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DNA flexibility studied by covalent closure of short fragments into circles

(ring closure probability/persistence length/cloned *Eco*RI restriction fragments/T4 DNA ligase)

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ABSTRACT **The ring closure probability, or *j* factor, has been measured for DNA restriction fragments of defined sequence bearing *Eco*RI cohesive ends and ranging in size from 126 to 4361 base pairs (bp). The *j* factor is defined as the ratio of the equilibrium constants for cyclization and for bimolecular association via the cohesive ends. The end-joining reactions are fast compared to covalent closure of the cohesive ends by T4 DNA ligase. The rate of ligase closure is shown to be proportional to the equilibrium fraction of DNA molecules with joined cohesive ends, both in cyclization and in bimolecular association reactions. The *j* factor changes by less than 10-fold between 242 and 4361 bp, whereas it decreases by more than 100-fold between 242 and 126 bp as the DNA reaches the size range of the persistence length (150 bp). As regards ring closure, short DNA fragments are surprisingly flexible. These data are in good agreement with predictions by others for the ring closure probability of a wormlike chain.**

The DNA molecule is highly compacted in biological structures. In the eukaryotic chromosome the first level of compaction occurs in the nucleosome, where DNA is wrapped around a core of histones with a radius of 45 Å (1). The double-stranded DNA genomes of large bacteriophages are condensed inside the phage heads, possibly in shells of coaxial spools, the smallest of which has a radius of about 60 Å (2). In these cases, duplex DNA is thought to be approximately circular and highly bent. Consequently, to understand the energetics of DNA packaging, it is necessary to know the thermodynamics of bending and twisting small DNA molecules into circular conformations. The molecular mechanisms of bending and twisting can also be studied by investigating the mechanisms of DNA motion (3, 4).

The chief aim of our work is to discover whether the bending of DNA into small circles, which requires severe bends and large bending energies, can be described by a standard model for DNA bending (3, 4) in which the DNA is treated as a thin elastic rod. The configuration of DNA in solution is then represented by the wormlike coil model which is characterized by a single bending parameter, the persistence length, P. In our ionic conditions, P is about 500 Å or about 150 base pairs (bp) (5). The ring closure probability has been computed as a function of molecular length for the wormlike coil model by Yamakawa and Stockmayer (6).

We give data here for the ring closure probability, or *j* factor, for DNA restriction fragments of defined sequence bearing *Eco*RI cohesive ends. The ring closure probability is defined (7) as the ratio of two equilibrium constants, $j = K_c/K_a$; K_c is the cyclization constant and K_a is the bimolecular equilibrium constant for joining two molecules. We follow Wang and Davidson (8, 9) in using the noncovalent joining of cohesive ends as the reaction that causes DNA cyclization or joining of half-molecules. In the present case the cohesive ends are identical and self-complementary with the base sequence 5′ A-A-T-T 3′.

The ring closure probability can be understood as the effective concentration of one end of a linear DNA molecule in the vicinity of the other end; "effective concentration" refers to the reactivity of the two DNA ends in comparison with the reactivity of half-molecules (8). The actual concentration of ends may not be the only factor that controls the reactivity of the ends in ring closure. If the linear DNA is sufficiently short such that the relative orientation of the two ends is correlated, this will affect the reactivity of the ends. Two kinds of orientation need be considered: angular orientation of the helix axes at the ends, and twist of the DNA helix (the polynucleotide backbones must be aligned for joining). Sufficiently long DNA molecules may be represented by the random coil model: a freely jointed chain with a segment length $2P$ and a Gaussian distribution of segment density. The ring closure probability of λb2b5 DNA (41 kb) has been measured and has been compared to the value expected for the random coil model (8, 9).

We show here that the ratio K_c/K_a can be obtained from the rates of covalent closure, by T4 ligase, of DNA circles and of half-molecules joined through cohesive ends. Dissociation of joined *Eco*RI cohesive ends is a fast reaction compared to ligase closure and as a result the rate of ligase closure is proportional to the equilibrium fraction of DNA molecules with joined cohesive ends. The steps involved in covalent closure of circles are as follows. Cyclization converts a linear DNA molecule (L) into a substrate (S) for covalent joining; T4 ligase (E) binds (S) and converts it to product (P), a covalently closed DNA circle (Fig. 1).

$$\mathrm{L} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{S} \qquad [1]$$

$$\mathrm{E} + \mathrm{S} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k_{34}} \mathrm{E} + \mathrm{P}. \qquad [2]$$

Hydrolysis of ATP in the ligation reaction is not shown. The ligase seals breaks in each of two DNA strands to give a covalently closed circle. Electrophoresis experiments, which resolve linear, closed circular, and open circular DNA molecules, indicate that T4 ligase normally seals the second strand rapidly after the first strand has been closed. We thus treat covalent closure as a single reaction. Application of the steady-state condition to both ES and S

$$\frac{d(\mathrm{ES})}{dt} = 0 \qquad [3a]$$

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Abbreviations: bp, base pair(s); P, persistence length.

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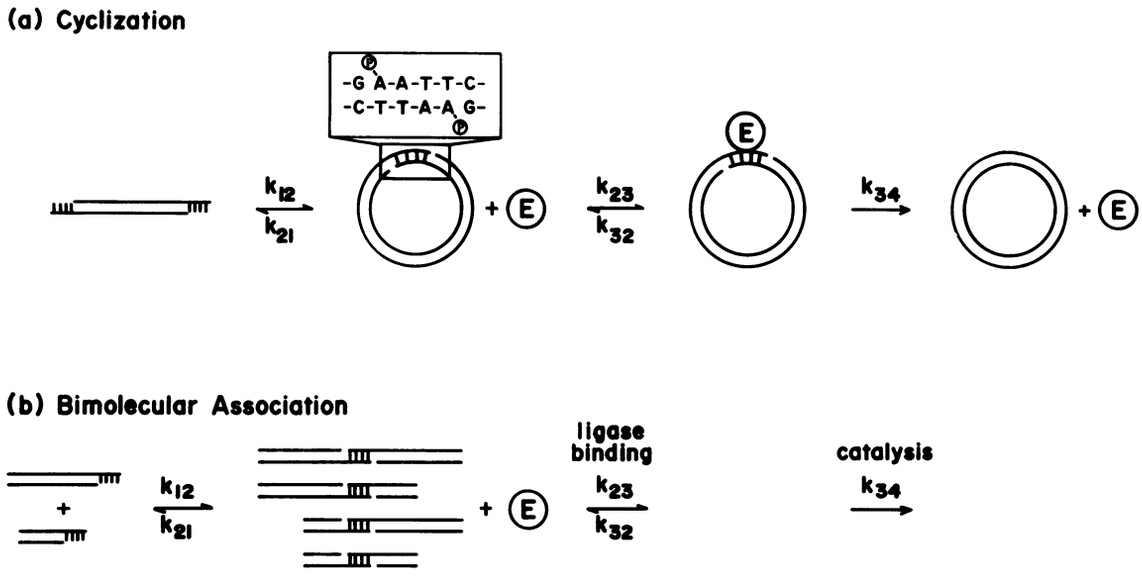

FIG. 1. (*a*) Schematic representation of cyclization, H-bond formation, ligase binding, and covalent joining. The four-base 5′-protruding *Eco*RI cohesive ends are represented by hatch marks; the detailed structure of the joined cohesive ends is shown above. Ⓟ represents a 5′-[^{32}P]phosphate in these experiments. (*b*) Bimolecular association. A mixture of two DNA fragments of different lengths, each containing one *Eco*RI cohesive end and one blunt end, can associate via H bonding in four different ways. The subsequent steps of ligase binding and covalent closure are not shown.

$$\frac{d(S)}{dt} = 0 \qquad (\text{for } f_S << 1) \qquad [3b]$$

yields

$$k_1 = \frac{k_{34}[k_{23}(E)]}{k_{34} + k_{32}} \cdot \frac{k_{12}}{k_{12} + k_{21} + \frac{k_{34}[k_{23}(E)]}{k_{34} + k_{32}}} \qquad [4]$$

in which k_1 is the measured first-order rate constant for covalent closure of circles

$$k_1 \equiv -\frac{1}{(D)}\frac{d(D)}{dt}, \qquad [4a]$$

(D) is the reactant DNA concentration

$$(D) \equiv (L) + (S), \qquad [4b]$$

and f_S is the fraction of (D) that is a substrate for closure by ligase,

$$f_S \equiv (S)/(D). \qquad [4c]$$

There are two limiting cases.

Case I. Joining of cohesive ends is a fast preequilibrium reaction.

$$k_1 = \frac{k_{34}[k_{23}(E)]}{(k_{34} + k_{32})} \cdot \frac{K_c}{1 + K_c} \qquad [k_{21} >> k_{23}(E)] \qquad [5]$$

This may also be written in the equivalent form

$$k_1 = \frac{k_{34}(E_0)\, K_c(1 - f_S)}{[K_m + (S)]} \qquad [5a]$$

in which (E_0) is the total ligase concentration and

$$K_m \equiv (k_{32} + k_{34})/k_{23}. \qquad [5b]$$

Case II. Dissociation of the cohesive ends is slow compared to covalent closure by ligase.

$$k_1 = k_{12} \qquad \left(\frac{k_{34}[k_{23}(E)]}{k_{32} + k_{34}} >> k_{21}\right). \qquad [6]$$

The measured second-order rate constant for joining half-molecules is given next for case I:

$$k_2 = \frac{k_{34}(E_0)\, K_a[1 - 2f_S + f_S^2]}{2[K_m + (S)]} \qquad [7]$$

$$k_2 \equiv -\frac{1}{(D)^2}\frac{d(D)}{dt}. \qquad [7a]$$

The half-molecules (A + B) are distinguishable in electrophoresis but have identical cohesive ends, so that A reacts with both A and B. The equilibrium constant for the reaction of A with A is $K_a/2$ and for A with B is K_a. We measure

$$K^* = \frac{[(AA) + (AB) + (BB)]}{[(A) + (B)]^2} = \frac{K_a}{2}. \qquad [8]$$

From *a priori* considerations, case I should apply. Measurements of the dissociation rates of oligonucleotide dimer helices indicate that $k_{21} > 10^3\ s^{-1}$ (10) whereas k_{23} normally does not exceed $10^8\ M^{-1}\ s^{-1}$ for the formation of an enzyme–substrate complex (11) and (E_0) is always less than 10^{-7} M here, so that $k_{23}(E) < 10\ s^{-1}$. Our experiments show directly that case I applies because k_1 and k_2 are directly proportional to (E_0) and to K_c and K_a, respectively, whereas for case II the observed rate constants should be independent of (E_0) and of K_c and K_a. Varying the DNA concentration shows that the condition $(S) << K_m$ also applies here so that j can be expressed as

$$j = K_c/K_a = k_1/2k_2 \qquad (\text{for } f_S < 0.03) \qquad [9]$$

from Eqs. **5a** and **7** in which k_1 and k_2 are normalized to the same (E_0). For $f_S > 0.03$, it is necessary to determine f_S and to correct k_1 and k_2 before using them to compute j.

MATERIALS AND METHODS

Source of DNA Molecules. DNA molecules used in this study are described in Table 1.

Plasmid DNA was prepared from lysozyme/Triton X-100 lysates, banded twice in CsCl/ethidium bromide density gradients, and extracted at least six times with butanol to remove the ethidium. The small ϕX174 insert DNA fragments were

Table 1. DNA molecules used in this study

Length, bp	Source
126	*Hae* III fragment 9 from ϕX174
242	*Hae* III fragment 7 from ϕX174
288	*Alu* I fragment from *Hae* III fragment 4 from ϕX174
318	*Hae* III fragment 5 from ϕX174
345	*Alu* I fragment from *Hae* III fragment 3 from ϕX174
366	*Alu* I fragment 4 from ϕX174
504	*Fnu*DII fragment 6 from ϕX174
611	*Hae* III fragment 4 from ϕX174
670	*Alu* I fragment 3 from ϕX174
880	*Hae* III fragment 3 from ϕX174
1015	*Alu* I fragment 1 from ϕX174
1361	*Hae* III fragment 1 from ϕX174
2302	*Eco*RI/*Pvu* II fragment from pBR322
4361	pBR322

Fragments derived from ϕX174 were ligated to 8-bp *Eco*RI linkers (Collaborative Research, Waltham, MA) and cloned into *Eco*RI site of the plasmid pBR325 (12). Lengths given for these molecules include the 8-bp linker. The 2302-bp *Eco*RI/*Pvu* II fragment from pBR322 (a gift from S. Scherer) was ligated to an *Eco*RI linker, cyclized, and propagated as a plasmid.

purified from vector DNA by digestion with *Eco*RI and sedimentation through 5–30% sucrose gradients. DNA fragments obtained this way are >95% pure by mass.

DNA fragments treated with calf intestinal alkaline phosphatase were ^{32}P-labeled at their 5′ ends by incubation with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Enzymes. T4 DNA ligase was a generous gift of Stewart Scherer and was >97% pure. One unit of ligase is defined here as 1.3 ng of protein. *Eco*RI endonuclease was kindly provided by John Carlson. Mung bean nuclease and T4 polynucleotide kinase were from P-L Biochemicals. Calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim.

Covalent Joining of Base-Paired Cohesive Ends by T4 DNA Ligase. Purified, radioactively labeled DNAs (total cpm, $\approx 10^5$; 0.2 μg/ml) in 60 μl of 10 mM Tris, pH 7.5/50 mM NaCl/10 mM $MgCl_2$/5mM dithiothreitol/0.25 mM rATP were equilibrated to 20°C (±0.5°C). Covalent joining was initiated by the addition of 1 μl of an appropriate dilution of T4 DNA ligase, followed by manual mixing. The kinetics of covalent joining were measured by withdrawal of 5-μl samples in which the ligase reaction was immediately quenched by either (*i*) 1:50 dilution into mung bean nuclease buffer or (*ii*) addition of EDTA to 50 mM and heating to 70°C for 10 min before gel electrophoresis (see below).

Assay for Fraction of Covalently Closed DNA Circles by Digestion with Single-Strand-Specific Nuclease. Mung bean nuclease is a single-strand-specific nuclease (13) that will render acid-soluble the ^{32}P-labeled 5′-phosphate group of *Eco*RI single-stranded cohesive ends. Because phosphate groups are protected from digestion when they are covalently joined to adjacent 3-OH groups, susceptibility of a population of such labeled molecules to release of label by mung bean nuclease is a direct assay for the fraction of molecules that have been covalently joined by ligase. Aliquots (5 μl) from ligase reaction mixtures were diluted 1:50 into 50 mM sodium acetate, pH 5.2/1 mM $ZnCl_2$/5% glycerol containing 10 units of mung bean nuclease. Incubation was for 1 hr at 37°C and was followed by acid precipitation and scintillation counting. Resistance of 5′-end-label to mung bean nuclease is a specific assay for covalent closure, as shown by the experiments in Table 2.

Characterization of Covalently Closed DNA Fragments by Agarose Gel Electrophoresis. Electrophoresis was in 1.0–2.5% agarose gels (concentration used depended upon fragment size). Gels were dried and the DNA was visualized by autoradiography. Quantitation was obtained by scanning densitometry and measurement of peak areas. The kinetics of covalent closure of labeled linear DNA fragments were followed by the disappearance of linear monomer and the concomitant appearance of a new band with lower mobility. The relative mobilities of these two bands were reversed when electrophoresis was carried out in the presence of ethidium bromide at 0.5 μl/ml. This fact strongly suggests that the new band is a covalently closed monomer circle. Fig. 2 shows a simple experiment that proves that the ligase reaction product is indeed a covalently closed monomer circle.

Conditions for Measuring the Kinetics of Covalent Joining. It is important that ligase activity remain constant during the time in which rates of covalent closure are determined. This is accomplished by measuring the initial rate of covalent closure (5–30% completion) over a period of time during which ligase activity decreases by less than 5%. Long-term ligase stability is checked by measuring the rate of covalent joining of a "reference" fragment every time a new rate measurement is made. Finally, all rates are measured at very low DNA concentrations so that $K_m >>$ [S] ([S] is the concentration of free DNA sites for ligation), and in addition dimer formation is avoided.

Joining of Half-Molecules. Two different substrates have been used to measure the bimolecular covalent joining of *EcoRI* cohesive ends by T4 DNA ligase. The first is a mixture of an equal number of two different molecules, 2294 and 2067 bp long, each having one *Eco*RI cohesive end and one blunt end. This population of molecules is covalently joined by ligase almost exclusively via the ^{32}P-labeled *Eco*RI cohesive ends, as determined by gel electrophoresis. Three experimentally distinguishable products are produced by ligase in the expected 1:2:1 ratio (Fig. 1). The second substrate is a mixture of 268- and 98-bp fragments, each with one *Eco*RI cohesive end and one blunt end. Covalent joining of both substrates was followed by the nuclease sensitivity assay and the products were examined by gel electrophoresis.

RESULTS

Covalent Joining at High Ligase Concentrations. Covalent joining of the ends of linear DNAs by ligase to form closed circular molecules is a fast reaction. All linear DNAs tested, ranging in size from 242 to 4361 bp, could be completely converted to covalently closed circles in <1 min at 20°C by addition of sufficient amounts of T4 DNA ligase. The equilibrium fraction

Table 2. Mung bean nuclease assay of covalent closure of DNA circles

Treatment	%*
1. None	0.8
2. Ligase	87
3. Ligase, then 70°C for 10 min	89†
4. Ligase, then 0.1% $NaDodSO_4$	86†
5. Ligase, then *Eco*RI	7.9‡

Ligase reactions were carried out on ice with 50 units of ligase for 10 min. Data shown are for 1361-bp DNA fragment. *Eco*RI digestion was at 37°C for 30 min with 10 units of *Eco*RI in 10 mM Tris, pH 7.5/100 mM NaCl/10 mM $MgCl_2$. The reaction was stopped by heating at 70°C for 10 min.

* % of total precipitated cpm remaining after mung bean nuclease digestion.

† Indicates that resistance to digestion is not due to ligase binding to ends.

‡ Reversal of the ligase covalent closure reaction returned DNA to susceptible state, indicating that covalent closure of the *Eco*RI site generates nuclease resistance.

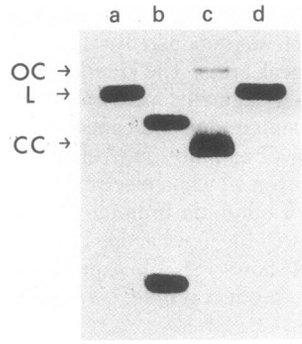

FIG. 2. Autoradiogram of agarose gel electrophoresis of ^{32}P-labeled DNA, showing ligase closure into monomer circle. Lanes: a, 611-bp linear DNA (L); b, material from lane a digested with *Hpa* I to yield 487- and 124-bp DNAs; c, 611-bp DNA after treatment with 50 units of ligase for 10 min on ice (OC, open circle; CC, covalently closed circle); d, material from lane c digested with *Hpa* I, yielding original monomer linear. This can only result from digestion of monomer circle. If ligation product were dimer circle, then digestion of head-to-head dimers with *Hpa* I would yield linear molecules of size different from that of monomer length.

of H-bonded circles under these conditions is probably <0.5%, based on data of Mertz and Davis (14) from electron microscopic counts of circular and linear species formed from simian virus 40 (5243 bp) linear molecules with *Eco*RI cohesive ends. The formation of H-bonded circles must then be a fast reaction, allowing ligase to drive the covalent joining of circles to completion in under a minute.

Kinetics of Covalent Joining of H-Bonded Circles. Formation of covalently closed DNA circles by T4 DNA ligase is a simple, apparent first-order reaction with respect to DNA concentration. This was true for all DNAs tested, ranging in size from 242 to 4361 bp, when the ligation reaction was done under conditions described in *Materials and Methods*. All covalent joining reactions could be monitored by either the nuclease sensitivity or gel electrophoresis assay. The two methods yielded identical kinetic data. Results from a typical covalent joining reaction are shown in Fig. 3.

Dependence of the Rate of Covalent Joining on Ligase Concentration. For covalent joining, k_1 is directly proportional to (E_0) in the joining reaction. Again, this result was found for all fragment sizes tested. Rate constants could be determined accurately over approximately a 10-fold range of ligase concentration. Fig. 4 displays data for the covalent closure of an 880-bp DNA circle.

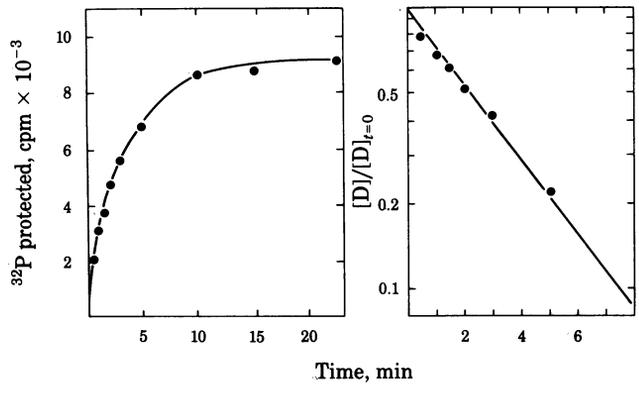

FIG. 3. Kinetics of ligase closure of H-bonded circles. Ten units of ligase was added to ^{32}P-end-labeled 1361-bp DNA, and mung bean nuclease-resistant radioactivity was measured. (*Left*) ^{32}P end label resistant to mung bean nuclease as a function of time. Total precipitable radioactivity was 10,100 cpm. (*Right*) Data plotted as logarithm of fraction of ligatable starting material remaining.

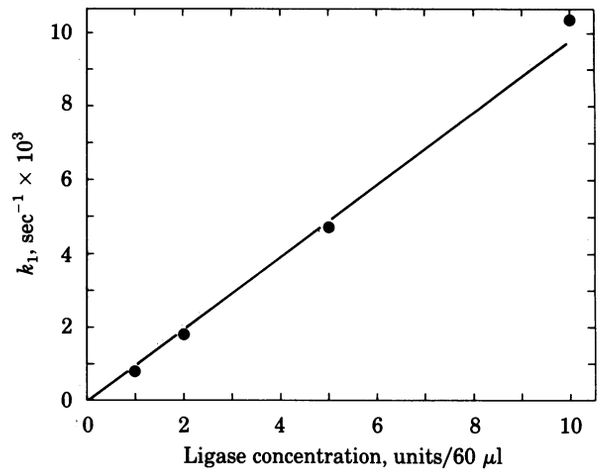

FIG. 4. Apparent first-order rate constant for ligase closure versus ligase concentration, expressed as the total number of units in a 60-μl reaction mixture. Data are for 880-bp DNA.

Test for Nonspecific Binding of Ligase to DNA. The ratio of DNA ligase to DNA substrate in these experiments was high and raises the possibility of nonspecific ligase binding to DNA. To test for nonspecific binding, unlabeled phage λ DNA was added to ligase reactions in as much as a 100-fold mass excess over ^{32}P-labeled reactant DNA. The amount of λ DNA (49 kb and added as the linear DNA) was more than 30-fold greater than the reactant DNA (1361 bp) and should have contributed few sites for ligation. No detectable change in k_1 or in the nature of the closed circular product was observed in these experiments. Because k_1 is known to be proportional to ligase concentration, we can conclude that nonspecific ligase binding is not a factor in these experiments. As expected, k_1 was independent of (S) in the range of DNA concentrations used in these experiments.

Rate of Bimolecular Covalent Closure and Determination of *j* Factor. The kinetics of ligase closure for bimolecular joining were apparent second order with respect to DNA concentration, in contrast to the cyclization reactions, and were proportional to ligase concentration. Values of k_2 for the two substrates described in *Materials and Methods* were the same within experimental error. The *j* factor of a particular DNA fragment is given by the ratio $k_1/2k_2$ (Eq. 9) when these rates are normalized to the same ligase concentration. Because k_2 is independent of fragment size in this range, only k_1 need be measured separately for each DNA fragment. Fig. 5 is a plot of *j* versus DNA fragment length determined at 20°C.

DISCUSSION

We conclude that the *j* factor, or effective ends concentration, is given by one-half the ratio of apparent rate constants for ligase closure in cyclization and bimolecular joining reactions. This will be true if joining of the cohesive ends, ligase binding, and covalent closure are identical reactions for cyclization and bimolecular joining. The observed rate constants for ligase closure, both for cyclization and for bimolecular joining, are directly proportional to ligase concentration; also, they show similar temperature dependences (unpublished data). In addition, the k_2 values for joining of two very different pairs of DNA molecules are found to be identical. This strongly suggests that the chemical steps involved in both reactions are identical despite the fact that the length of DNA flanking the joining sites

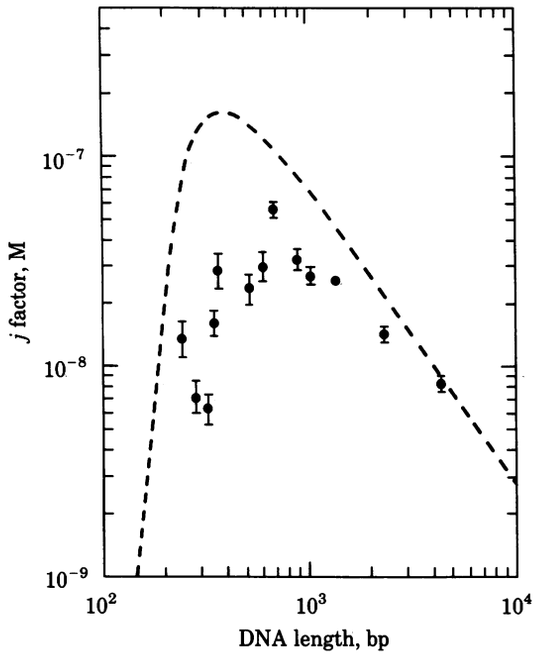

FIG. 5. *j* factor versus DNA length, determined at 20°C. Fragment lengths were 242, 288, 318, 345, 366, 504, 611, 670, 880, 1015, 1361, 2302, and 4361 bp; their origin is described in Table 1. A 126-bp fragment is not shown on this graph because its *j* factor is at least 100-fold lower than that of the 1361-bp reference fragment. The dashed curve is the angle-independent ring closure probability calculated from equation 62 of Yamakawa and Stockmayer (6) for a persistence length of 500 Å.

differs by 1 order of magnitude. Moreover, the rate of ligase closure in a cyclization reaction was found to be unaffected by a 100-fold excess of a linear phage DNA, indicating that nonspecific ligase binding to DNA is not a problem here.

There are two striking features of the results presented in Fig. 5. DNA fragments ranging in length from 242 to 4361 bp (approximately 1.5 to 30 *P*) differ in ring closure probability by less than a factor of 10 in agreement with earlier results of Mertz and Davis (14). Molecules of only a few persistence lengths are quite flexible. It is remarkable that the maximal ring closure probability occurs close to *P*. Perhaps just as striking as the broad plateau in *j* factor between 4361 and 242 bp is the sharp decrease in this quantity between 242 and 126 bp.

Both of these results are in close agreement with the theoretical predictions of Yamakawa and Stockmayer (6) and of Olson (15, 16). Yamakawa and Stockmayer derived the ring closure probability for a wormlike coil end-joined in a lowest bending energy configuration, which is a noncircular cusp shape. Olson developed a molecular model of DNA flexibility in which minor rotations about the two phosphodiester bonds of the sugar–phosphate backbone give rise to bending and twisting of the helix. Both models give similar ring closure probabilities in this size range.

The *j* factor appears to be a nearly smooth function of DNA length alone (Fig. 5). However, for fragments below 500 bp this is not the case. If the number of bp in the DNA fragment is not an integral multiple of the helix repeat, then the need to twist the DNA helix in order to make strand ends meet may decrease the *j* factor significantly for sizes less than 500 bp.

This paper is dedicated to Norman Davidson on his 65th birthday. We thank Carol Post, Stewart Scherer, Dr. Tom St. John, Jonathan Widom, and Dr. B. H. Zimm for their discussions and Dr. P. J. Hagerman for sending us his manuscript before publication. We also thank Elaine Morita and Dr. C. A. Christiansen for their help in an earlier phase of this project. This research was supported by Grant 2ROI GM 1998821 from the National Institutes of Health.

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