Fang & Cech, 1993; Guo et al., 1993). In one study of a four-stranded G-quartet structure, Watson−Crick duplex formation was shown to be faster than parallel-stranded tetramer formation for the deoxyoligonucleotide d(GGGGTTTT) (Sarma et al., 1992).

In the present study, size exclusion chromatography was used to resolve single strand from tetramer as a function of time, and kinetics of dissociation and association were determined. Both the phosphorothioate and phosphodiester oligonucleotides demonstrated similar, surprisingly slow kinetics. Dissociation of the phosphorothioate oligonucleotide d(TTGGGGTT) in PBS, which contains a low concentration of potassium (about 4 mM), appeared first order. At 37 °C, the $t_{1/2}$ was approximately 60 days. Extrapolating from the Arrhenius plot (Figure 2b), the $t_{1/2}$ at 90 °C is 6.5 s. Dissociation of short oligonucleotide duplexes is

typically at least an order of magnitude faster than this even at room temperature (Riesner & Romer, 1973). The phosphodiester tetramer dissociated more slowly and associated more rapidly than the phosphorothioate. This may be due to the diastereoisomers at each phosphate linkage in the phosphorothioate oligonucleotide. As the phosphorothioate oligonucleotide is a heterogeneous mixture of 7^2 compounds, the observed rate constants for the phosphorothioate tetramer likely represent a weighted average of a spectrum of rate constants for the many diastereomers.

Association of d(TTGGGGTT) to the parallel-stranded tetramer was determined to be fourth order with respect to strand concentration for both phosphorothioate and phosphodiester oligonucleotides. The mechanism that we propose (Figure 4a) is consistent with this observed fourth-order dependence on strand concentration and the negative activation energy for tetramer association. In this mechanism, tetramer formation from two dimers is rate limiting, and single strands and dimers are in rapid pre-equilibrium, favoring single strand. Plausible base-pairing schemes for dimer intermediates involving guanine−guanine pairs have been proposed (Schultze et al., 1994). The data are also consistent with a mechanism involving a four-stranded intermediate stabilized by one or more G-quartets. The four-stranded intermediate is analogous to the partial helix invoked to explain negative activation energies for nucleic acid duplex formation. Formation of the stable tetramer from this partial quartet structure would be rate limiting. This mechanism would require a four-body collision, however, while the mechanism that we propose involves the more likely scenario of a series of two-body collisions.

The slow kinetics of tetramer association and dissociation make equilibrium measurements practically impossible. As shown in Figure 5, the apparent melting temperature changed dramatically as the heating rate was slowed. At a heat rate of 1 deg min^{-1} at strand concentrations and buffer conditions described, the apparent melting temperature of the phosphorothioate tetramer was 73 °C. The actual melting temperature calculated from thermodynamic parameters derived from the rate constants is 23 °C. Adequate time must be allowed for sample equilibration when attempting to use equilibrium techniques to analyze thermodynamic parameters (Williamson, 1994). For practical reasons, this is all but impossible for the G-quartet structures analyzed in this study.

The present work was prompted by the discovery that the tetramer form of the phosphorothioate oligonucleotide d(TTGGGGTT) has anti-HIV activity in cell-based assays at concentrations below 1 μM (Buckheit et al., 1994; Wyatt et al., 1994). Although the tetrameric form is not thermodynamically stable at this concentration, the extremely slow dissociation rate of the tetramer assures that the active structure is present during the 6-day period of the antiviral assay. The studies detailed here indicate that under ionic conditions found *in vivo*, and in the absence of catalyzed dissociation of the tetramer, the tetramer will be sufficiently stable for use as a therapeutic agent. The elimination half-life of phosphorothioate oligonucleotides has been measured in animals (Zhang et al., 1995; Crooke et al., 1996) and is approximately 24−48 h. If the pharmacokinetics of the tetramer are similar to those of other phosphorothioate oligonucleotides, pharmacokinetic clearance will occur much more rapidly than tetramer dissociation.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing data on the salt dependence of dissociation and association rates of the phosphorothioate tetramer, the dependence of dissociation rate on concentration of sodium and potassium, and the dependence of association rate on the concentration of sodium and potassium (2 pages). Ordering information is given on any current masthead page.

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