Topological Transformations of Synthetic DNA Knots†

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ABSTRACT: Two synthetic DNA molecules that can be knotted have been employed as substrates for *E. coli* DNA topoisomerases I and III. Both molecules contain 104 nucleotides, including sequences that can form two single-turn helical domains, connected by single-stranded oligo(dT) linkers in an X-Y-X′-Y′ pairing motif. One of the knots can be ligated to form cyclic molecules with the topologies of a circle, a trefoil knot with negative nodes, or a figure-8 knot. Cyclic molecules constructed from the other molecule can form a circle, a figure-8 knot, and trefoil knots with either positive or negative nodes. The topologically negative nodes in these knots are derived from right-handed B-DNA, and the positive nodes are derived from left-handed Z-DNA. The topoisomerases can catalyze the interconversion of the different topological forms of these molecules, as a function of solution conditions and the extent to which they favor B-DNA or Z-DNA. The enzymes appear to catalyze a single strand-passage event at a time. The topoisomerases can catalyze strand passage events involving both positive and negative nodes as substrates. Gel retention experiments show that both knots can bind up to four molecules of *E. coli* DNA topoisomerase I. The thermal denaturation of the domains of a trefoil knot closely related to these knots suggests that the two helical domains are uncoupled, so the single-stranded linkers in the knots are not taut. Chemical ligation experiments yield a distribution of products similar to those of enzymatic ligation, showing that the ATP cofactor in DNA knot ligation does not appear to skew the products markedly. Knots that are stressed by being placed in unfavorable solution conditions have been shown to be a highly sensitive system for detecting topoisomerase activity.

Knotted nucleic acids are found frequently in biological systems [e.g., Liu *et al.* (1976, 1980), Griffith and Nash (1985), Wasserman and Cozzarelli (1986), and Mizuuchi *et al.* (1980)]. In addition, knotted topology has been the subject of synthetic chemical interest for about 40 years (Ambs, 1953; Frisch & Wasserman, 1961; Walba, 1985; Sauvage, 1990), culminating in Sauvage's synthesis of a trefoil knot (Dietrich-Buchecker & Sauvage, 1989; Dietrich-Buchecker *et al.*, 1990). The plectonemic nature of the DNA double helix makes it an ideal material for the construction of single-stranded synthetic knots that can be used as model systems for learning about the properties of these unusual molecular topologies (Seeman, 1992). We have reported recently the construction of trefoil (3_1) knots and of "amphicheiral" figure-8 (4_1) knots from synthetic DNA molecules (Mueller *et al.*, 1991; Du & Seeman, 1992, 1994; Wang *et al.*, 1993; Seeman *et al.*, 1993). The topologically negative nodes in these knots are derived from double-helical half-turns of right-handed B-DNA, and the positive nodes result from double-helical half-turns of left-handed Z-DNA (Wang *et al.*, 1979). Sequences that can form Z-DNA are also capable of forming B-DNA, in the absence of Z-promoting conditions (Pohl & Jovin, 1972; Rich *et al.*, 1984). Consequently, by varying the solution conditions used during ligation, it is possible to make these different knots from the same synthetic strand of DNA. We refer to a segment of DNA that is capable of forming Z-DNA as a "proto-Z domain". An unknotted cyclic molecule is a common byproduct of reactions that produce knots (Wang *et al.*, 1993).

The ability to synthesize molecules of different topologies from the same strand suggests that it might be possible to interconvert them by DNA topoisomerases. If solution conditions are established that favor one topological form over another, a single-strand-specific DNA topoisomerase ought to be able to catalyze the interconversion of these species in the absence of an energy source. Most of the knots we have made (Mueller *et al.*, 1991; Du & Seeman, 1992; Wang *et al.*, 1993) contain 104 nucleotides, with a total of 22 or 23 nucleotide pairs in two double-helical domains; the rest of the DNA is present in four single-stranded regions containing dT_{14} or dT_{15} that connect the helical domains. The cyclic sequence of these knots is X-T-Y-T-X′-T-Y′-T-, where X and X′ pair together to form approximately one double-helical turn of DNA, Y and Y′ do likewise, and T represents an oligo(dT) linker. We have shown that knots can be constructed from 66-mers and 70-mers that have the same pairing motif, but with oligo(dT) linkers only five or six nucleotides long (Wang *et al.*, 1993). Thus, the 104-mer knots are not very tight, and there is expected to be enough slack to provide the single-stranded binding region required (Kirkegaard & Wang, 1985) by *Escherichia coli* DNA topoisomerase I (topo I). A second single-stranded topoisomerase molecule, *E. coli* DNA topoisomerase III (topo III), is also known to catalyze single strand passage events (Srivenugopal *et al.*, 1984), and we have explored its catalysis of strand passage in knots as well.

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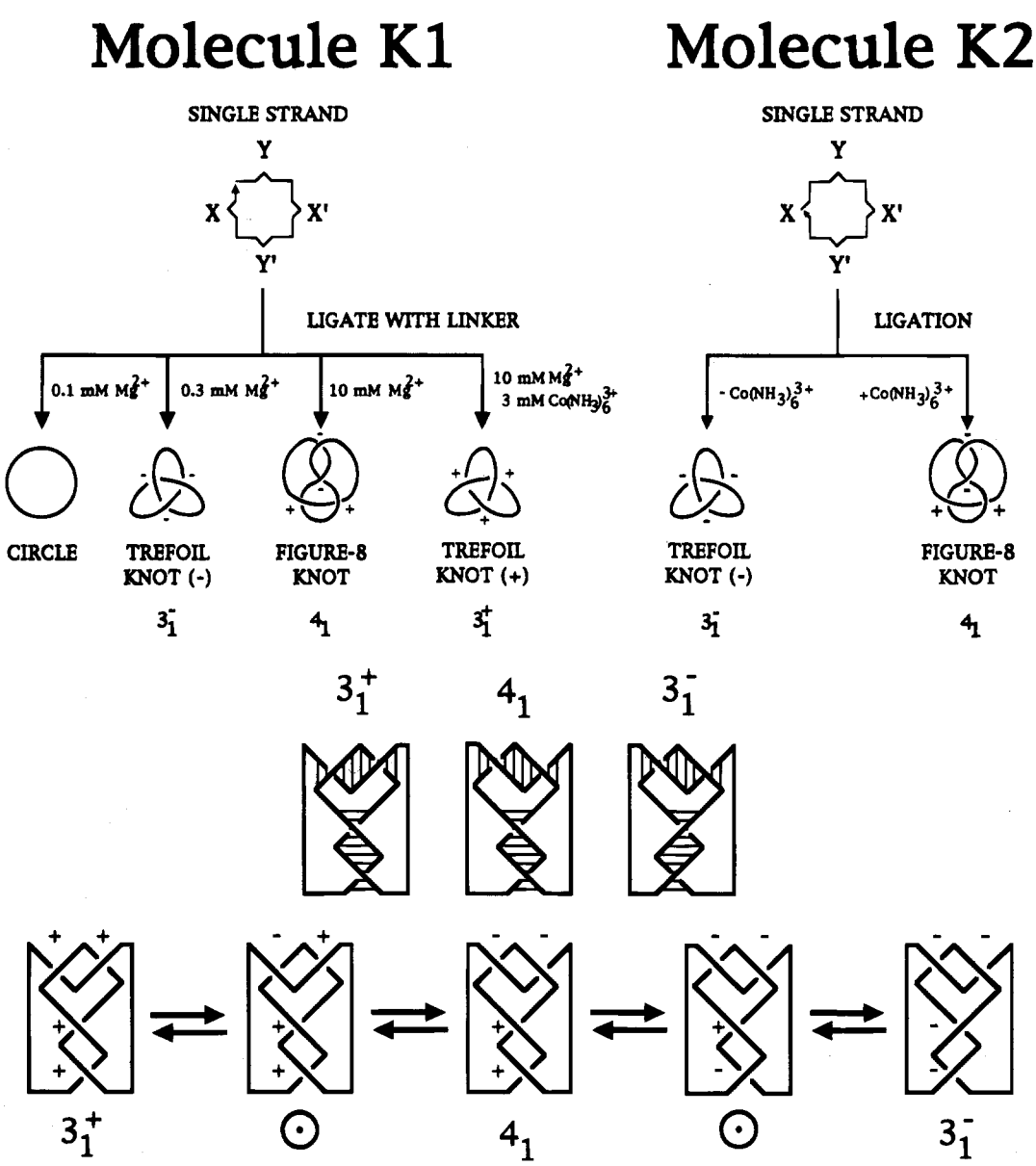

FIGURE 1: Knotted systems used as substrates. (a, top panel) Synthesis of knotted molecules under different solution conditions. The cyclization of K1 is shown on the left, and the cyclization of K2 is shown on the right. The region X is a single turn complementary to X′, and Y is likewise a single turn complementary to Y′; they are indicated by bulges on the square single strand, and the linkers form the corners of the square. The X and Y domains of K1 are each proto-Z sequences that can be converted to Z-DNA under conditions of different Z-promoting intensity; only the Y domain of K2 can be converted to Z-DNA (see Table 1). The arrowheads on the single strands indicate their 3′ ends, in the linker region of K1 and in the X region of K2. K1 is ligated using a linker, whereas the 5′ and 3′ ends of K2 are juxtaposed by base pairing. Under the conditions indicated, the different products are formed from these molecules: In the case of K1, 0.1 mM Mg^{2+} generates the circle, 0.3 mM Mg^{2+} produces the trefoil knot with negative nodes (formed from B-DNA), 10 mM Mg^{2+} generates the figure-8 knot by converting a single domain to Z-DNA, and the combination of 10 mM Mg^{2+} with 3 mM $Co(NH_3)_6^{3+}$ produces the trefoil knot with positive nodes. In the case of K2, the absence or presence of $Co(NH_3)_6^{3+}$ (ca. 5−10 mM) in the ligation reaction produces the trefoil knot with negative nodes or the figure-8 knot, respectively. (b, bottom panel) Topological transformations that accompany strand passage events in this system. At the top of this panel are the three knots discussed here, the trefoil knot with positive nodes (3_1^+), the figure-8 knot (4_1), and the trefoil knot with negative nodes (3_1^-). The nucleotide pairs that give rise to the nodes are indicated between strands. The same knots are shown in the bottom portion of the panel, interspersed by circles drawn with the node structures of dumbbells. The lines indicating the base pairs have been removed for clarity. The + and − signs near the nodes indicate their topological signs. The equilibria indicated between structures are catalyzed by the topoisomerases studied here. The 3_1^+ knot on the left has all positive signs, and the signs of a single node at a time are switched from positive to negative in each of the structures as one proceeds toward the right of the panel. Changing the sign of a single node in the 3_1^+ knot produces a circle (dumbbell), and changing a second node in the same domain produces a 4_1 knot. Changing the sign of another positive node in the 4_1 knot produces the circle (dumbbell) on the right, and changing the sign of the last node generates the 3_1^- knot. It is important to realize that the two circles shown may interconvert without the catalytic acitivity of a topoisomerase. Non-dumbbell circular structures are formally possible, but they are not shown.

We have performed topological transformations on two different 104-mer knotable molecules containing the two-helix motif. Figure 1a shows that it is possible to make four topologies from one of these DNA molecules, termed K1 here (S. M. Du, B. D. Stollar, and N. C. Seeman, in preparation); it contains two proto-Z domains that can be converted to Z-DNA under Z-promoting conditions of different strengths. Note that one domain of K1 can be converted to Z-DNA by the presence of 10 mM Mg^{2+}, without a special Z-promoting reagent. The same strand can

Table 1: Sequences of the Molecules Studied[a]

molecule	region	sequence
K1	5′ linker complement	CTCT
	linker 1	T_5
	domain 1 (5′)	$(^{m5}CG)_4(CG)_2$
	linker 2	T_{12}
	domain 2 (5′)	$^{m5}CG^{m5}CA(^{m5}CG)_2{}^{m5}CA^{m5}CG$
	linker 3	T_{12}
	domain 1 (3′)	$(CG)_2(^{m5}CG)_4$
	linker 4	T_{12}
	domain 2 (3′)	$^{m5}CGTG(^{m5}CG)_2TG^{m5}CG$
	linker 5	T_5
	3′ linker complement	CTGGAC
K2	5′ end (B-domain)	CTCT
	linker 1	T_{14}
	proto-Z domain (5′)	$(CG)_6$
	linker 2	T_{15}
	B domain (intact strand)	AGAGGTCCAGT
	linker 3	T_{14}
	proto-Z domain (3′)	$(CG)_6$
	linker 4	T_{15}
	3′ end (B domain)	ACTGGAC
K3	5′ end (B domain 1)	CTCT
	linker 1	T_{15}
	B domain 2 (5′)	CGTAGCCGCAT
	linker 2	T_{15}
	B domain 1 (intact strand)	AGAGGTCCAGT
	linker 3	T_{15}
	B domain 2 (3′)	ATGCGGCTACG
	linker 4	T_{15}
	3′ end (B domain 1)	ACTGGAC

[a] ^{m5}C signifies a nucleotide containing 5-methylcytosine.

serve as a precursor to a circle, a trefoil knot with negative nodes (3_1^-) or positive nodes (3_1^+), or a figure-8 (4_1) knot, containing two positive and two negative nodes. A second molecule we have used in topological transformation experiments contains only a single proto-Z domain; this molecule is termed K2 here, and is identical to a molecule reported earlier (Du & Seeman, 1992). K2 can form a trefoil knot with negative nodes, as well as a figure-8 knot, but it is incapable of forming a trefoil knot with positive nodes. In some experiments, we have also used 88-mer or 80-mer versions of K2, in which each oligo(dT) linker has been shortened by 4 or 6 nucleotides, respectively. We have also used a control 104-mer fashioned from the same motif, K3: This molecule contains no proto-Z domains, so the only knot it forms is a trefoil with negative nodes; its ligation to form a knot or a circle has been reported previously (Mueller *et al.*, 1991). The sequences of these knots are shown in Table 1. Unless otherwise indicated, the term "trefoil knot" used below refers to a trefoil knot containing negative nodes (3_1^-) derived from B-DNA.

Figure 1b shows that a series of four individual strand passage operations can interconvert the two trefoil knots and the figure-8 knot, *via* circular intermediates. The figure suggests that the strand passage operations occur in the regions of base pairing, but this representation should be regarded strictly as a graphic device, because the site of strand passage is unknown. Topo I is known to bind to the single strand−double strand junction of a looped molecule (Kirkegaard & Wang, 1985), and it is known to cleave a gapped molecule at this junction (Kirkegaard *et al.*, 1984). We are able to demonstrate that each reaction shown in Figure 1b can be catalyzed by topo I or topo III. The reactions are controlled by the variation of solution conditions.

MATERIALS AND METHODS

Synthesis and Purification of DNA. All DNA molecules in this study have been synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support, and deprotected, using routine phosphoramidite procedures (Caruthers, 1985). Molecules are synthesized with a 5′-phosphate added chemically, using 2-[[2-[(4,4′-dimethoxytrityl)oxy]ethyl]sulfonyl]ethyl 2-cyanoethyl *N,N*-diisopropylphosphoramidite, from Glen Research (Sterling VA). DNA strands are purified by denaturing gel electrophoresis.

Enzymatic Reactions. A. Kinase Labeling. Two picomoles of an individual strand of DNA is dissolved in 10 μL of a solution containing 66 mM Tris·HCl, pH 7.6, and 10 mM $MgCl_2$ and mixed with 1 μL of 2.2 μM [γ-^{32}P]ATP (10 mCi/mL) and 3 units of polynucleotide kinase (U.S. Biochemicals) for 8 h at 37 °C. The reaction is stopped by heating the solution to 90 °C. We find that previous chemical phosphorylation results in increased specific activity of the radioactively labeled material, because it is not necessary to follow radioactive phosphorylation with a second phosphorylation using unlabeled ATP, thereby avoiding exchange of labeled phosphate with unlabeled phosphate.

B. Ligations. Ligations are performed with a solution containing 100 nM DNA, dissolved in 20 μL of kination buffer, which is brought to 66 μM ATP. $MgCl_2$ and $Co(NH_3)_6Cl_3$ are added as indicated. The 10 units of T4 polynucleotide DNA ligase (U.S. Biochemicals) is added, and the ligation proceeds at 16 °C for 10−16 h.

C. Exonuclease III Treatment. One hundred units of exonuclease III (U.S. Biochemical) is added directly to the ligation mixture, and the reaction is allowed to proceed for 2 h at 37 °C. Exonuclease III from *E. coli* K12 BE 257/pSGR3 was obtained at various times from New England Biolabs, U.S. Biochemical, and Promega Biotech. Exonuclease III from *E. coli* SR80 was obtained from Bethesda Research Laboratories.

D. Topoisomerase I Treatment. E. coli DNA topoisomerase I has been prepared as described previously (Tse-Dinh & Beran-Steed, 1988). Following annealing in a buffer containing 10 mM Tris·HCl, pH 7.9 (at 25 °C), 50 mM NaCl, and 1 mM dithiothreitol, the sample is treated with 0.4 pmol of topo I at 37 °C for 1 h, except as noted. The reaction is stopped by phenol extraction if necessary. Magnesium chloride or $Co(NH_3)_6Cl_3$ is added to the buffer as indicated.

E. Topoisomerase III Treatment. E. coli DNA topoisomerase III has been prepared as described previously (DiGate & Marians, 1988, 1989) and was the generous gift of Dr. Kenneth J. Marians. The same reaction conditions are used as for topo I, except as noted.

Chemical Ligations. One picomole of purified, desalted oligomer is cooled to 4 °C in 10 μL of 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)−NaOH, pH 5.7, and 20 mM $MgCl_2$ for at least 4 h. Upon addition of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (CDI) (final concentration, 0.4 M), the mixture is kept at 4 °C for 24 h. The samples are precipitated by alcohol, dried, and analyzed by denaturing gel electrophoresis. Triton X-100 detergent, 0.1%, is added to the reaction buffer to improve the efficiency of the reaction.

Thermal Transition Profiles. Response to increased temperature has been measured on a Gilford Response II

UV−vis spectrophotometer at 268 nm. The unimolecular melting temperature has been measured both in 1 mM sodium phosphate, pH 7, and in the presence of 10 mM Mg^{2+} and 10 mm Tris·HCl. Temperature is incremented at the rate of 0.1 °C/min.

Denaturing Polyacrylamide Gel Electrophoresis. Gels contain 8.3 M urea and 10% acrylamide [19:1, acrylamide/bis(acrylamide)], except as noted. The running buffer consists of 89 mM Tris·HCl, pH 8.0, 89 mM boric acid, and 2 mM EDTA (TBE). The sample buffer consists of 10 mM NaOH and 1 mM EDTA containing 0.1% xylene cyanol FF tracking dye. Gels are run on a Hoefer SE 600 electrophoresis unit at 60 °C (31.25 V/cm, constant voltage); gels are then dried onto Whatman 3MM paper and are exposed to Kodak X-OMAT AR film for up to 15 h.

Gel Retention Assay. Sixty femtomoles of DNA knot is incubated with varied amounts of topo I at 4 °C for 30 min in 0.5× TBE before loading on a gel. Topo I is not active under these conditions in the absence of Mg^{2+}. Gels containing 4% acrylamide (29:1, acrylamide/bisacrylamide) are buffered with 44.5 mM Tris·HCl, pH 8.0, 44.5 mM boric acid, and 1 mM EDTA. The sample buffer is the same as the running buffer, except that it contains 5% glycerol and 0.1% xylene cyanol FF tracking dye. Electrophoresis is performed on a Hoefer SE-600 unit at 4 °C for 4 h at 8.3 V/cm. The gels are electrophoresed for 4 h under the same conditions prior to loading. Gels are dried onto Whatman 3MM paper and are exposed to Kodak X-OMAT AR film for ν ν to 15 h.

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Topoisomerases Can Catalyze Strand-Passage Events in Knots. In order to demonstrate that a topoisomerase can catalyze transformations of these knots, we have treated K2 in the form of a 4_1 knot with topo I. The presence of $Co(NH_3)^{3+}$ is necessary to convert the proto-Z domain of K2 to Z-DNA (Du & Seeman, 1992). In its absence, B-DNA is favored for this domain, but the conversion of the two positive nodes of the Z-DNA to the two negative nodes of B-DNA requires catalysis by a topoisomerase. The molecule is expected to be highly stressed in the absence of a Z-promoting reagent and, hence, a good potential substrate for a topoisomerase. Figure 2a shows the time course of the topo I treatment of K2 in the presence of 10 mM Mg^{2+}, but in the absence of $Co(NH_3)^{3+}$. The time course illustrates that the 4_1 knot is converted first to the circle and then to a 3_1 knot. The circle appears to accumulate before substantial amounts of 3_1 knot are seen. Thus, in this system, topo I seems to catalyze a single strand-passage event at a time. By the end of 4 h, all of the 4_1 knot is seen to be converted to circle or to 3_1 knot. The release of strain appears to serve as the driving force for the conversion of the 4_1 knot to the circle; the facilitation of an undistorted B-DNA double helix appears likely to be responsible for the conversion of the circle to the 3_1 knot. Figure 2b shows that similar results are obtained with topo III. In general, topo III appears to process knots more efficiently than topo I, under the conditions used.

Solution Conditions Determine the Products of Knot Topoisomerizations. The importance of solution conditions to reactions catalyzed by topoisomerases is highlighted in Figure 3a. It is possible to convert a K2 circle substrate

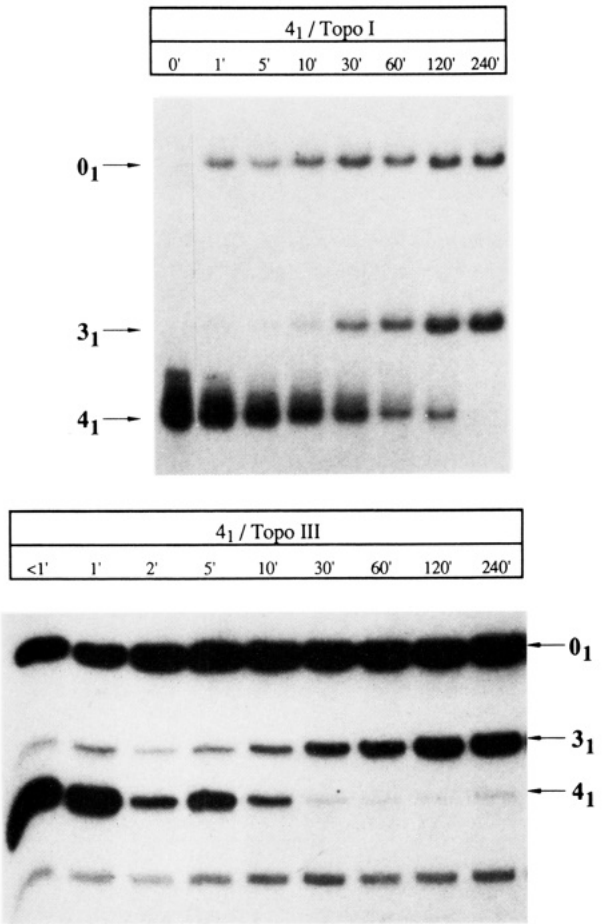

FIGURE 2: Time courses showing the conversion of K2 from a figure-8 knot to a trefoil knot. (a, top panel) Conversion catalyzed by topo I. This is the autoradiogram of a denaturing gel. Thirty femtomoles of DNA has been incubated with 400 fmol of topo I. The bands corresponding to the figure-8 (4_1) knot, the trefoil knot (3_1), and the circle (0_1) are indicated on the left, and the times are indicated for each lane. Note that the circle appears to be a precursor for the trefoil knot. At the end of 4 h, all of the stressed figure-8 knot has been converted either to circle or to trefoil knot. The percentages of material in each lane are (4_1 knot, 3_1 knot, circle): 1 min, 88.2, 1.4, 10.4; 5 min, 84.0, 2.0, 14.0; 10 min, 67.0, 7.0, 26.0; 30 min, 59.1, 16.5, 24.4; 60 min, 26, 41, 33; 120 min, 22.7, 46.7, 30.6; 240 min, 0.0, 58.0, 42.0. (b, bottom panel) Conversion catalyzed by topo III. Thirty femtomoles of DNA has been incubated with 500 fmol of topo III at 52 °C, which is a much more effective temperature for topo III. The bands corresponding to the figure-8 (4_1) knot, the trefoil knot (3_1), and the circle (0_1) are indicated on the right, and the times are indicated for each lane. The leading band is due to linear molecules that result from breakdown of the cyclic species, which occurs at roughly 0.8%/h. The percentages of material in each lane are (4_1 knot, 3_1 knot, circle): <1 min, 55.4, 3.2, 41.4; 1 min, 54.0, 4.5, 41.5; 2 min, 19.1, 3.1, 77.8; 5 min, 28.2, 4.4, 67.4; 10 min, 11.7, 8.4, 79.9; 30 min, 1.9, 19.9, 78.2; 60 min, 0.6, 25.5, 73.9; 120 min, 0.6, 37.3, 62.1; 240 min, 1.5, 38.2, 60.3; 420 min, 2.1, 40.6, 57.3; 600 min, 1.8, 38.9, 59.3. Note that the figure-8 knot is largely gone after 10 min, and has virtually disappeared after a half hour.

partially to a 3_1 knot by incubating it in the presence of 1 mM Mg^{2+} for 60 min, but incubating it in the presence of 10 mM Mg^{2+} yields a greater amount of 3_1 knot. The addition of 1 mM $Co(NH_3)_6^{3+}$ to the reaction mixture produces some 4_1 knot, but adding 5 mM of this Z-promoting reagent produces almost exclusively 4_1 knot. The 3_1 knot is not a very good substrate for topo I, but a small amount of it can be converted to circle at very low magnesium concentrations; such low magnesium concentrations are

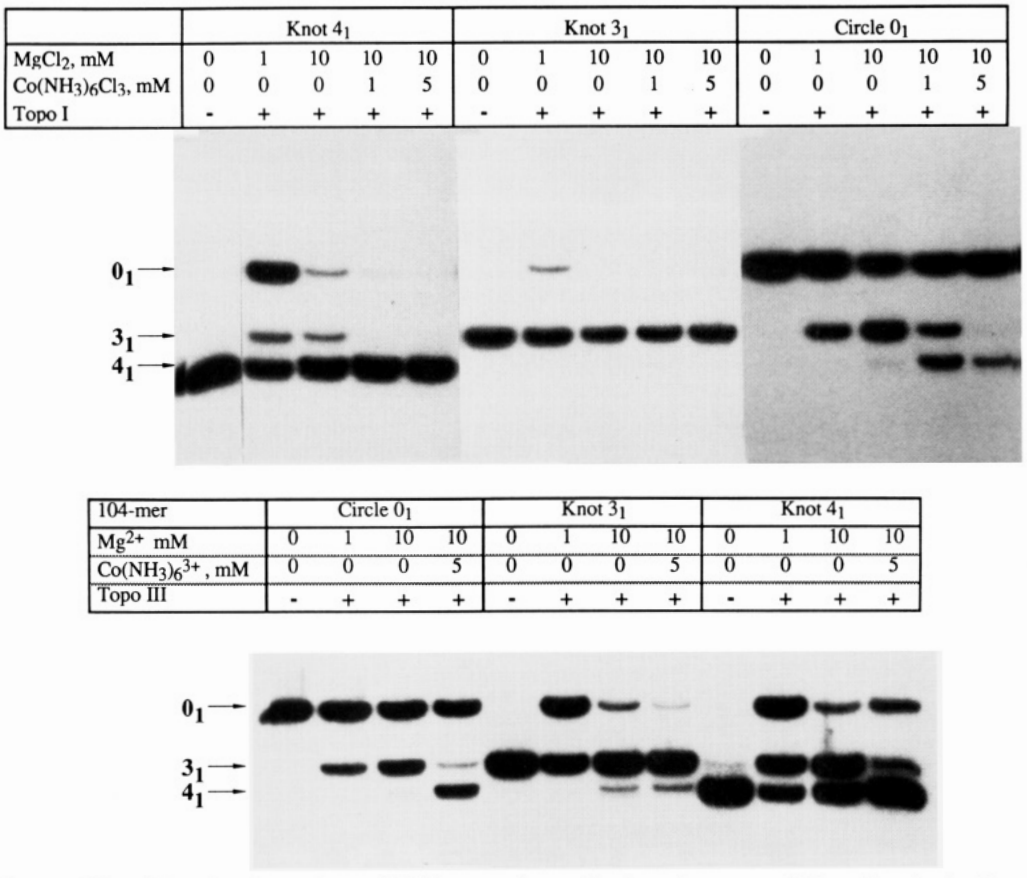

FIGURE 3: Solution conditions determine the products of K2 knot topoisomerizations. (a, top panel) Topo I catalysis of topoisomerizations. This is an autoradiogram of a denaturing gel. The bands corresponding to the figure-8 (4_1) knot, the trefoil knot (3_1), and the circle (0_1) are indicated on the left. This is a composite of three denaturing gels. The leftmost lane of each panel contains untreated markers (60 fmol) which were used as substrates for 400 fmol of topo I. The different solution conditions are indicated at the top of the panel. A solution containing 1 mM Mg^{2+} is expected to destabilize the trefoil knot, because its anionic phosphate groups are more tightly packed than those in a circle. In each case, higher ratios of circle to trefoil knot are seen under this condition than in the case of 10 mM Mg^{2+}. Note that the production of circle from trefoil knot in the central panel requires the conversion of a negative node to a positive node. At low concentrations of $Co(NH_3)_6Cl_3$, both the trefoil and the figure-8 knot can be derived from the circle, but at higher concentrations the figure-8 knot is the only product. (b, lower panel, bottom panel) Topo III catalysis of topoisomerizations. The same conventions apply as in (a), although treatment with low amounts of $Co(NH_3)_6Cl_3$ has been omitted, and the directions of the panels have been reversed. In the presence of this more vigorous enzyme, the products of the circle are similar to those seen with topo I, but the trefoil knot is a much better substrate. Large amounts are converted to circle in low $MgCl_2$ concentrations, and a little figure-8 knot is produced by higher ionic strength and Z-promotion. Small amounts of the figure-8 knot are converted to circle and then to trefoil knot even under Z-promoting conditions.

expected to favor the circle over the knot, because the charges are less densely packed, on average, in the circle. Dependence on temperature and ionic strength has been observed in the knotting and unknotting of fd bacteriophage DNA by topo I (Liu *et al.*, 1976). It is important to realize that this particular strand passage entails the conversion of a negative node to a positive node. Topo I is incapable of catalyzing the conversion of the trefoil knot to a circle (and then to a 4_1 knot) when its proto-Z domain is stressed by the presence of $Co(NH_3)_6^{3+}$. Although the presence of $Co(NH_3)_6Cl_3$ favors the formation of the figure-8 knot, its presence apparently does not destabilize the trefoil knot sufficiently to generate the circle, suggesting that a circle may be an obligatory intermediate between the trefoil and the figure-8 knot. Nevertheless, topo I is capable of catalyzing the conversion of the 4_1 knot to circle and 3_1 knot, as shown in Figure 3a, and also as seen above in Figure 2a. Conversion to trefoil knot, rather than circle, is seen to be favored by higher Mg^{2+} concentrations.

Figure 3b shows that the same reactions involving K2 are catalyzed by topo III, often more efficiently. Circle is readily transformed to trefoil knot when treated with topo III in the presence of Mg^{2+}. The presence of $Co(NH_3)_6^{3+}$ in the solution yields the figure-8 knot as the predominant product from the circle. Likewise, trefoil knot is partially converted to circle in low Mg^{2+} concentration, and less is converted at higher concentrations of Mg^{2+}. Surprisingly, a small amount of figure-8 knot is seen in the absence of $Co(NH_3)_6^{3+}$, but this has been noted before (Wang *et al.*, 1993). Conversion of trefoil to figure-8 knot is slightly more efficient in the presence of $Co(NH_3)_6^{3+}$, but as with topo I, it is not well catalyzed. Figure 3b shows again that it is much easier to convert figure-8 knot to circle or trefoil knot than it is to convert trefoil knot to figure-8 knot. Topo III appears to be more efficient than topo I in these reactions.

It is important to distinguish K2 products obtained upon treatment by the two enzymes from products obtained under different conditions. When topo I is used at 52 °C, stronger bands are seen for the circle and trefoil knot when the figure-8 knot is treated in the presence of 10 mM Mg^{2+}. A much stronger circle band and a trace of trefoil are seen when 5 mM $Co(NH_3)_6^{3+}$ is added to that mixture. Likewise, some circle is seen when trefoil knot is treated in the presence of 10 mM Mg^{2+}. The strong product bands seen in Figure 3a

	Circle 0_1				Knot 4_1				Knot 3_1^-				Knot 3_1^+			
Mg^{2+}, mM	0	0.1	10	10	0	0.1	10	10	0	0.1	10	10	0	0.1	10	10
$Co(NH_3)_6^{3+}$, mM	0	0	0	2	0	0	0	2	0	0	0	2	0	0	0	2
Topo I	-	+	+	+	-	+	+	+	-	+	+	+	-	+	+	+

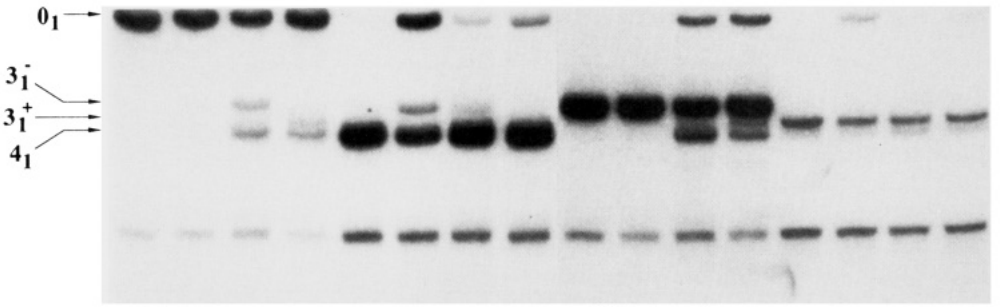

FIGURE 4: Solution conditions determine the products of K1 knot topoisomerizations catalyzed by topo I. This autoradiogram of a denaturing gel contains four panels illustrating the effects of solution conditions on K1 in its different topological forms. The bands corresponding to the figure-8 (4_1) knot, the trefoil knot with positive nodes (3_1^+), the trefoil knot with negative nodes (3_1^-), and the circle (0_1) are indicated on the left. The leading band is due to linear molecules that result from breakdown of the cyclic species. The conditions used are similar to those used to produce the different topologies in ligation reactions (see Figure 1a). The circle can be converted to a mixture of the (3_1^-) knot and (4_1) knot at high ionic strength, and a trace of (3_1^+) knot is seen when $Co(NH_3)_6Cl_3$ is added. In fact, it appears that traces of (3_1^+) knot are produced under those conditions from every precursor; this reaction corresponds to the conversion of a negative node to a positive node. The (3_1^-) knot is a far better substrate for topo I than was the (3_1^-) knot derived from K2. This may have to do with its relative instability, due to the strong propensity of both domains to undergo the B→Z transition. The figure-8 knot is the intermediate structure here, and appears to exhibit some instability under all conditions.

when circle is treated at 37 °C decrease to traces at 52 °C (data not shown). Conversely, some product bands seen in Figure 3b disappear when topo III is used at 37 °C. The figure-8 knot product of treating the circle with 10 mM Mg^{2+} and 5 mM $Co(NH_3)_6^{3+}$ disappears, as does the trefoil product when the figure-8 knot is treated under similar conditions (10 mM $Co(NH_3)_6^{3+}$ instead of 5 mM). Likewise, the circular product obtained by treating the trefoil knot in the presence of 10 mM Mg^{2+} disappears (data not shown). Thus, there are no major differences in the products obtained by treatment with the two enzymes, when the different temperature conditions are taken into account.

Some light may be shed on topo I activity by analyzing the transformations that it can catalyze in the 80-mer version of K2 at 37 °C. This molecule is designed to contain the same nucleotide pairs as 104-mer K2 (Wang *et al.*, 1993), but its linkers consist of only dT_8 or dT_9, rather than dT_{14} or dT_{15}. By contrast to the larger version of K2, topo I does not catalyze some of the same reactions on this knot. Only the conversion of 4_1 knot to circle in the absence of $Co(NH_3)_6^{3+}$ (and not further to trefoil knot) can be catalyzed efficiently by topo I, although a trace of circle is produced when the trefoil knot is treated in 1 mM Mg^{2+} (data not shown). Topo I is a 97-kDa polypeptide (Tse-Dinh & Wang, 1986), so its size may limit its mode of action on these relatively small substrates. The linker lengths in 80-mer molecules, dT_8, approach the minimal size of single-stranded DNA that can be utilized by topo I as a cleavage site (Tse-Dinh *et al.*, 1983). Binding to dT_{14} is stronger than to dT_8 (Y.-C. Tse-Dinh, unpublished).

Figure 4 illustrates the action of topo I on K1. This molecule is capable of forming both 3_1^+ and 3_1^- knots. The 3_1^- knots are the conventional trefoil knots we have discussed above, but the 3_1^+ knots possess two domains that contain Z-DNA (Du et al., 1995). The conditions for synthesis of these knots and the 4_1 knot are shown in Figure 1a. The circle can be converted to a mixture of 3_1^- and 4_1 knots at 10 mM Mg^{2+} concentrations, and a very small amount of 3_1^+ knot can be detected in the presence of $Co(NH_3)_6^{3+}$. The 4_1 knot is converted to 3_1^- knot and circle at lower [Mg^{2+}], but very little, if any, 3_1^+ knot is seen upon the addition of $Co(NH_3)_6^{3+}$. The 3_1^- knot can be converted to circle and figure-8 knot at 10 mM Mg^{2+}, and to circle, figure-8 knot, and a trace of 3_1^+ knot upon the addition of $Co(NH_3)_6^{3+}$. The 3_1^+ knot is largely inert, although it can be converted to circle at 0.1 mM Mg^{2+}.

Topo I Shows Gel Retardation When Complexed with Knots. The interaction of topo I and the synthetic knots can be followed by gel retention assays. Figure 5 shows these assays for three different knots. The panel on the left of Figure 5 illustrates a titration of topo I with molecule K3, as a trefoil knot. This knot has neither Z domains nor proto-Z domains. Four distinct species can be seen as the concentration of topo I is increased. Similar results are seen when K2 is used, either as the trefoil knot (central panel) or as the figure-8 knot (right panel). Only two retained species are seen when the 88-mer version of K2 is used (data not shown).

Stressed Knots Are Sensitive Indicators of Topoisomerase Activity. A DNA knot placed in a solution environment that favors a different topology is a stressed knot. Stressed knots are an extremely sensitive monitor of the presence of topoisomerase activity. Figure 6 illustrates this point with commercial preparations of exonuclease III, in which molecule K2 is used as a substrate. As noted above, the absence of $Co(NH_3)_6^{3+}$ destabilizes the 4_1 knot constructed from this molecule. The preparation of exonuclease III from *E. coli* K12 (Rogers & Weiss, 1980) has many similarities to the preparation of topoisomerase I (Tse-Dinh & Beran-Steed, 1988). When the 4_1 knot is treated with exonuclease III from this source, in the absence of a Z-promoting reagent, it can be seen that a substantial portion of it is converted to circle.

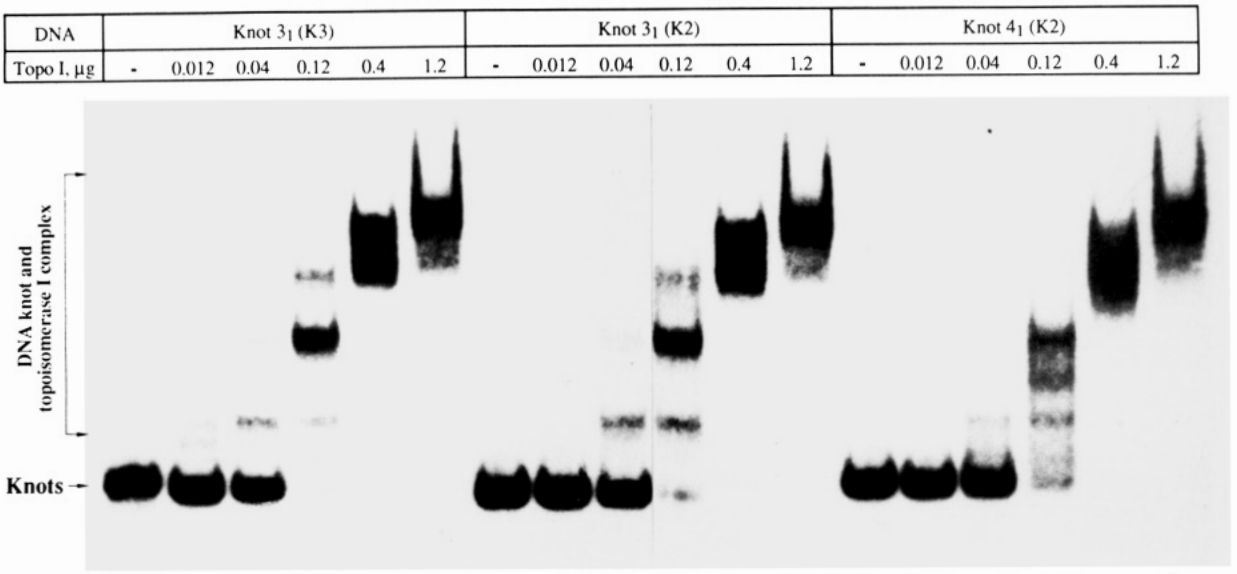

FIGURE 5: Gel retention lassays showing the interaction of topo I and K2 and K3 knots. The three panels of this autoradiogram of a non-denaturing gel correspond, from left to right, to K3 trefoil knot, to K2 trefoil knot, and to K2 figure-8 knot. Note that the migrations of all unbound 104-nucleotide knots are similar under non-denaturing conditions. In each panel, the gel is labeled by the knot employed. Sixty femtomoles of DNA knot is incubated with the amount of topo I indicated at the top of each panel. The increasing quantities correspond to enzyme/knot ratios of roughly 2:1, 6:1, 20:1, 60:1, and 200:1. Four prominent species can be seen for each of these knots. The second band from the bottom is trailed by a smear; this may result from the dissociation−reassociation of the protein during migration, a phenomenon that has been noted previously (Jang & Stollar, 1990).

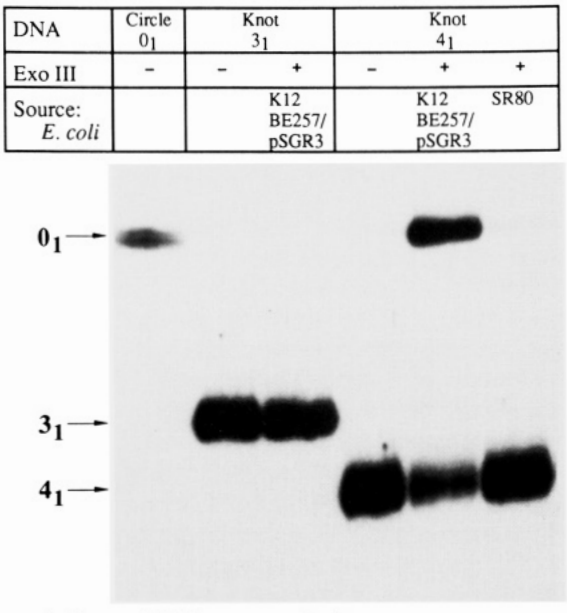

FIGURE 6: Stressed K2 knots treated with commercial preparations of exonuclease III. This is an autoradiogram of a denaturing gel. The mobilities corresponding to the circle (0_1), the trefoil knot (3_1), and the figure-8 knot (4_1) are indicated on the left. The origins of exonuclease III are indicated at the top of the panel. Note that exonuclease III from only one strain of *E. coli* is able to convert the figure-8 knot to a circle in non-Z-promoting conditions.

This result is not seen when the knot is treated with exonuclease III from a different source, indicating that topoisomerase activity is not an inherent property of exonuclease III. It is worth pointing out that exonuclease III, the primary component in this system, is a DNA-binding protein; its presence may enhance the sensitivity of the assay.

Chemical Ligation Can Be Used To Test the Role of the ATP Cofactor in Knot Ligation. All of the knots we have reported above have been prepared as described previously (Mueller *et al.*, 1991; Du & Seeman, 1992, 1994; Wang *et al.*, 1993) by using T4 DNA ligase to seal a nick in a single strand. This enzyme has an ATP cofactor, and it is instructive to ask whether the topoisomer product distribution is skewed by this energy source. It is a particularly meaningful question to ask here, because the topoisomerases used in this work utilize no energy source, so they might yield a different product distribution in the solution conditions used for ligation. We have examined this question by comparing enzymatic ligation of the K2 molecule with chemical ligation (Ashley & Kushlan, 1990) of the same molecule (Figure 7). Enzymatic ligation in the presence of 10 mM $Co(NH_3)_6^{3+}$ leads to 42% circle, 5% trefoil and 53% figure-8 knot. Chemical ligation leads to 7% circle, 25% trefoil knot, and 67% figure-8 knot. In both cases, the figure-8 knot is the dominant product, but the circle is more prominent in the enzymatic ligation. Enzymatic ligation of K2 in the absence of $Co(NH_3)_6^{3+}$ produces 46% circle and 54% trefoil knot, versus 28% circle and 72% trefoil knot. The same is true of molecule K3, with 38% circle and 62% trefoil knot with enzymatic ligation and 9% circle and 91% trefoil knot from chemical ligation. Thus, there is qualitative agreement between the results obtained in the presence and the absence of the ATP energy source. The presence of 0.1% Triton X-100 detergent in the ligation mixture improves the efficiency of chemical ligation in a dramatic fashion; comparison of lanes 9 and 10 in Figure 7 illustrate this point.

It is important to point out that the chemical ligations shown in Figure 7 contain 20 mM Mg^{2+}, in contrast to the enzymatic ligations. When the enzymatic ligations are performed in the presence of 20 mM Mg^{2+}, little difference is seen: Ligation of K2 in the absence of $Co(NH_3)_6^{3+}$ yields 12% circle, 82% 3_1 knot, and 6% 4_1 knot; ligation of K2 in the presence of $Co(NH_3)_6^{3+}$ yields 35% circle, 8% 3_1 knot, and 57% 4_1 knot (data not shown). Thus, enzymatic ligation in the presence 20 mM Mg^{2+} generates product distributions similar to those seen when chemical ligation is performed with this concentration of Mg^{2+}. Enzymatic ligation in the presence of the buffer and detergents used to promote

	1	2	3	4	5	6	7	8	9	10
DNA	M	K2					K3			
Triton X-100	-	-	-	-	+	+	-	-	-	+
$Co(NH_3)_6Cl_3$	-	-	-	+	-	+	-	-	-	-
Ligation	-	-	E	E	C	C	-	E	C	C

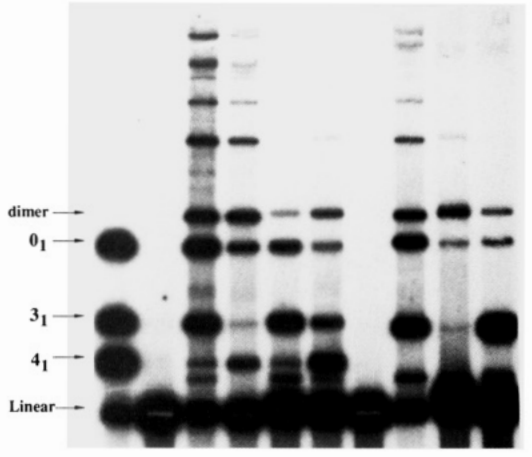

FIGURE 7: Enzymatic and chemical ligation of DNA knots. This is an autoradiogram of an 8.5% denaturing gel. The species indicated on the left are, from top to bottom, the linear dimer, the circle (0_1), the trefoil knot (3_1), the figure-8 knot (4_1), and the linear monomer. The molecule ligated in lanes 2−6 is K2, and the molecule ligated in lanes 7−10 is K3. The presence of the detergent Triton X-100 or of the Z-promoting reagent $Co(NH_3)_6Cl_3$ is indicated above each lane; the mode of ligation is also shown, "E" for enzymatic ligation and "C" for chemical ligation. Comparison of lanes 9 and 10 indicates the usefulness of adding the detergent to the chemical ligation reaction. When it is added, the chemical ligation reaction appears to be similar in effectiveness to the enzymatic ligation reaction. Note, too, that the distributions of products are similar between the two reactions.

chemical ligation does not affect the product distribution markedly (data not shown).

The Thermal Transition Profile of a 104-mer Knot Shows That the Domains Are Not Tightly Coupled. The thermal behavior of these knots provides information on the cooperativity of the two helical domains. Figure 8 indicates the thermal transition profile of the trefoil knot constructed from molecule K3. This is the only knot that we have yet been able to construct in quantities sufficient to measure a thermal transition by optical means. In the presence of 10 mM Tris HCl and 10 mM Mg^{2+}, the midpoint of the transition is roughly 78 °C, but the complete transition cannot be visualized at this high temperature. In 1 mM sodium phosphate buffer, pH 7, the midpoint of the transition is seen to be 56.5 °C. It is possible to estimate the van't Hoff ΔH by measuring the slope of the curve at the midpoint of the transition (Marky & Breslauer, 1987). Following this procedure leads to $\Delta H_{vH} = 91$ kcal/mol. Likewise, at the midpoint, $\Delta G = 0$, so from the standard thermodynamic relationship, $0 = \Delta G = \Delta H - T_m\Delta S$, $\Delta S = \Delta H/T_m$. This yields a value of $\Delta S_{vH} = 277$ kcal/(mol deg). These values are similar to those expected for the melting of the individual double-helical 11-mers in the sequence of K3 (Doktycz *et al.*, 1992). By contrast, $\Delta H = 200$ kcal/mol and $\Delta S = 546$ cal/(mol deg) would be predicted for a 22-mer of this sequence melting as a single cooperative unit (A. S. Benight, private communication). Thus, the thermal behaviors of the two domains appear to be independent. This observation is

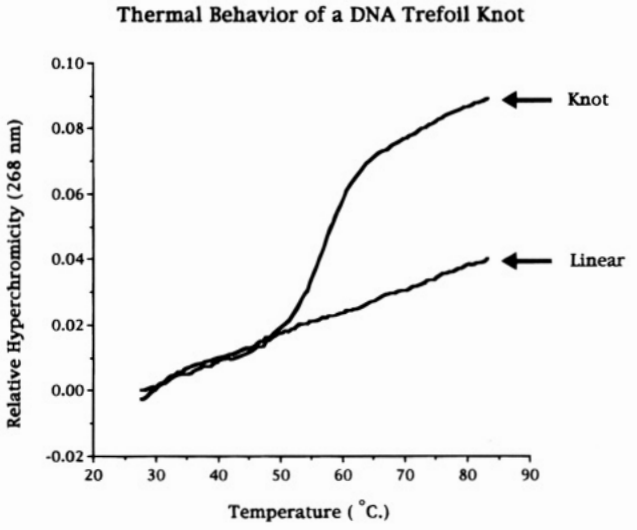

FIGURE 8: Thermal transition of trefoil knot K3. The temperature is indicated along the abcissa, whereas the ordinate indicates the relative hyperchromicity measured at 268 nm. The data have been smoothed by a 21-point interpolation routine. The function on the ordinate corresponds to $[OD(T) - OD(T_o)]/OD(T_o)$, where $T_o = 25°$. The cooperative transition corresponding to the trefoil knot formed from molecule K3 is indicated. Shown for comparison is the thermal behavior of the linear molecule K3, which is clearly not cooperative.

in agreement with the finding that the single strands are loose enough for topo I to bind to the knots and to catalyze transformations. It is possible to interpret the melting data to suggest that only a single domain has melted; distinguishing between these two alternatives is beyond the scope of the current work, and does not impinge on the independent behavior of the two domains.

DISCUSSION

Topo I and Topo III Catalyze Single Strand Passage Operations on Synthetic Knots. The products generated by the topoisomerases with synthetic knots as substrates can be explained by their usual single strand passage activities. The accumulation of circular intermediates in the time course experiments suggests that both enzymes act by performing a single strand passage, followed by release of DNA. Neither enzyme has an energy source associated with it, so they function to catalyze the topological equilibration of the various knotted and circular species, as directed by solution conditions. It is unlikely that we have reached equilibrium in any of the reactions; nevertheless, the products generated by the action of the topoisomerases indicate the relative stabilities of various species under particular ionic conditions.

The gel retention assays shed light on the interactions of the topoisomerases with the knots. The usual conditions under which the reactions were run correspond to about 10:1 ratios of topoisomerase to knot, although higher ratios (e.g., 60:1) are more effective. Nominally catalytic ratios (below unity) produce no topoisomerization of the knots. The 10:1 ratios correspond roughly to the conditions in Figure 5 in which two molecules are bound to a knot. Thus, even though two steps are being catalyzed in Figure 2a and two enzyme molecules are present, the steps appear to be successive. It seems likely that the topoisomerases are bound to the single-stranded regions, because the gel retention indicates saturation at four molecules per knot, and because of the single-stranded binding shown previously by Kirkegaard and Wang

(1985). This suggestion is supported by the independent melting of the two domains of K3, which indicates that the single-stranded linkers are sufficiently loose that the two helical domains behave as separate units. The mechanism by which one protein molecule can catalyze strand passage when one to three other molecules are simultaneously bound is certainly not apparent, unless the enzyme molecules can coordinate in some fashion. More structural data, such as crystallography (Lima *et al.*, 1994), will be needed to elucidate these questions.

Topo I and Topo III Can Catalyze Strand Passage Operations Involving Both Positive and Negative Nodes. A key finding of this work is that positive and negative nodes can both serve as substrates for these topoisomerases. The experiment that shows strand passage at negative nodes is the conversion of 3_1^- knots to circles. Topo I can catalyze this reaction on the K2 3_1^- trefoil at very low Mg^{2+} concentrations, which destabilize the knot (Figure 3a). The conversion of this knot to a circle necessarily entails a strand passage operation that operates on a negative node (Figure 1b). The work of Kirkegaard and Wang (1985) indicating that topo I can relax positively supercoiled DNA is a precedent for this finding. Likewise, topo III can catalyze this same reaction (Figure 3b). Further support for the ability of topo I to convert negative nodes to positive nodes is seen in the generation of a trace of K1 3_1^+ knot from the K1 circle and K1 3_1^- knot (Figure 4). Figure 1b illustrates that the conversion of a negative node to a positive one is required to effect this transformation.

The conversion of positive nodes to negative nodes can be invoked to explain all of the other products seen in the topological transformations we have reported. The conversion of the figure-8 knot first to the circle and then to the 3_1^- knot clearly entails the conversion of positive nodes to negative ones. The generation of figure-8 knot from the circle can result from either of the circles that flank it in Figure 1b. The circle on the left is converted to a figure-8 knot by changing a positive node to a negative node, whereas the circle on the right is converted to a figure-8 knot by changing a negative node to a positive one. Regardless of the knotted precursor, we expect the circle on the left to be the likelier circle (dumbbell) formed by K2, since its proto-Z domain contains exclusively G−C nucleotide pairs. Thus, we expect the figure-8 knot to be generated from the circle by conversion of a positive node to a negative node.

The Distribution of Knotted Products Generated by DNA Ligase Is Not Distorted Markedly by the ATP Cofactor. The role of the ATP cofactor is a concern in the formation of closed circular DNA (Shore *et al.*, 1981), knots, and other unusual DNA topologies (Seeman *et al.*, 1993; Fu *et al.*, 1994; Zhang & Seeman, 1994). One would like to know whether the ATP distorts the product distribution from what it would be in a reaction that did not have this energy source present. In the system under investigation here, the synthetic DNA knots, we have seen that the knots of K2 treated with topo I, under their ligation conditions, are not converted to another topoisomer. This is also largely true with topo III, although some transformation is catalyzed by this enzyme. The chemical ligation experiments conducted here on K2 and K3 support this finding. Thus, the knots formed by enzymatic ligation of these molecules are not molecules in high-energy states, if maintained in ligation conditions. It is important that we have discovered that the presence of a detergent in the chemical ligation reaction greatly improves the yield. We expect that chemical ligation in the presence of the detergent will provide a relatively inexpensive means to produce the large quantities of knots necessary for physical characterization.

Synthetic Knots May Be Used To Assay for the Presence of Strand Passage Activities. The sensitivity of the K2 figure-8 knot to the presence of a topoisomerase contaminant in some commercial preparations of exonuclease III indicates that these molecules could be used to seek single-stranded topoisomerase activities in other extracts. In order to do so, one should be certain to place the knot under conditions where it is stressed. For example, the figure-8 knot K2 requires the presence of a Z-promoting reagent, such as $Co(NH_3)_6^{3+}$; in its absence, the circle or trefoil knot is the preferred form, and a single-stranded topoisomerase will convert it to the favored topology.

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