Characterization of a G-Quartet Formation Reaction Promoted by the β-Subunit of the *Oxytricha* Telomere-Binding Protein†

Guowei Fang and Thomas R. Cech*

Department of Chemistry and Biochemistry, Howard Hughes Medical Institute, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: Telomeres, the ends of linear chromosomes, typically consist of tandem repeats of a simple guanine-rich sequence. Telomeric DNA is able to form intermolecular G-quartet structures. The β-subunit of the *Oxytricha* telomere-binding protein acts as a molecular chaperone to promote the formation of dimers and specific higher order complexes of telomeric DNA stabilized by G-quartets; these reactions occur under physiological conditions *in vitro*. In the present article, we show that, at saturating protein concentrations (≥200 nM), β-mediated G-quartet formation is a first-order reaction with respect to DNA concentration, with $k \approx 1\ h^{-1}$ at 37 °C. In contrast, the protein-independent reaction is a second-order reaction. The β-subunit enhances the rate of G-quartet formation by 10^5–10^6-fold at a telomeric DNA concentration of 20 nM. The β-mediated higher order complexes are identified as parallel four-stranded tetramers of telomeric DNA (G4-DNA). Poly-L-lysine also promotes formation of the tetramers, but not dimers. These DNA structures were studied by irreversible thermal melting experiments and probed by annealing to different complementary strands. Guanine residues important for structure formation were analyzed by methylation interference experiments. On the basis of these data, models for the β-mediated structures are proposed, and possible mechanisms for the β-mediated reaction are discussed. In addition, we found that the β-subunit promotes the annealing of two complementary strands into a duplex, as do many other basic proteins. However, not all proteins with annealing-promoting activity are active in the formation of G-quartet structures. The activity of the telomere protein in promoting the formation of telomeric DNA structures may enable chromosome–chromosome association or the regulation of telomerase activity *in vivo*.

Telomeres, the ends of linear chromosomes, are essential for the stability and the complete replication of eukaryotic chromosomes [reviewed in Zakian (1989) and Blackburn (1991, 1992)]. Telomeric DNA typically consists of tandem repeats of a simple sequence with one strand rich in guanine residues [for example, T_2AG_3 in humans, T_4G_4 in the ciliated protozoa *Oxytricha* and *Stylonychia*, T_2G_4 in *Tetrahymena*, and TG_{1-3} in *Saccharomyces*; reviewed in Blackburn and Szostak (1984) and Zakian (1989)]. The G-rich strand is always oriented in a 5′ to 3′ direction toward the end of the chromosome. A single-stranded overhang of two repeat units protrudes at the 3′-end of this G-rich strand in several evolutionarily divergent organisms examined, including *Oxytricha*, *Stylonychia*, *Euplotes*, *Tetrahymena*, and the slime mold *Didymium* (Klobutcher et al., 1981; Henderson & Blackburn, 1989). Recently, *Saccharomyces* telomeres have been shown to acquire single-stranded telomeric DNA late in the S phase (Wellinger et al., 1993). Therefore, 3′ single-stranded overhangs appear to be a general feature of telomeres.

Telomeric DNA has been shown to form higher order structures *in vitro*. Oligonucleotides containing two repeats of *Oxytricha* or *Tetrahymena* telomeric sequences can form dimers through G-quartet structures, and oligonucleotides containing four repeats of telomeric sequences can fold intramolecularly into G-quartet structures (Sundquist & Klug, 1989; Williamson et al., 1989). In addition, guanine-rich oligonucleotides, including those with telomeric sequences, can form parallel four-stranded structures (G4-DNA) held together by G-quartets (Sen & Gilbert, 1988, 1990, 1992). In a G-quartet, four guanines are arranged in a square-planar unit, with each guanine serving as both hydrogen-bond acceptor and donor in a Hoogsteen base pair (Figure 1). Successive layers of G-quartets stack on each other and form the structures. The main features of the G-quartet model were confirmed and extended by X-ray crystallographic and NMR studies (Kang et al., 1992; Smith & Feigon, 1992; Aboul-ela et al., 1992; Wang & Patel, 1992; Cheong & Moore, 1992). Intermolecular G-quartet formation is a very slow process even at high DNA concentrations, so the biological relevance of the G-quartet structures remains unclear.

Telomeric DNA interacts with proteins in many organisms. In *Oxytricha nova*, a heterodimeric protein consisting of 56-kDa (α) and 41-kDa (β) subunits binds specifically to the single-stranded $T_4G_4T_4G_4$ overhang at the termini of macronuclear chromosomes (Gottschling & Cech, 1984; Gottschling & Zakian, 1986; Price & Cech, 1987, 1989; Raghuraman & Cech, 1989; Hicke et al., 1990; Gray et al., 1991). The α subunit binds to telomeric DNA by itself. The α- and β-subunits undergo DNA-dependent dimerization and bind to telomeric DNA cooperatively, forming an extremely stable telomeric complex (ternary complex) (Gray et al., 1991; Fang & Cech, 1993a; Fang et al., 1993). Deletion analysis shows that the amino-terminal two-thirds of the β-subunit is necessary and sufficient for the β-subunit to participate in the ternary complex (Fang et al., 1993).

Recently, we found that the β-subunit promotes G-quartet formation under physiological conditions *in vitro* (Fang & Cech, 1993b). The reaction occurs with oligonucleotides ending in the *Oxytricha* ($T_4G_4T_4G_4$) or *Tetrahymena* ($T_2G_4T_2G_4$) telomeric sequences; the sequence preceding these telomeric repeats can be nontelomeric, single- or double-

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* Author to whom correspondence should be addressed.

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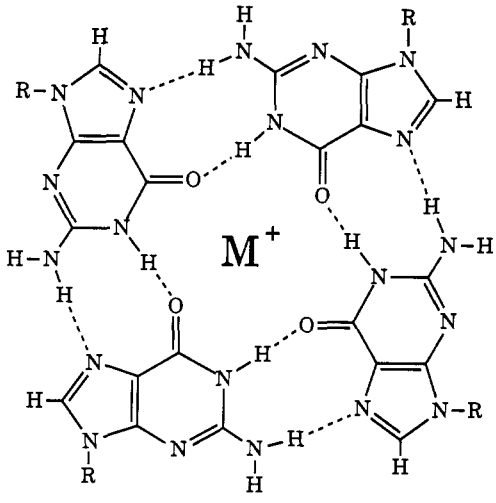


FIGURE 1: G-quartet structure. Four guanine residues are arranged in a square-planar array. Each guanine serves as both hydrogen-bond acceptor and donor in a Hoogsteen base pair (Gellert et al., 1962). Monovalent cations are selectively bound in the central cavity formed by successive layers of G-quartets.

stranded. The intrinsic ability of an oligonucleotide to form G-quartets is a prerequisite for the β-mediated reaction, and the β-subunit enhances the rate of this thermodynamically favored transition. At lower protein concentrations, the β-subunit acts without remaining stably bound to the DNA; at higher protein concentrations, which drive the formation of protein–DNA complexes, proteinase K treatment reveals that the same set of DNA structures is formed. Thus, the β-subunit acts as a molecular chaperone to promote nucleic acid structure formation. Protein deletion analysis indicates that the carboxy terminal region, which is highly basic and which bears a slight sequence similarity to histone H1 (Hicke et al., 1990), is important for the β-mediated reaction. Therefore, the ability to promote G-quartet formation and the ability to form the ternary complex require different domains of the β-subunit, and represent two different activities of the β-subunit. The finding that a telomere protein promotes G-quartet formation by telomeric DNA strongly suggests that the G-quartets are biologically relevant structures.

In the present article, we characterize this β-mediated reaction and propose models for the β-mediated structures. We find that the β-mediated and the protein-independent reactions have different mechanisms; the former reaction follows first-order kinetics with respect to DNA concentration, and the latter follows second-order kinetics. The β-mediated reaction occurs at nanomolar DNA concentrations with a $t_{1/2}$ of 60–70 min at saturating β concentrations. This represents a rate enhancement of 10^5–10^6-fold at 20 nM DNA. In addition to dimers, the β-mediated DNA structures include two types of tetramers (G4-DNA; Sen & Gilbert, 1988), which differ with respect to the register in which the blocks of guanines are aligned.

MATERIALS AND METHODS

Oligonucleotides. OXY2 (5′-TTTTGGGGTTTTGGGG-3′), OXY2T (5′-AAGACGACATCGCTCAGCCAGA-CATTTTGGGGTTTTGGGG-3′), OXY2LT (5′-AAGAC-GACATCGCTCAGCCAGACAGACGACATCGCTCA-GCCAGATTTTGGGGTTTTGGGG-3′), OXY2T′ (5′-AA-GACGACATCGCTCAGAGTCGACATGTAAT-TACCTTTTTGGGGTTTTGGGG-3′), 5′-OXY2T (5′-TTTTGGGGTTTTGGGGAGTCGACATGTAATTACCT-3′), MDOXY2 (5′-AGCGGATAACAATTTCACACAGG-ATTTTGGGGTTTTGGGGCTATTGAATTCGTC-GTGACTGGGAAAACCATGGCG-3′), cT15 (5′-TGTCTG-GCTGAGCGA-3′), cT (5′-TGTCTGGCTGAGCGATGT-CGTCTT-3′), cOXY2 (5′-CCCCAAAACCCCAAAA-3′), cOXY2T (5′-CCCCAAAACCCCAAAATGTCTGGCT-GAGCGATGTCGTCTT-3′), ds1 (5′-AAGACGACATCG-CTCAGCCAGACAACCAAGTTCACACTCC-3′), and ds2 (5′-AAGACGACATCGCTCAGCCAGACAGGAGT-GTGAACTTGGT-3′) were made on an Applied Biosystems 380B DNA synthesizer. Oligonucleotides were purified and ^{32}P-labeled as described (Fang & Cech, 1993b).

Proteins. The telomere-binding protein and its deletion mutant βC232 were produced in *Escherichia coli* containing recombinant plasmid expression vectors (Gray et al., 1991) and purified to homogeneity using a protocol developed by J. Ruggles and S. Schultz, as described by Fang et al. (1993). Rabbit heart cytochrome *c*, poly-L-lysine (10 kDa), and L-lysine were purchased from Sigma.

DNA Structure-Formation Reactions. 5′-^{32}P-labeled oligonucleotides were incubated with or without protein in 10 μL of 30 mM HEPES (pH 7.5), 1 mM EDTA, and 80 mM NaCl for 4–25 h at 37 °C unless otherwise indicated. SDS and proteinase K were then added to final concentrations of 0.5% and 150 ng/μL, respectively, and the mixture was incubated for 30 min at 37 °C. Samples were prepared for gel electrophoresis by adding 2 μL of 50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol and were loaded onto a 10% polyacrylamide (55:1 acrylamide/bisacrylamide) gel that had been prerun for 30 min. Electrophoresis was carried out for 4–8.5 h at 15 mA and 4°C in 1 mM EDTA, 45 mM Tris–borate, and 45 mM boric acid at pH 8.3. The gel was dried and subjected to autoradiography. For quantitative study of the rate of G-quartet formation (Figures 2 and 3), all incubations were performed in presiliconized Eppendorf tubes (National Scientific Supply Company, Inc.).

For the complementary strand-annealing experiment (Figure 10), 5′-^{32}P-labeled ds1 and unlabeled ds2 were denatured separately by boiling for 5 min and cooling to room temperature; ds1 and ds2 were then mixed and incubated with or without α, β, βC232, cytochrome *c* or lysine in 10 μL of 30 mM HEPES (pH 7.5), 1 mM EDTA, and 80 mM NaCl for 5 min at 37 °C. Final DNA concentrations were 2 nM ds1 + 2 nM ds2. Thirty microliters of stop solution (330 nM unlabeled ds1, 50 ng/μL proteinase K, 0.33% SDS, 8% glycerol, 0.04% bromophenol blue, and 0.04% xylene cyanol in Tris–EDTA buffer) were added, and the mixture was incubated for 10 min at 37 °C. Samples were analyzed in a 10% nondenaturing gel as described above. As a control, 1 μM α or β was digested with proteinase K for 20 min at 37°C prior to incubation with ds1 and ds2.

Dimethyl Sulfate Methylation Interference. 5′-^{32}P-labeled OXY2T (25 pmol) was denatured for 5 min at 95 °C in 10 μL of Tris–EDTA buffer. Dimethyl sulfate (DMS) was added to 0.1%. Methylation proceeded for 10 min at room temperature and was stopped by the addition of 2 μL of stop solution (2.5 M β-mercaptoethanol, 0.1 M EDTA, and 0.5% SDS in Tris-EDTA buffer). The reaction mixture was diluted by the addition of 90 μL of Tris–EDTA buffer, and the methylated DNA was purified through a G-50 Sephadex spin column (Boehringer-Mannheim). Methylated OXY2T (200 nM) was then incubated with 1 μM β or 0.5 μM poly-L-lysine (10 kDa) in 35 μL of 50 mM HEPES (pH 7.5), 2 mM EDTA, and 115 mM NaCl for 20 h at 37 °C. Proteins were digested

by proteinase K, and different products (M, D, and T) were separated in a 10% nondenaturing gel. Radioactive bands were cut out and eluted into 800 μL of Tris–EDTA buffer with 250 mM NaCl. After ethanol precipitation, methylated OXY2T was cleaved by 1 M pyrrolidine for 15 min at 90°C and then dried in a speed vacuum (Savant). DNA was resuspended in 15 μL of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol and analyzed by 12% polyacrylamide/8 M urea gel electrophoresis.

RESULTS

β-Subunit Converts Dimerization to a First-Order Reaction. Oligonucleotide OXY2T has two repeats of T_4G_4 at the 3′-end preceded by a 24-nucleotide nontelomeric tail sequence. The β-subunit was incubated with increasing amount of OXY2T, and then digested by proteinase K to disrupt DNA–protein complexes. The products were analyzed by nondenaturing gel electrophoresis (Figure 2A, five lanes on the left). The two shifted bands with faster migration rates are termed D products; they have been identified as dimers of the oligonucleotide stabilized by G-quartets (Fang & Cech, 1993b). The three shifted bands with slower migrating rates are termed T products. The faster migrating band in the T products is called T1, and the slower migrating doublet is T2.

Figure 2 shows the DNA concentration dependence of G-quartet formation. In the presence of the β-subunit, both D and T products were detected at low OXY2T concentrations (20 nM to 1 μM). In the absence of the β-subunit, only D products were detected, and they formed only at much higher OXY2T concentrations (5–15 μM). These products comigrated with the D products formed in the presence of the β-subunit (Figure 2A). The amount of D or T products formed at different OXY2T concentrations was quantitated, and the logarithm of product concentration was plotted against the logarithm of total OXY2T concentration. At saturating concentration of β, the plots were linear with slopes of 0.90, 0.92, and 0.93 for D products formed after 1-, 2-, and 4-h incubations, respectively, and slopes of 0.98, 1.05, and 1.01 T for products (Figure 2B,C; data not shown for the 2-h incubation). Additional experiments confirmed that the slopes were independent of incubation time (from 15 min to 6.5 h, ~10% OXY2T molecules had reacted by 15 min and ~50% molecules had reacted by 6.5 h; data not shown). Therefore, the rate-limiting steps for β-mediated formation of both D and T products are first-order with respect to OXY2T concentration. In the absence of the β-subunit, the plots for D products were also linear, with slopes of 1.91, 1.91, and 1.85 for 1-, 3-, and 72-h incubations, respectively (Figure 2D). These plots monitor the initial reaction, since less than 4% of the OXY2T molecules had reacted during the first hour of incubation and less than 13% had reacted by 72 h. Additional plots of different incubation times (8, 25, and 48 h) had similar slopes (between 1.79 and 1.89; data not shown). Thus, the rate-limiting step for the formation of D products in the absence of β follows second-order kinetics with respect to OXY2T concentration. We conclude that the β-mediated and the protein-independent reactions have different mechanisms.

β-Subunit Enhances the Rate of G-Quartet Formation by 10^5–10^6-Fold. Figure 3A shows the kinetics of β-mediated G-quartet formation. At 20 nM OXY2T and 200 nM β, D and T products formed rapidly, with a $t_{1/2}$ of ~60 min. The reaction plateaued after 6 h when about one-half of the DNA had reacted; the kinetic analysis is restricted to this reactive component. The T2 products formed faster than T1; however, at later time points T2 disappeared while T1 increased monotonically with time. Quantitative analysis of the formation of products D and T by a PhosphorImager showed that the apparent rate constant (k_{200}) was 0.68 h^{-1} (Figure 3B). Analysis of the reaction kinetics at different β concentrations showed that the reaction rate approaches saturation at 100 nM protein concentration (k_{100} = 0.49 h^{-1}) (Figure 3B and Table I). At lower β concentrations, the reaction was substantially slower. For example, lowering of the β concentration by 2-fold reduced the rate constant by 10-fold (k_{50} = 0.05 h^{-1}). Thus, there is a sharp transition in the dependence of the rate constant on the β concentration, which does not fit the Michaelis–Menten steady-state reaction model. At low concentration, the β-subunit sticks efficiently to the walls of the Eppendorf tubes (data not shown), which may explain the steep decline in activity with decreasing protein concentration. However, we cannot exclude the possibility that there is cooperativity between β molecules in promoting G-quartet formation.

The rate constant did not change with OXY2T concentration (20–1000 nM) at saturating β concentrations (200–1000 nM) (Table I). This is in contrast to the second-order dependence that would be expected if the rate-limiting step involved collision of β·OXY2T complexes.

In the absence of β-subunit, OXY2T (20 nM) did not form G-quartets, even after 96-h incubation (Figure 3A). The detection sensitivity of the PhosphorImager was $^1/_{200}$ of the total OXY2T signal in each sample. Thus, the second-order rate constant of the protein-independent reaction is less than 2.6×10^3 M^{-1} h^{-1} [calculated as $k = (0.1\text{ nM}/96\text{ h})/(20\text{ nM})^2$]. At a higher OXY2T concentration (15 μM), the reaction is still very slow. After a 72-h incubation, only 7.5% of the OXY2T molecules had reacted (data not shown). In addition, the formation of D products was biphasic. The fast reaction (<8 h) accounted for 5% OXY2T. The second-order rate constant for this reaction phase is 3.9×10^2 M^{-1} h^{-1}. The rate constant for the slow-phase reaction is 32 M^{-1} h^{-1}. Formation of D products at different OXY2T concentrations (5, 10, 20, and 30 μM) all showed biphasic kinetics (data not shown). The average rate constant for the fast phase reaction determined at these OXY2T concentrations is $(4.0 \pm 0.6) \times 10^2$ M^{-1} h^{-1}, and the average rate constant for the slow-phase reaction is 29 ± 2.5 M^{-1} h^{-1}. After a 72-h incubation, only 3–13% of OXY2T molecules had reacted. We do not know whether still slower reaction phases exist besides the two phases characterized here.

On the basis of the average rate constant of 4.0×10^2 M^{-1} h^{-1}, the initial rate for G-quartet formation in the absence of protein is 1.6×10^{-4} nM h^{-1} at 20 nM OXY2T. In the presence of 200 nM β, the reaction rate at the same OXY2T concentration is 14 nM h^{-1}. Thus, there is a 10^5-fold rate increase by the saturating amount of β at 20 nM OXY2T. However, this comparison undervalues the rate enhancement due to β, because it is based on the fast phase of the protein-independent reaction, which is characteristic of only the fastest 5% of the DNA molecules. When compared with the slow phase of protein-independent G-quartet formation, the rate enhancement by β is greater than 10^6-fold. In addition, at lower OXY2T concentrations the extent of rate enhancement is expected to be greater, because the order of the reaction with respect to DNA concentration is higher for the protein-independent reaction than for the β-mediated reaction.

β-Mediated Structures Have Different Kinetic Stabilities. The β-mediated structures were studied by irreversible thermal

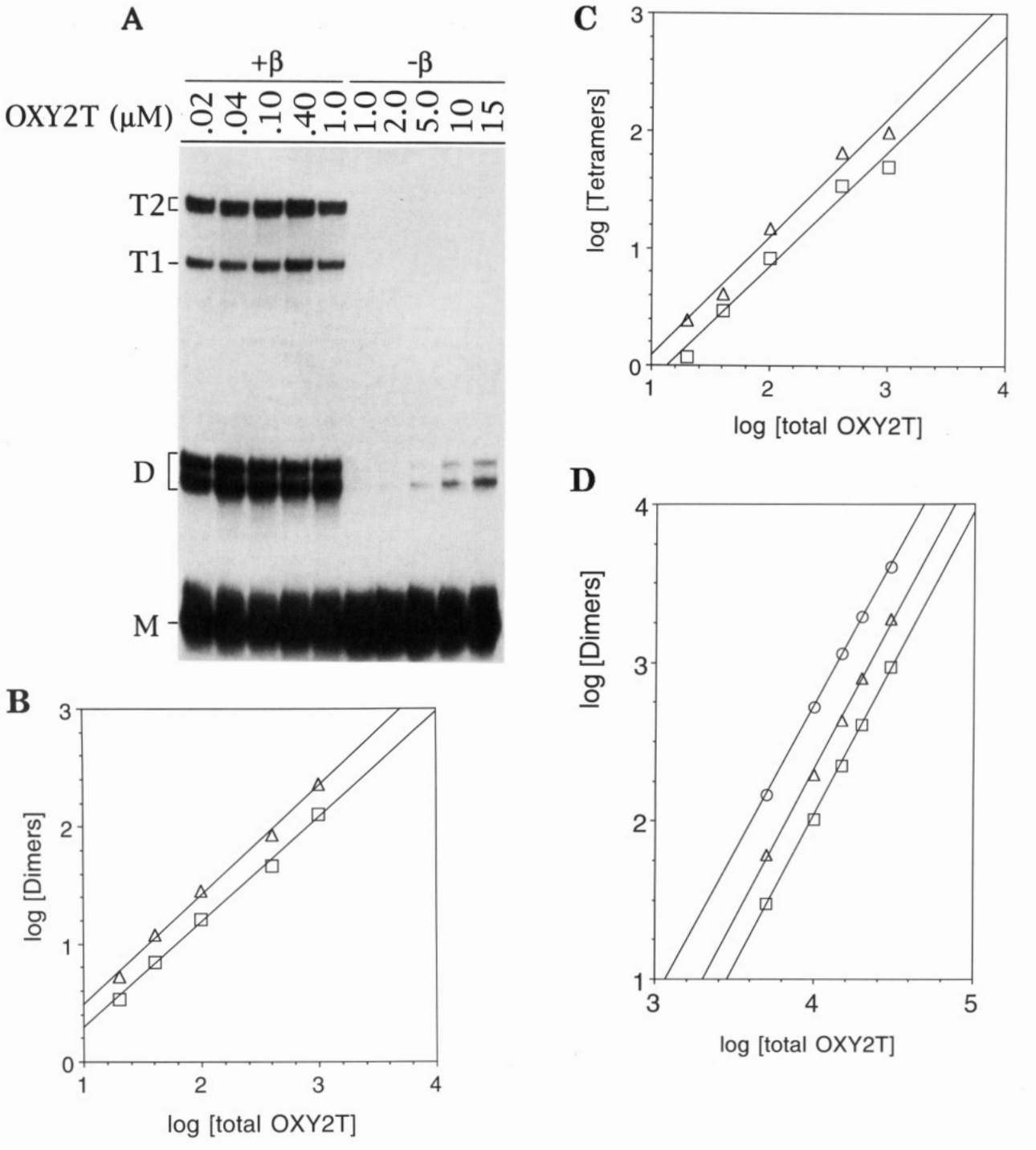


FIGURE 2: β-Mediated and protein-independent structure formation at different OXY2T concentrations in 1 mM EDTA, 80 mM NaCl, and 30 mM HEPES (pH 7.5). (A) Different amounts of OXY2T were incubated for 4 h with 1 μM β or for 25 h without β. All samples were incubated by submerging in water in a water bath set at 37 °C. Each sample contained the same amount of 5′-^{32}P-labeled OXY2T and enough unlabeled OXY2T to give the DNA concentration indicated. Samples incubated with β were then digested with proteinase K for 30 min at 37 °C. All samples were analyzed by 10% nondenaturing polyacrylamide gel electrophoresis. M, D, and T (T1 and T2) were thus named because they are monomer, dimers, and tetramers of the telomeric oligonucleotide, respectively (see text). Upon longer gel electrophoresis, the faster migrating band in the D products resolved into two species, a major and a minor band (data not shown). (B and C) Plots of dimer (B) and tetramer (C) formation as a function of total OXY2T concentration in the presence of β. All DNA concentrations are nanomolar. After OXY2T was incubated with 1 μM β at 37 °C for 1 (□) or 4 (△) h, the protein was digested with proteinase K, and samples were analyzed by gel electrophoresis. The amount of dimers and tetramers formed was quantitated by a PhosphorImager (Molecular Dynamics). (D) Plots of dimer formation as a function of total OXY2T concentration in the absence of β. OXY2T was incubated for 1 (□), 3 (△), or 72 (○) h. In plots B–D, the solid lines represent the best linear fits to the data, and the slopes of these lines are reported in the text.

melting experiments (Figure 4). OXY2T was incubated with the β-subunit. After digestion by proteinase K, the β-mediated products were incubated for 5 min at the temperature indicated, cooled to room temperature, and then analyzed by nondenaturing gel electrophoresis. Since OXY2T (20 nM) does not form G-quartets in the presence of proteinase K-digested β-subunit even after several hours (Fang & Cech, 1993b), structures that melted at high temperatures would not reform at room temperature before gel electrophoresis.

D and T2 products melted at lower temperatures than T1 (Figure 4A). We define the T_i (irreversible melting temperature) as the temperature at which 50% of a population

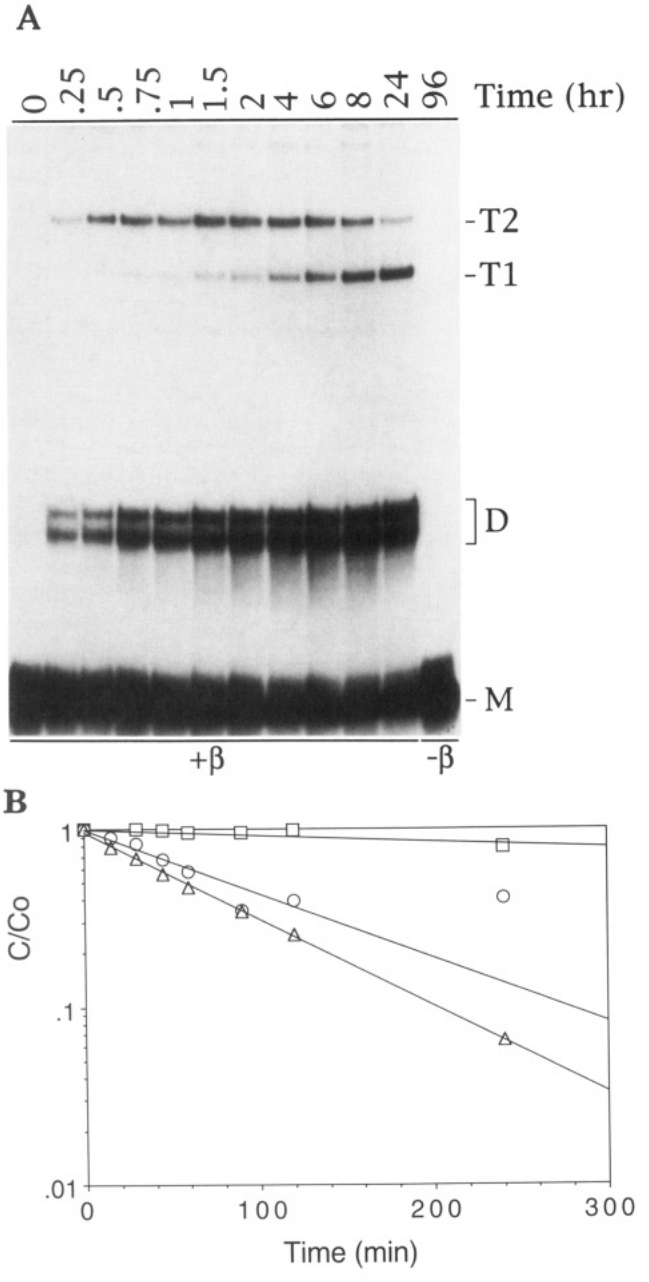


FIGURE 3: Kinetics of β-mediated G-quartet formation. (A) OXY2T (20 nM) was incubated with or without β (200 nM) at 37 °C for the times indicated. Samples were then digested with proteinase K and analyzed by 9% nondenaturing polyacrylamide (29:1 acrylamide/bisacrylamide) gel electrophoresis. (B) Quantitation of rates for β-mediated G-quartet formation. The fraction of monomeric OXY2T remaining (C/C_0) was quantitated by a PhosphorImager. The reaction of 20 nM OXY2T reached a plateau of 9.6 nM for products D plus T after a 6-h incubation with 200 nM β (△) or after a 24-h incubation with 100 nM β (○) and a plateau of 2.5 nM after a 24-h incubation with 50 nM β (□). Thus, the extent of reaction at each time was normalized to these end points. The solid lines represent the best linear fits to the data, and the rate constants derived from these lines are reported in the text.

of molecules is melted after 5 min of incubation. This experiment measured the kinetic stability of the β-mediated products since an increase in incubation time at the indicated temperature resulted in a larger fraction of the products melted. D and T2 products had the same T_i (55 °C) (Figure 4B), suggesting that a similar amount of interactions holds these structures together. If the dissociation of the structures were dominated by the energetic contribution of the G-quartets, the thermal melting study would suggest that D and T2 have

Table I: First-Order Rate Constants for β-Mediated G-Quartet Formation (D and T Products)

OXY2T (nM)	β (nM)	k (h^{-1})[a]
20	25[b]	0.02 ± 0.01[c]
20	50[b]	0.05 ± 0.03[c]
20	100[b]	0.49 ± 0.06[c]
20	200	0.68 ± 0.01[d]
20	500	0.59 ± 0.03[d]
20	1000	0.66 ± 0.20[d]
20	2000	0.76 ± 0.08[c]
20	4000	0.74 ± 0.09[c]
20	8000	0.85 ± 0.09[c]
100	200	0.63 ± 0.11[c]
100	1000	0.60 ± 0.08[c]
1000	1000	0.70 ± 0.13[c]

[a] The rate constants were determined in 30 mM HEPES (pH 7.5), 1 mM EDTA, and 80 mM NaCl at 37 °C by the method described in Figure 3B. [b] At these protein concentrations, β efficiently sticks to the walls of the tubes. Therefore, the rate constants may be undervalued. [c] The error range was based on the best and worst fits of a straight line to the data in a semilog plot such as that shown in Figure 3B. [d] The error range was based on replicate experiments performed on separate days.

the same number of layers of G-quartet. Product T1 had a higher T_i (65 °C) (Figure 4B), which could be explained if this structure contains more layers of G-quartet than D and T2. The relative order of T_i remained the same for products D, T1, and T2 at longer incubation times (data not shown).

The β-mediated structures were also studied by annealing to different complementary strands (Figure 5A). The β-subunit or poly-L-lysine (which promotes the formation of structures T1 and T2; Fang & Cech, 1993b) was incubated with OXY2T to promote G-quartet formation. Proteins were then removed, and the structures were annealed with oligonucleotides complementary to different regions of OXY2T (Figure 5B). Oligonucleotide cT, complementary to the nontelomeric tail sequence, did not disrupt either the D or T products. The monomeric OXY2T shifted by forming a duplex with cT, as did the D and T products. Thus, the nontelomeric tail sequence is not involved in structure formation. On the other hand, oligonucleotide cOXY2T, complementary to the whole length of OXY2T, disrupted both D and T products. When oligonucleotide cOXY2, complementary to $T_4G_4T_4G_4$, was incubated with the products, only T2 was disrupted, and D and T1 were unaffected. In addition, the mobility of D and T1 did not change in the presence of cOXY2, while monomeric OXY2T shifted its position in the nondenaturing gel. Thus, cOXY2 is able to anneal to monomeric OXY2T and to T2, but not to D and T1.

T Products Represent Tetramers of Telomeric DNA. As already mentioned, D products are dimers of telomeric DNA. The stoichiometry of the T products was determined by two different approaches. In a mixing experiment (Sundquist & Klug, 1989; Sen & Gilbert, 1990; Kim et al., 1991; Fang & Cech, 1993b), two different DNA oligonucleotides (OXY2T and OXY2LT) were used. Both have two repeats of T_4G_4 at their 3′-ends, but OXY2LT has a longer nontelomeric tail sequence and therefore reduced electrophoretic mobility. Poly-L-lysine was used in this experiment because it promotes the formation of products T1 and T2, but not D (Figure 5B). When OXY2T and OXY2LT were incubated with poly-L-lysine separately, each formed T1 and T2 products (Figure 6A, lanes 1 and 5, bands a and c, e and h). When OXY2T and OXY2LT were mixed together and then incubated with poly-L-lysine, a new set of bands with different mobility was detected (Figure 6A, lanes 2–4, bands b, d, f, and g). It was difficult to interpret this experiment since we did not know

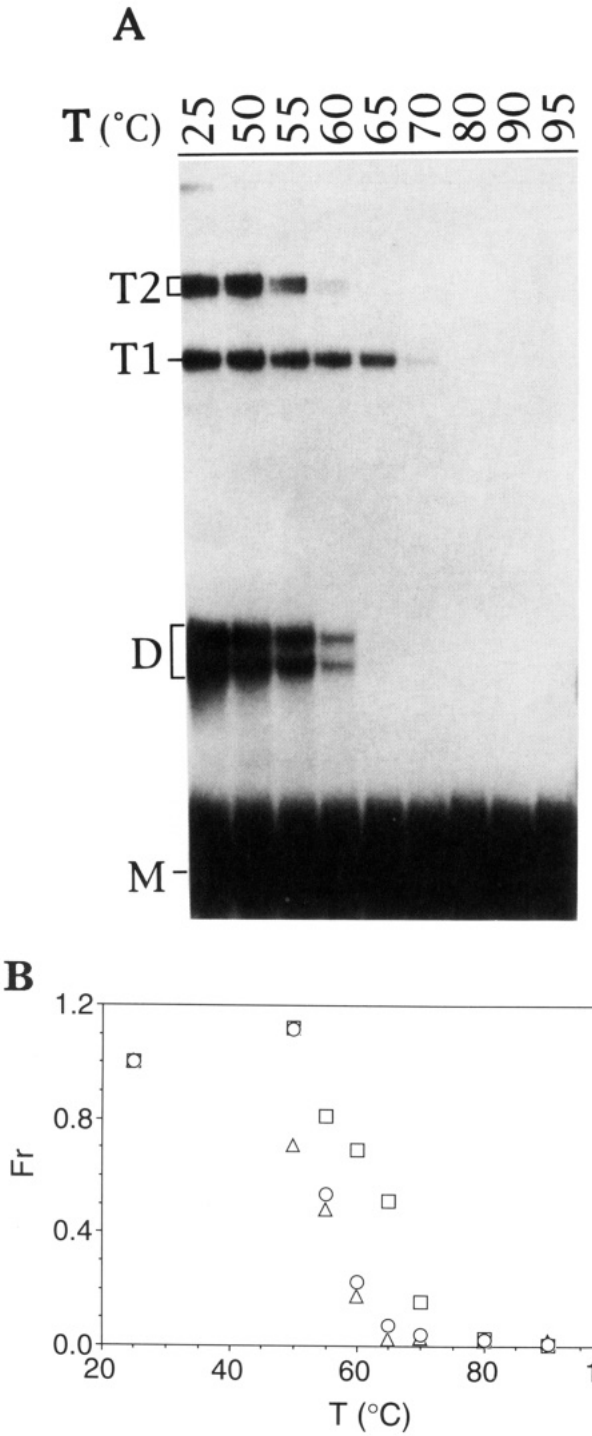


FIGURE 4: Thermal melting study of the β-mediated D and T structures. (A) OXY2T (20 nM) was incubated with β (1 μM) in 100 mM NaCl for 5 h at 37 °C. After proteinase K digestion, samples were incubated for 5 min at the temperature indicated and then analyzed by nondenaturing gel electrophoresis. (B) Quantitation of the experiment in panel A: △, D products; □, T1 product; ○, T2 products. Fr represents the fraction of G-quartet structures remaining.

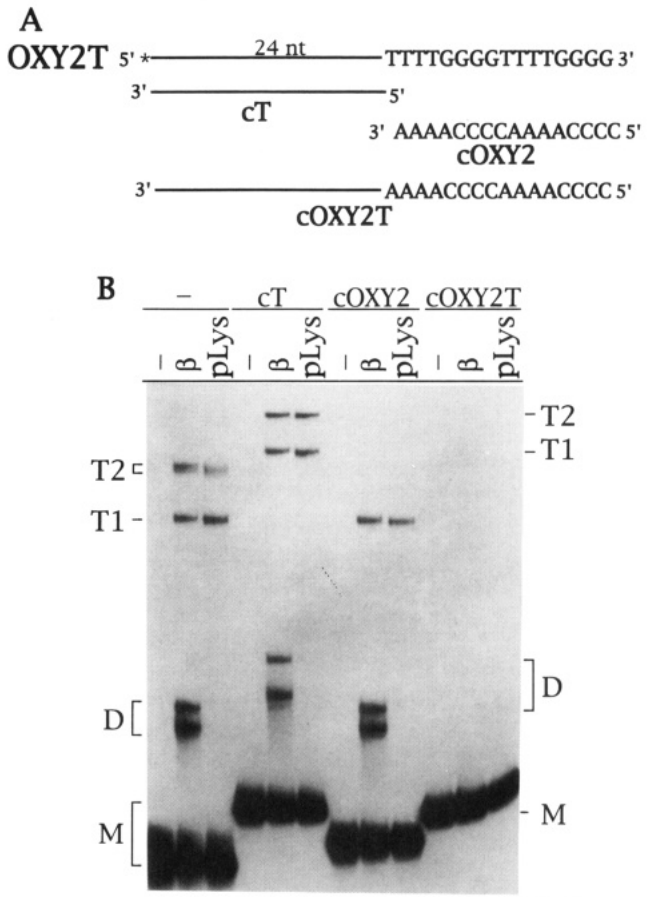


FIGURE 5: D and T structures studied by annealing with oligonucleotides complementary to different regions of OXY2T. (A) Oligonucleotides used in this experiment. An asterisk indicates a 5′-^{32}P label. (B) ^{32}P-labeled OXY2T (200 nM) was incubated with β (1 μM) or poly-L-lysine (0.5 μM). After proteinase digestion, unlabeled cT, cOXY2, cOXY2T (each at 400 nM), or Tris–EDTA buffer (three lanes on the left) was added and the mixture incubated for 30 min at 37 °C. Samples were then analyzed by nondenaturing gel electrophoresis.

which bands corresponded to T1 and which to T2. In addition, different T1 and T2 bands might comigrate.

Oligonucleotide cOXY2 preferentially disrupts the T2 products (Figure 5B). To simplify the banding patterns in the mixing experiment, T products were formed and cOXY2 was then added (Figure 6B). As expected, the T2 bands disappeared. OXY2T and OXY2LT each gave rise to a single T1 band. When the two oligonucleotides were mixed together and then incubated with poly-L-lysine, three bands with intermediate mobility were detected (Figure 6B, lanes 2–4, bands b–d). Thus, the T1 product represents a tetramer of telomeric DNA. Bands b–d had the electrophoretic mobility expected for tetramers of $(OXY2T)_3{\cdot}(OXY2LT)_1$, $(OXY2T)_2{\cdot}(OXY2LT)_2$, and $(OXY2T)_1{\cdot}(OXY2LT)_3$, respectively (Figure 6C). The asymmetric distribution of band intensities could be due to different rate or equilibrium constants for tetramer formation with different oligonucleotides. Disruption of the T2 products by incubating T1 and T2 at 60 or 65 °C prior to gel electrophoresis gave the same result (data not shown).

Once the mobilities of the products related to T1 had been determined (Figure 6B), it was possible to subtract this distribution from the pattern in Figure 6A (which represents T1 products plus T2) and thereby infer the distribution of products related to T2. Bands d and e were darker in lane 2 of Figure 6A than in lane 2 of Figure 6B. The extra intensity in Figure 6A was explained by comigration of T2 bands with T1. Bands f and g were T2 products since they migrated slower than the T1 band of $(OXY2LT)_4$ (Figure 6A, band e). Thus, four T2 bands (d–g) with intermediate mobility were detected. Although the simplest explanation was that T2 bands represent pentamers of telomeric DNA, the data were also consistent with T2 bands being asymmetric tetramers [i.e., four OXY2T strands are not equivalent in the tetramers; for examples, see Figure 6C and Kim et al. (1991)]. The T2 bands consisted of two species with slightly different elec-

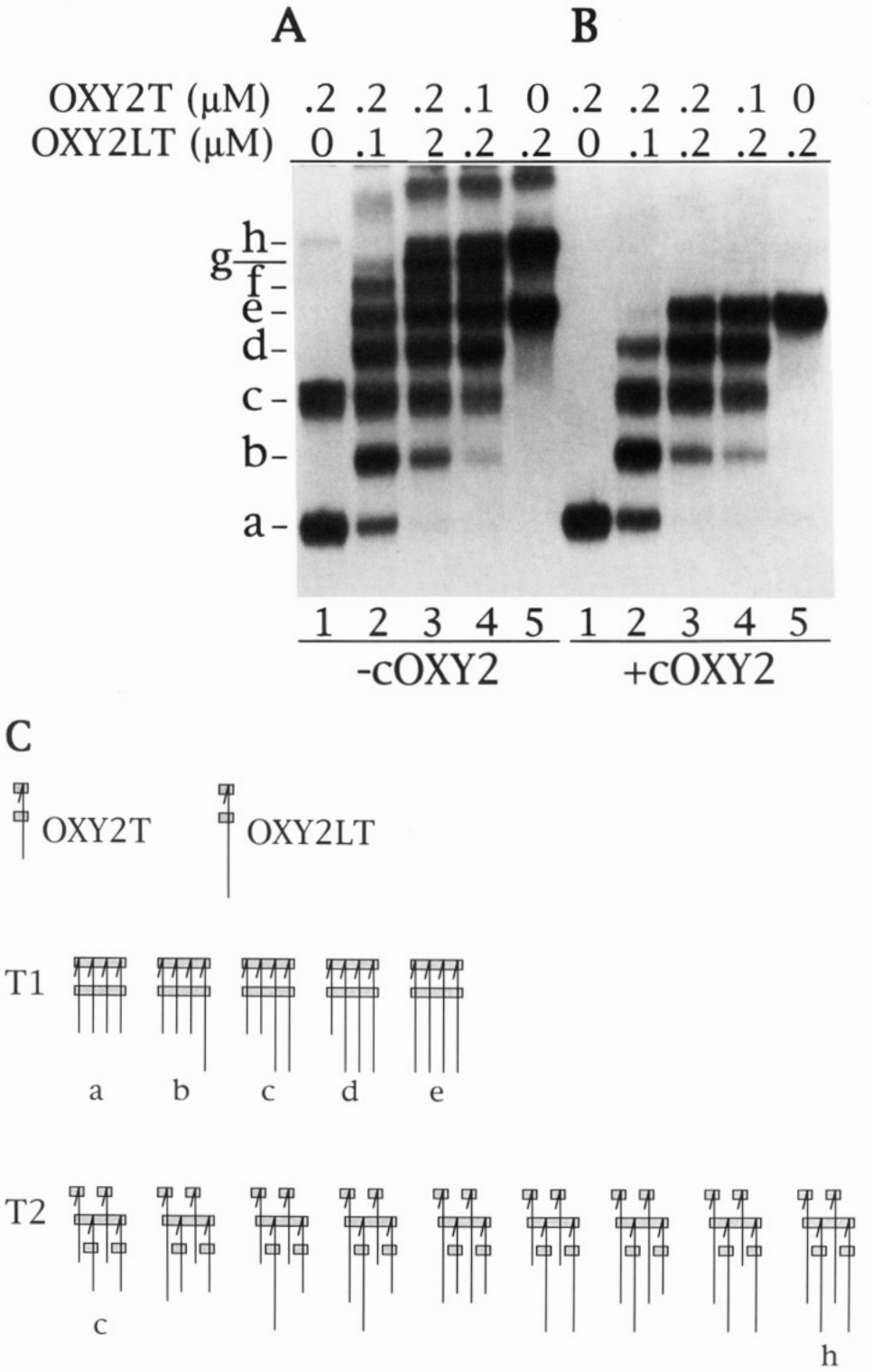


FIGURE 6: Stoichiometry of the T products determined by mixing experiments. (A and B) 5′-^{32}P-labeled OXY2T and/or OXY2LT were incubated with poly-L-lysine (0.5 μM) for 6 h at 37 °C. After digestion with proteinase K, samples (10 μL) were incubated with 2 μL of Tris–EDTA buffer (A) or 2 μL of 10 μM unlabeled cOXY2 (B) for 40 min at 37 °C and then analyzed by nondenaturing gel electrophoresis for 6.5 h at 4 °C. (C) Schematic diagram showing a symmetric tetramer (T1) and one example of an asymmetric tetramer (T2) in the mixing experiments. Structures shown here are two-dimensional projections of the tetramers. The names of the bands in panels A and B corresponding to the structures in this panel are noted below the structures. Each short vertical line represents an OXY2T molecule and each longer line an OXY2LT. The small dotted boxes represent G_4 sequences in the telomere region that are not involved in G-quartets, and the larger dotted boxes represent these same sequences forming four layers of G-quartets. In the asymmetric $(OXY2T)_4$ (T2, structure c), two OXY2T strands were paired in a staggered register with respect to the other two strands. In the mixing experiments, seven different species with mobilities intermediate between those of $(OXY2T)_4$ and $(OXY2LT)_4$ are expected, some of which might comigrate in the nondenaturing gel.

trophoretic mobilities (Figure 6A, lane 1), which further complicated the interpretation of the mixing experiments.

OXY2LT also formed a band migrating slower than the T2 products (Figure 6A). The stoichiometry of this product has not been determined. This structure was disrupted by cOXY2 annealing (cf. Figure 6A,B).

The second approach to determine the stoichiometry of T products involved annealing an oligonucleotide that was

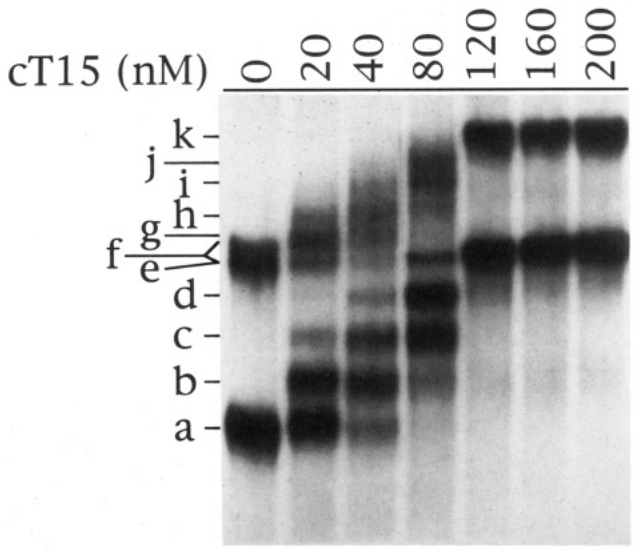


FIGURE 7: Stoichiometry of the T products determined by a tail-annealing experiment. 5′-^{32}P-labeled OXY2T (200 nM) was incubated with poly-L-lysine (0.5 μM) for 6 h at 37 °C. After digestion with proteinase K, samples were incubated with an increasing amount of unlabeled cT15 for 40 min at 37°C and then analyzed by nondenaturing gel electrophoresis for 8.5 h at 4 °C. Band e represents product T1 annealed with four cT15 molecules in lanes 80–200. Band f represents the T2 doublet in lanes 0 and 20.

complementary to part of the OXY2T nontelomeric tail sequence at substoichiometric concentrations (tail-annealing experiment) (Sen & Gilbert, 1988; Kim et al., 1991). This causes a mobility shift for the T1 and T2 products in the nondenaturing gel. The number of shifted bands was counted and the stoichiometry determined. cT15 (complementary to a 15-nucleotide sequence in the OXY2T tail region) was used in this experiment because it gave the best separation of shifted bands. With an increasing amount of cT15 added to the products, there was a gradual change in the banding pattern (Figure 7). Bands a–e migrated faster than the T2 products (band f), and therefore they represent T1 products annealed with different numbers of cT15 molecules. We detected four shifted T1 products (bands b–e), which are assigned to the annealing of 1–4 cT15 molecules. Thus, T1 represents a tetramer of telomeric DNA. Similarly, five shifted T2 bands (bands g–k) were detected. These data were consistent with the results from the mixing experiments. Again, the T2 structures could be pentamers or asymmetric tetramers. The methylation interference experiment supported the conclusion that T2 products are tetramers with the telomeric sequence out of register (see Discussion).

Tetramers T1 and T2 Have Different Methylation Interference Patterns. Methylation interference was performed to analyze the guanine residues important for the formation of the G-quartet structures. OXY2T was methylated with dimethyl sulfate (DMS) and then incubated with the β-subunit or poly-L-lysine. After digestion of the proteins by proteinase K, each of the products was gel-purified and its methylation pattern analyzed (Figure 8).

Monomeric OXY2T was uniformly methylated by DMS at the N7 of guanine residues (No Protein lane). No methylation in the telomeric guanine residues was tolerated in the D and T1 products, while methylation of the guanine residues in the tail sequence did not interfere with structure formation (lanes D1, D2, and T1). Thus, all of the guanine residues of the telomeric sequence in the D and T1 structures are involved in structure formation. On the other hand, telomeric guanine residues in the T2 structures were uniformly methylated, and therefore methylation in this region was tolerated (T2 lanes). However, the extent of methylation in the telomeric guanine residues in T2 was much less than that in monomeric OXY2T (cf. lanes M and T2), which suggests that a fraction of these guanine residues is involved in structure formation. Methylation interference experiments performed with the β-subunit and with poly-L-lysine gave the same results.

The Presence of a Nontelomeric Tail and the Location of $(T_4G_4)_2$ Are Important for G-Quartet Formation. We examined different DNA molecules (Figure 9A) in the β-mediated and protein-independent reactions. As shown previously, the β-subunit promoted G-quartet formation at low OXY2T concentrations (20 nM in this experiment) (Figure 9B, lanes a and b). In the absence of protein, OXY2T formed dimers through G-quartets at higher DNA concentrations (15 μM in this experiment) (lanes c, Figure 9B). OXY2, which has two repeats of T_4G_4 without any tail sequence (Figure 9A), failed to form detectable G-quartet structures under our protein-free conditions; the β-subunit did not promote higher order structure formation with any efficiency (Figure 9B), although a small yield of products became visible upon prolonged exposure of the gel (data not shown). Thus, the presence of a nontelomeric tail is important for both the β-mediated and protein-independent reactions.

We also tested whether the location of the guanine-rich sequence affects G-quartet formation. 5′-OXY2T, which has two repeats of T_4G_4 at the 5′-end followed by a 19-nucleotide tail sequence (Figure 9A), formed G-quartets by itself, and the β-subunit promoted this reaction at a much lower DNA concentration (Figure 9B). The β-mediated product and the protein-independent product comigrated in the nondenaturing gel, consistent with the data from experiments performed with OXY2T. Interestingly, there was only one major product, which might be a dimer of 5′-OXY2T on the basis of its electrophoretic mobility.

If the guanine-rich sequence was placed in the middle of an oligonucleotide (MDOXY2, Figure 9A), no G-quartet structure was detected in either the β-mediated or protein-independent reaction (Figure 9B). Since MDOXY2 has longer tail sequences (24 nucleotides at the 5′-end and 35 nucleotides at the 3′-end), we tested whether the length of the tail sequence affects G-quartet formation. OXY2T′ has two repeats of T_4G_4 at the 3′-end preceded by a 36-nucleotide nontelomeric tail sequence that is different from the tail sequence of OXY2T (see Materials and Methods). Like OXY2T, OXY2T′ formed G-quartets in the absence of protein, and β promoted this reaction at lower DNA concentrations (Figure 9B). OXY2LT, which contains a 44 nucleotide tail sequence, gave the same results (Figure 6; Fang & Cech, 1993b; data not shown). Thus, the inability of MDOXY2 to form G-quartets is not due to the longer tail sequences. We concluded that the location of the guanine-rich sequence in an oligonucleotide contributes to its ability to form G-quartets.

Basic Proteins Including β Promote Strand-Annealing Reactions. The β-subunit promotes G-quartet formation. A prerequisite for G-quartet formation is that two or four strands of telomeric DNA have to be brought together. We tested whether the β-subunit can bring different DNA strands together to promote the annealing of complementary strands. Two nontelomeric oligonucleotides complementary to each other in their 3′-regions (Figure 10A) were denatured separately and then mixed together with the β-subunit. After 5 min of incubation, the annealing reaction was stopped, and samples were analyzed by nondenaturing gel electrophoresis (Figure 10B). The β-subunit promoted the annealing of two

complementary oligonucleotides. In addition, the annealing-promoting effect required the structural integrity of the β-subunit, since proteinase-digested β-subunit did not promote the strand-annealing reaction.

It has been shown that other basic proteins, such as cytochrome c and poly-L-lysine, also promote G-quartet formation (Fang & Cech, 1993b). We then analyzed these proteins in the strand-annealing experiments (Figure 10B). Cytochrome *c* was active in promoting the strand-annealing reaction, although it was less potent than the β-subunit. Interestingly, the α-subunit of the telomere-binding protein and a β-deletion mutant, βC232, which consists of the first 232 amino acids of the β-subunit, were active in promoting strand annealing, although both of them are inactive in promoting G-quartet formation (Fang & Cech, 1993b). The amino acid lysine and proteinase-digested α-subunit did not facilitate strand annealing. We conclude that a wide variety of basic proteins promote the annealing of complementary single-stranded DNA and that G-quartet formation is a more specific reaction in the sense that it is facilitated by a more select group of proteins.

DISCUSSION

G-quartet structures are extremely stable (Raghuraman & Cech, 1990; Sen & Gilbert, 1990; Zahler et al., 1991; Smith & Feigon, 1992; Wang & Patel, 1992). However, the association of guanine-rich sequences into intermolecular G-quartets requires high DNA concentrations and long incubation times (Sundquist & Klug, 1989; Sen & Gilbert, 1990). Thus, G-quartet formation is a kinetically slow reaction with a high activation energy barrier. We show that the β-subunit of the *Oxytricha* telomere-binding protein acts as a molecular chaperone to promote G-quartet formation, even at nanomolar DNA concentrations. At 20 nM OXY2T and saturating β concentrations, the rate enhancement is 10^5–10^6-fold over that of the protein-independent reaction. The β-mediated structures are dimers and tetramers of telomeric DNA.

The β-subunit does not promote a novel reaction. Rather, it enhances the rate of a thermodynamically favored transition. The intrinsic ability of an oligonucleotide to form G-quartets is a prerequisite for β to promote structure formation (Fang & Cech, 1993b). Under the conditions used here, OXY2 fails to form detectable G-quartet structures at high DNA concentrations, and the β-subunit promotes structure formation inefficiently. Similarly, MDOXY2, which has two repeats of T_4G_4 in the middle of the oligonucleotide, does not form G-quartets in either the presence or the absence of β. On the other hand, OXY2T, OXY2T′, OXY2LT, and 5′-OXY2T can all form G-quartet structures in the absence of protein at high DNA concentrations, and the β-subunit promotes these reactions at lower DNA concentrations. Thus, the presence of a nontelomeric tail and the location of the guanine-rich sequence contribute to the intrinsic ability of an oligonucleotide to form G-quartets. Only when the guanine-rich sequence is present at the 3′-end of an oligonucleotide are both dimers and tetramers formed in the presence of the β-subunit. We speculate that this specificity of the β-mediated reaction might be relevant to the biological functions of telomeres, which have guanine-rich repeats at their 3′-ends.

Mechanisms for Telomeric DNA Dimerization. The β-mediated and protein-independent reactions follow different mechanisms. At saturating protein concentrations, the β-mediated reaction is first order with respect to DNA concentration; the protein-independent reaction is second-order.

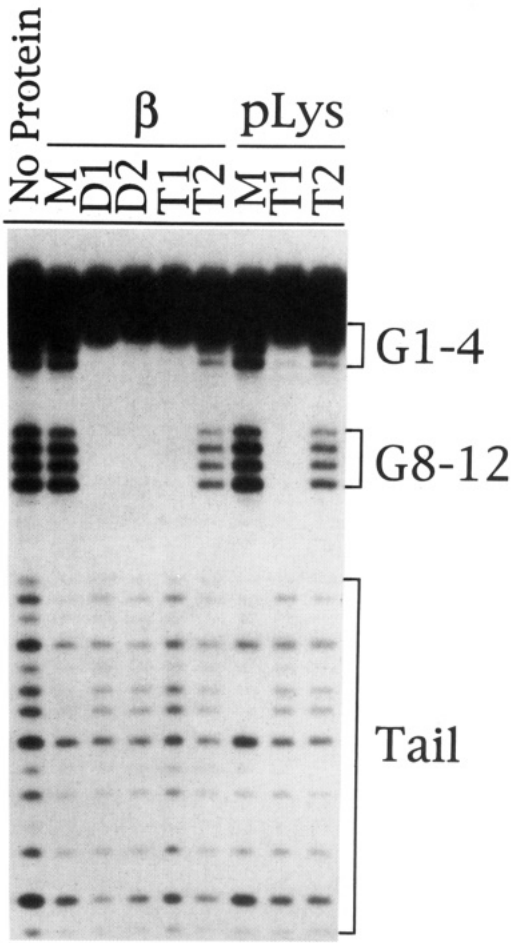


FIGURE 8: DMS methylation interference experiment. OXY2T was methylated by DMS in Tris–EDTA buffer and then incubated with β (1 μM) or poly-L-lysine (0.5 μM). After digestion with proteinase K, monomeric OXY2T and OXY2T complexes were purified in a nondenaturing gel. Methylated OXY2T was cleaved by pyrrolidine, and methylation patterns were analyzed in a 12% polyacrylamide/8 M urea gel. Lanes M, D (D1 and D2), and T (T1 and T2) represent the methylation interference patterns for monomer, dimers, and tetramers of OXY2T. D1 is the faster migrating band in the D products. D2 is the slower migrating band in the D products. The nucleotide numbering in OXY2T is from its 3′-end.

The second-order kinetics of the protein-independent dimerization reaction implies that bimolecular collision is the rate-limiting step (Scheme I). The rate constant for the fast reaction phase is $(4.0 \pm 0.6) \times 10^2\ M^{-1}\ h^{-1}$, which is 250 times greater than the rate constant for parallel four-stranded G-quartet formation reported by Sen and Gilbert (1990). This great difference in the reaction rates between the formation of dimers and tetramers could explain the absence of tetramers in the protein-independent reaction under our conditions. Our second-order rate constant for G-strand dimerization is smaller than that observed for helix formation between two complementary oligonucleotides by a large factor ($\sim 10^6$–10^8; Porschke & Eigen, 1971; Porschke et al., 1973). This suggests that most collisions between telomeric DNA strands are nonproductive in G-quartet formation.

The β-subunit greatly accelerates the rate of G-quartet formation. Although we certainly have not established the mechanism of protein facilitation, it is still worth considering the existing data in terms of a provisional model (Scheme I). For the purpose of simplicity, this model assumes that the β-subunit and OXY2T form a 1:1 complex, although we cannot exclude other possible mechanisms involving DNA–protein complexes with different stoichiometry. The β-subunit binds

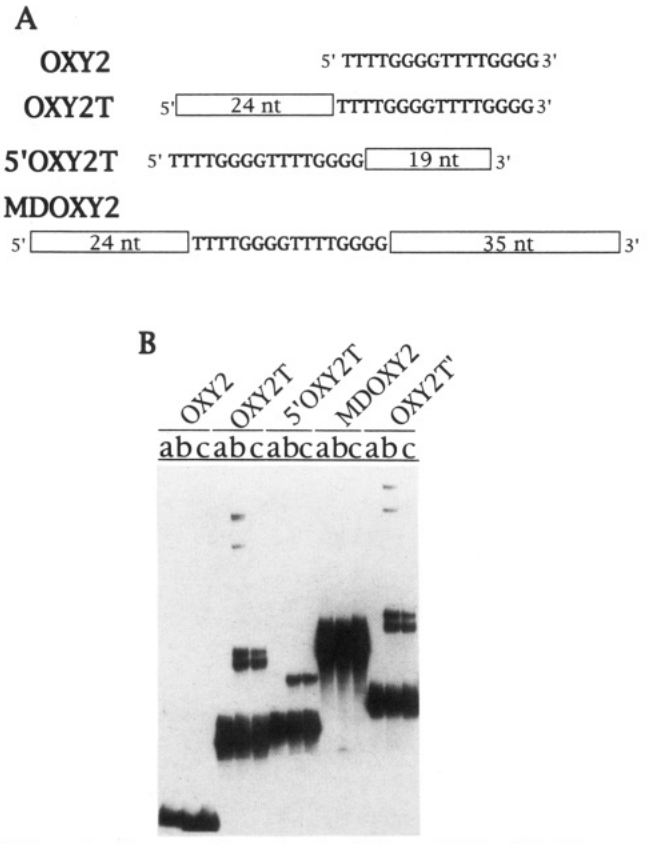


FIGURE 9: G-quartet formation by different DNAs. (A) Oligonucleotides analyzed in this experiment. (B) Oligonucleotides incubated with or without β. After proteinase K digestion, samples were analyzed by nondenaturing gel electrophoresis. Lanes a, 20 nM DNA incubated in 80 mM NaCl. Lanes b, 20 nM DNA + 1 μM β incubated in 80 mM NaCl. Lanes c, 15 μM DNA incubated in 100 mM NaCl.

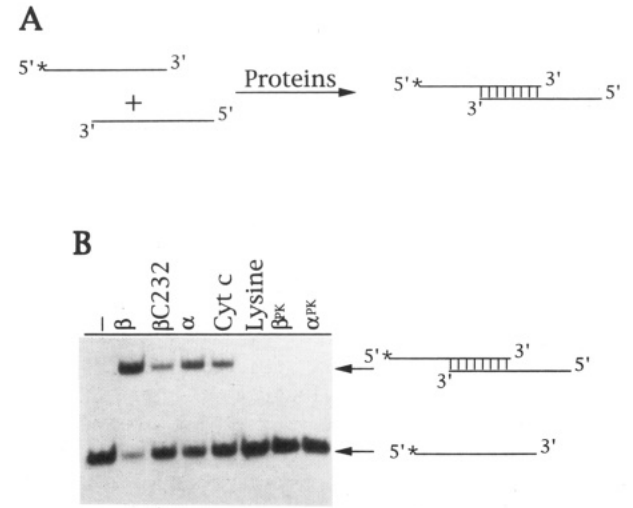
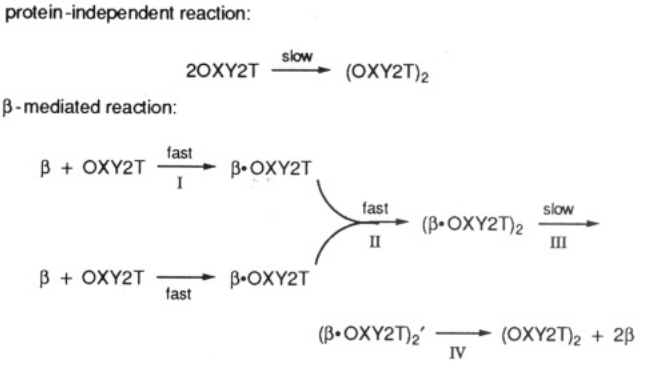


FIGURE 10: Complementary strand annealing promoted by basic proteins. (A) Schematic diagram of the strand-annealing reaction. An asterisk indicates a 5'-^{32}P label. (B) ^{32}P-labeled ds1 (2 nM) and unlabeled ds2 (2 nM) were incubated with or without proteins (1 μM) or lysine (30 μM) for 5 min at 37 °C. After the annealing reaction was stopped by dilution with Tris–EDTA buffer and proteinase K digestion, samples were analyzed by nondenaturing gel electrophoresis. α^{PK} and β^{PK} indicate α (1 μM) and β (1 μM) digested with proteinase K prior to incubation with ds1 and ds2.

to OXY2T to form a β·OXY2T complex (step I). Two such complexes then dimerize (step II). [Since the β-subunit by itself does not form a stable dimer (Fang & Cech, 1993a), we propose that dimerization occurs after β·OXY2T complex formation.] Next, there is an OXY2T isomerization step in

Scheme I



which intramolecular and intermolecular G–G Hoogsteen base pairs are formed (step III). The OXY2T dimers formed are then released from the DNA–protein complex (step IV), and the β-subunit can be involved in another round of reactions. A similar reaction scheme involving four β·OXY2T complexes could be proposed for β-mediated tetramer formation.

At saturating β concentrations, the formation of dimers and tetramers follows first-order kinetics with respect to OXY2T concentration. Thus, the rate-limiting step cannot be the dimerization of two β·OXY2T complexes (step II). The first-order rate constants do not increase with either DNA or protein concentration when $[\beta] \geq 200$ nM (Table I). Thus, formation of the β·OXY2T complex (step I) does not limit the reaction rate at saturating protein concentrations. Since the release of OXY2T dimers at saturating β concentrations requires proteinase K digestion, we do not monitor the release of dimers in our experiments (step IV). We propose that some conformational change is rate-limiting and accounts for the rate constant of ~ 1 h^{-1} at saturating β concentrations. More specifically, this step could be the formation of intramolecular or intermolecular G–G Hoogsteen base pairs within a $(\beta\cdot\text{OXY2T})_2$ complex (step III). Alternatively, isomerization of OXY2T to form intramolecular Hoogsteen base pairs could occur in the β·OXY2T complex prior to dimerization and could be rate-limiting (not shown in Scheme I). It is expected that at lower β concentrations the rate-limiting step would shift from step III (isomerization) to step I or II (complex formation).

The dimerization step (step II), which is rate-limiting in the absence of protein, must be greatly accelerated by β. A number of mechanisms may contribute, ranging from the neutralization of backbone negative charges to the formation of protein–protein interactions, both of which could facilitate the close approach of two DNA strands. Some of the mechanisms previously considered for protein-facilitated strand annealing may be applicable. For example, it has been proposed that the *Escherichia coli* single-stranded DNA binding protein and the T4 gene 32 protein promote strand annealing by destabilizing intramolecular secondary structures (Alberts & Frey, 1970; Christiansen & Baldwin, 1977). The annealing activity of heterogeneous nuclear ribonucleoprotein A1 might be attributable to its mediation of frequent, but transient, associations between complementary strands (Pontius & Berg, 1990). Rec A protein is thought to bind to two strands simultaneously and thereby increase the effective concentration of nucleic acids (Zlontnick et al., 1990).

Models for Different Structures. The β-mediated D structures are dimers of telomeric DNA (Fang & Cech, 1993b). We show here that the T1 structure is a tetramer. Although T2 products could be either symmetric pentamers

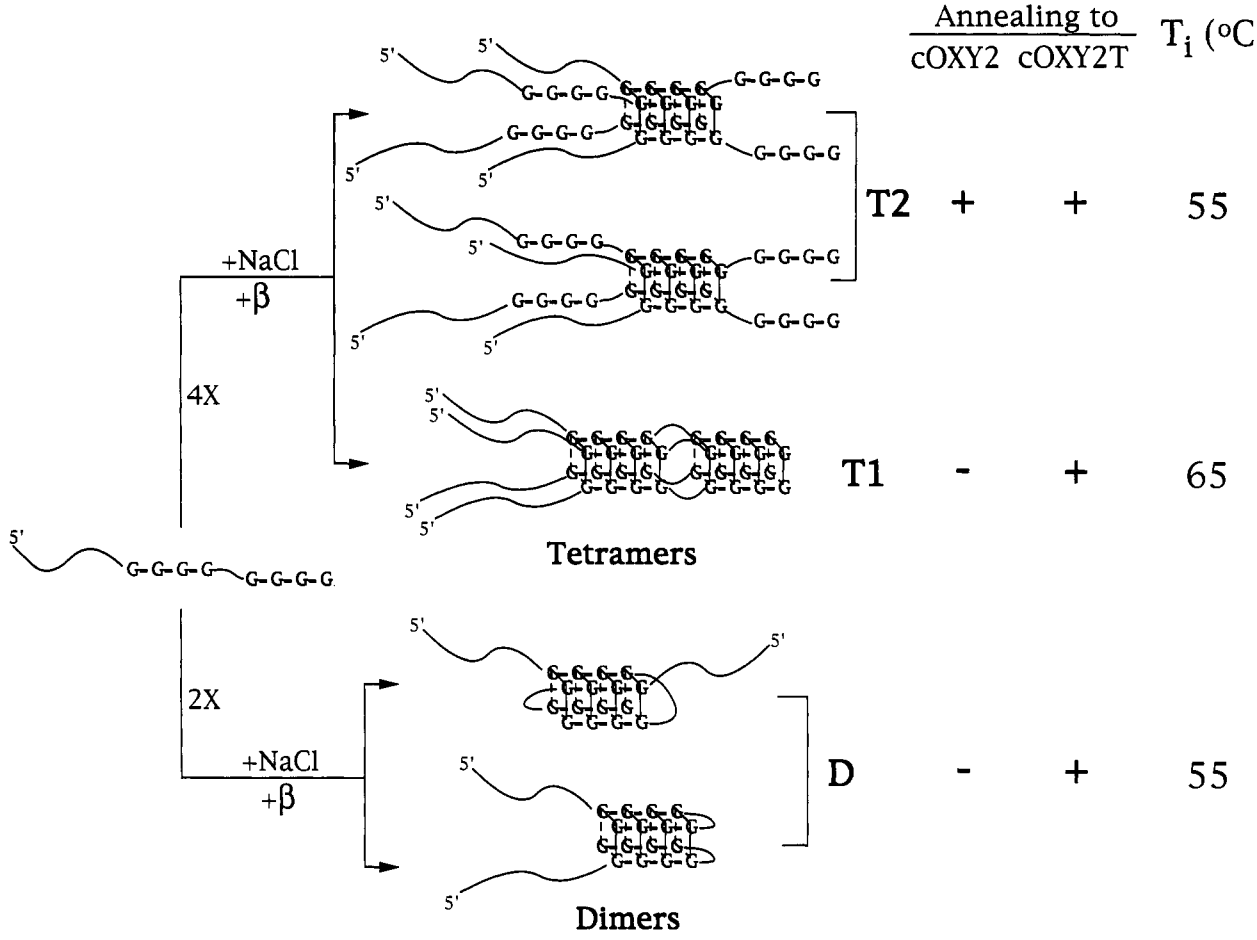


FIGURE 11: Models for structures D and T. The β-subunit promotes the association of telomeric DNA into dimers (D products) and tetramers (T products) through G-quartets. The two OXY2T strands in the two dimers have different orientations, and the four OXY2T strands in the two tetramers have different registers. The existence of dimers with different arrangements of parallel and antiparallel strands and the existence of the in-register tetramer (T1) are supported by X-ray crystallographic and NMR studies (Kang et al., 1992; Smith & Feigon, 1992; Aboul-ela et al., 1992; Wang & Patel, 1992; Cheong & Moore, 1992). The out-of-register tetramers (T2) were identified by the methylation interference experiments. In the T2 structures, the two OXY2T strands that use the same G_4 repeat in G-quartet formation (first vs second repeat) can be either across the diagonal or adjacent to each other. In addition, it is possible to have three OXY2T using the first repeat of G_4 and one OXY2T using the second repeat in the formation of G-quartet structures, and *vice versa*. These different strand arrangements could give rise to multiple T2 bands in the nondenaturing gel. T_i is the irreversible melting temperature. Adapted from Fang and Cech (1993b).

or asymmetric tetramers on the basis of the mixing experiments and the tail-annealing experiment, the following data support the tetramer explanation. (1) T2 was resolved into two species with slightly different mobilities in the nondenaturing gel, which is difficult to reconcile with a symmetric pentamer. (2) The methylation interference experiment showed that methylation at the telomeric guanine residues can be tolerated to some extent without disrupting T2 formation. This is best explained by tetramers with telomeric DNA arranged in an out-of-register manner, thereby arranged asymmetrically (see below). (3) Selective stabilization by different monovalent cations is a hallmark of G-quartet structures (Williamson et al., 1989; Sen & Gilbert, 1990; Kang et al., 1992). Monovalent cations bind in the central cavity generated by the successive layers of G-quartets (Figure 1). Na^+ and K^+ ions fit well into the size of the central cavity and therefore stabilize the structures. Li^+ is too small to bind tightly in the central cavity, and telomeric DNA does not form G-quartets in LiCl. If the T2 products were pentamers, the central cavity generated by five guanine residues would be expected to be larger than the one generated by four guanine residues. Therefore, T2 would have a different ion selectivity than D and T1. Contrary to this prediction, D, T1, and T2 products have similar ion selectivities for Li^+, Na^+, K^+, and Rb^+ (G. Fang and T. R. Cech, unpublished data). Thus, it is very unlikely that T2 represents symmetric pentamers.

On the basis of the existing data, we propose structural models for the different products (Figure 11). D structures are dimers of telomeric DNA (Fang & Cech, 1993b; Sundquist & Klug, 1989; Williamson et al., 1989). Two OXY2T molecules are held together by four layers of G-quartets. Since all of the telomeric guanine residues are involved in the G-quartet structures, no methylation is tolerated in this region. In the telomeric sequence, only thymine residues are available to base pair with cOXY2, and no stable helix can be formed between cOXY2 and D structures. The nontelomeric tail sequence is not involved in structure formation, and therefore it is available for annealing to oligonucleotide cT. cOXY2T can form a stable helix with the tail sequence in the dimers, which can then zip toward the G-quartets and destroy the structures. The different mobilities of the two forms of D in the nondenaturing gel are proposed to result from different orientations of the nontelomeric tail sequence (Figure 11). Other relative orientations of two OXY2T molecules are also possible and cannot be excluded on the basis of our experimental data.

The T1 structure is modeled as a parallel four-stranded tetramer (G4-DNA; Figure 11) similar to the one proposed

by Sen and Gilbert (1988, 1990). The four strands are equivalent to each other, and the tetramer is a symmetric structure, consistent with the data from the mixing experiments and the tail-annealing experiment. Four OXY2T strands are held together by eight layers of G-quartets, and therefore T1 has a higher irreversible melting temperature (65°C) than the D structures (55 °C). Like the D structures, all of the telomeric guanine residues are involved directly in G-quartet formation, and no methylation is tolerated in the telomeric sequence. This also explains the results of experiments in which cT, cOXY2, and cOXY2T were annealed to T1.

T2 structures are proposed to be tetramers of telomeric DNA with an out-of-register alignment (Figure 11; Sen & Gilbert, 1992; Wang & Patel, 1992). They are asymmetric structures, which explains the reason for more than three bands with intermediate mobility in the mixing experiments (Figure 6C) and more than four shifted bands in the tail-annealing experiment (Figure 7). In this model, two OXY2T strands use the first repeat of G_4 and the other two strands use the second repeat to form four layers of G-quartets. Since the proposed T2 structures have the same number of G-quartet layers as the D structures, they have the same irreversible melting temperature (55 °C). Although individual OXY2T strands in T2 molecules could have different alignments (Figure 11), T2 as a population has 50% of the OXY2T strands not using the first block of G_4 in the formation of G-quartets and 50% not using the second block. Methylation at these guanine residues does not disrupt the T2 structures. Thus, partial methylation of the telomeric guanine residues can be tolerated. In the T2 structures, 12 consecutive nucleotides on two OXY2T strands and 8 consecutive nucleotides on the other two OXY2T strands are available to form a stable helix with cOXY2, which can zip toward the G-quartets and destroy the structures by branch migration. These out-of-register tetramers are particularly interesting because they contain blocks of guanine residues that are not involved in structure formation; they could be the starting point for still higher order structures through additional G-quartets. The slowest migrating band in lane 5 of Figure 6A might represent one of these structures.

In summary, we have shown that the β-subunit of the *Oxytricha* telomere-binding protein accelerates G-quartet formation by 10^5–10^6-fold at 20 nM DNA. The structures formed are dimers and tetramers (in-register or out-of-register) of telomeric DNA. G-quartet structures have been proposed to be involved in chromosome–chromosome association and in the negative regulation of telomere replication by telomerase (Zahler et al., 1991). Thus, understanding the β-mediated structures may help to elucidate the biological functions of telomeres.

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