Gapped DNA and Cyclization of Short DNA Fragments

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ABSTRACT We use the cyclization of small DNA molecules, ~200 bp in length, to study conformational properties of DNA fragments with single-stranded gaps. The approach is extremely sensitive to DNA conformational properties and, being complemented by computations, allows a very accurate determination of the fragment's conformational parameters. Sequence-specific nicking endonucleases are used to create the 4-nt-long gap. We determined the bending rigidity of the single-stranded region in the gapped DNA. We found that the gap of 4 nt in length makes all torsional orientations of DNA ends equally probable. Our results also show that the gap has isotropic bending rigidity. This makes it very attractive to use gapped DNA in the cyclization experiments to determine DNA conformational properties, since the gap eliminates oscillations of the cyclization efficiency with the DNA length. As a result, the number of measurements is greatly reduced in the approach, and the analysis of the data is greatly simplified. We have verified our approach on DNA fragments containing well-characterized intrinsic bends caused by A-tracts. The obtained experimental results and theoretical analysis demonstrate that gapped-DNA cyclization is an exceedingly sensitive and accurate approach for the determination of DNA bending.

INTRODUCTION

Complexing of DNA with DNA-binding proteins is often associated with significant bending of the double helix. These bends may play an important role in DNA functioning and in the action of bound proteins. DNA is also known to be intrinsically bent in cases of special sequences, most notably A-tracts (Hagerman, 1990; Koo et al., 1986; Marini et al., 1982), and by some DNA-binding ligands, most notably by peptide nucleic acids (PNAs) (Kuhn et al., 2004; Protozanova et al., 2002). It is therefore very significant to have convenient and accurate methods to measure these bends in solution. Over the years, different approaches have been developed to address the issue. Intrinsic and induced DNA bending in solution is analyzed on the basis of DNA cyclization experiments (Balagurumoorthy et al., 1995; Crothers et al., 1992; Davis et al., 1999; Kahn and Crothers, 1992, 1993, 1998; Lyubchenko et al., 1991; Tchernaenko et al., 2003a,b; Ulanovsky et al., 1986), by fluorescence resonance energy transfer (reviewed in Hillisch et al., 2001; Parkhurst et al., 2001), by microscopy (Cherny et al., 1998; Le Cam et al., 1994; Mysiak et al., 2004; Shlyakhtenko et al., 2000), and by electrophoretic mobility (Hardwidge et al., 2002; Lee et al., 2003; Wu and Crothers, 1984). Each of these approaches, however, has certain limitations. The approaches based on measurements of the electrophoretic mobility and the microscopy approaches yield only limited accuracy of the bend evaluation. Fluorescence resonance energy transfer, which is becoming increasingly popular, does not allow measurement of distances larger than 10 nm; therefore, it is mainly suitable for bends close to 180°.

Methods based on DNA cyclization are more generally applicable. The approach based on the multimerization-cyclization of oligonucleotides 20–40 bp in length, intrinsically curved (Ulanovsky et al., 1986) or bound with proteins (Balagurumoorthy et al., 1995; Lyubchenko et al., 1991), gives only semiquantitative estimation of the bend angles (see Podtelezhnikov et al., 2000 for details). A more sophisticated approach, developed by Kahn and Crothers (1992), is based on the cyclization of intrinsically bent DNA fragments which also have a site for an inducible bend. The method yields not only the bend angle, but also the torsional deformation of the binding site and corresponding changes of bending and torsional rigidities associated with the bending. It requires, however, considerable experimental work (to reduce it, a modification of the experimental procedure was suggested by Zhang and Crothers, 2003). In addition, to obtain the conformational data, the approach requires computer optimization of the three parameters of the bent site, which is not a simple task.

Approaches based on cyclization of short DNA fragments are especially attractive because they do not require special equipment and can yield exceptional accuracy of the bend angle determination, since the cyclization efficiency of short DNA fragments is extremely sensitive to their conformational properties. Measurements of the cyclization efficiency for DNA fragments within the 200–400 bp length range have been used for more than 20 years to study DNA conformational properties and have yielded valuable information about the double helix. The cyclization experiments gave an elegant proof of the helical nature of the double-stranded DNA (Shore and Baldwin, 1983) and very accurate estimates of the DNA helical repeat, DNA bending and torsional rigidities (Crothers et al., 1992; Horowitz and Wang, 1984; Shore and Baldwin, 1983; Taylor and Hagerman,

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1990). In addition, they made it possible to study DNA intrinsic bends (Crothers et al., 1992; Roychoudhury et al., 2000) and to estimate the contribution of the DNA intrinsic curvature to the observed persistence length of the double helix (Vologodskaia and Vologodskii, 2002).

To determine the DNA conformational parameters for a particular fragment from the cyclization experiments, one has to measure the fragment's *j*-factor. The *j*-factor specifies the efficiency of the fragment cyclization (Jacobson and Stockmayer, 1950) and equals the effective concentration of one end of the chain in the vicinity of the other end in the appropriate angular and torsional orientation (see Shore and Baldwin, 1983 for example). Joining DNA ends, either cohesive or blunt, is a slow process, so its rate is not limited by the rate of diffusion of one end with respect to the other (Wang and Davidson, 1968). Therefore, the *j*-factor also can be expressed over the ratio of the corresponding kinetic constants of irreversible ligation of DNA ends (Shore and Baldwin, 1983; Shore et al., 1981). On the other hand, the *j*-factor value is completely defined by the conformational parameters of the DNA fragment: the minimum energy conformation of its axis, the distribution of the bending and torsional rigidities along the fragment, and its total equilibrium twist. The *j*-factor value can be accurately computed for both homogeneous and sequence-dependent models of the double helix, if the corresponding parameters are known (Hagerman, 1990; Koo et al., 1990; Podtelezhnikov et al., 2000). For the homogeneous wormlike chain, a very convenient equation exists (Shimada and Yamakawa, 1984).

In the existing form, however, the cyclization approach suffers from a serious drawback when it is applied to study DNA bends. The *j*-factor value of a short DNA fragment strongly depends on the preferable torsional orientation of the fragment ends. In its closed circular form, the double helix has to make an integer number of turns. This results in extra torsional stress in small DNA circles. The stress causes oscillations of the *j*-factor with the fragment length. The period of the oscillations corresponds to DNA helical repeat (Shore and Baldwin, 1983). The exact value of the helical repeat depends on the DNA sequence and, therefore, should be considered as an adjustable parameter during the analysis of the *j*-factor data. This means that *j*-factor measurements have to be performed for a series of fragment lengths to cover at least one period of oscillations.

An additional problem emerges when sharp bends induced by proteins have to be measured. The double helix is very rigid and, as a result, small circular molecules adopt conformations close to a perfect circle. Thus, the ends of a fragment which has a conformation such as shown in Fig. 1, *top*, cannot be joined and ligated without perturbing this conformation. The bend angle has to be reduced to facilitate the cyclization.

To overcome the limitations of the cyclization approach, we decided to modify it. To facilitate cyclization for DNA conformations similar to the one shown in Fig. 1, *top*, we incorporated a gap in the vicinity of one end of the fragment. Since single-stranded DNA is very flexible, the gap should serve as a swivel. Fig. 1, *bottom*, illustrates how even a strongly bent fragment can be cyclized with such a swivel. We expected that the single-stranded region would make all torsional orientations of the fragment ends energetically equivalent, so that the cyclization efficiency would become independent of the twist of the duplex part of the fragment. As a result, it would be sufficient to use one fragment length to estimate the value of the bend angle from the cyclization experiment.

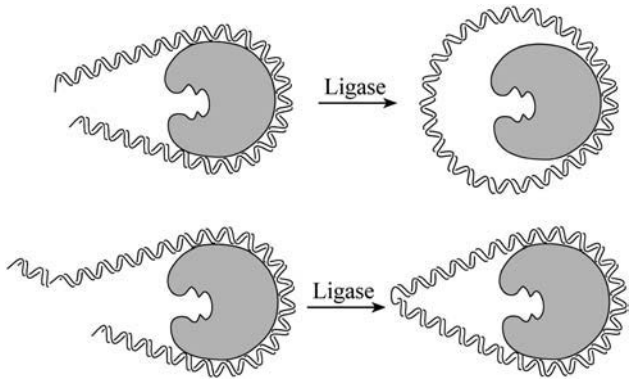

FIGURE 1 Conformations of a short DNA fragment in DNA-protein complexes. (*Top*) A short DNA fragment wrapped around a protein. If the bend angle, α, is close to 180°, the cyclization perturbs the DNA conformation in the complex. (*Bottom*) A gapped-DNA fragment of the same length can be closed without perturbing its conformation and, therefore, provides a more accurate determination of α.

For the implementation of this idea, two important conditions have to be fulfilled: i), the gap has to provide equal probability of any torsional orientation of the DNA ends; and ii), the bending rigidity of the gap has to be isotropic, independent on its torsional deformation, and known precisely.

Although conformational properties of gapped DNA were the subject of a few studies in the last decade (Guo and Tullius, 2003; Mills et al., 1994, 1999; Protozanova et al., 2004; Rivetti et al., 1998), we needed to perform our own investigation to choose a gap which satisfies these conditions. The method based on DNA cyclization is perfectly suitable for this.

Here we report on the successful implementation of the program. Using site-specific nicking enzymes, we obtained fragments with precisely located gaps. Using cyclization of the gapped-DNA fragments of different lengths, we have shown that the 4-nt-long gap satisfies the above conditions. Comparing the measured value of *j*-factor of the gapped DNA with theoretical computations, we determined the bending rigidity of the gap. Thus, we have shown that the cyclization of the gapped-DNA fragment can be a convenient and exceedingly accurate approach to determine intrinsic and induced DNA bends. For the gapped-DNA fragment of 200 bp in length, we have calculated the dependence of the *j*-factor on the bend angle introduced in the middle of the fragment. The approach has been verified by determining the intrinsic bend in fragments with phased A-tracts.

MATERIALS AND METHODS

DNA fragments

The fragments without A-tract sequence

Eight DNA fragments from 196 bp to 203 bp were incorporated between *Hind*III sites of pUC19 vector to obtain a set of eight plasmids (the 200-bp-long fragment is shown in Fig. 2). Each fragment consists of the common region (150 bp between *Pst*I and the second *Hind*III site) and the region subjected to changes. The changed sequences, ~50 bp in length, inserted between *Xba*I and *Pst*I sites, were obtained from the one predecessor sequence by insertions/deletions of single basepairs. The recognition sites for the nicking endonucleases are located within this variable region.

First, the *Hind*III and *Pst*I-ended subfragment, common for all eight fragments, was assembled by annealing six phosphorylated synthetic oligonucleotides and then was cloned into the pUC19 vector. The obtained plasmid was used as a vector to make eight different plasmids by inserting variable subfragments between *Xba*I and *Pst*I sites. The resulting insert carries an extra *Hind*III site near its left end, which was used to cut the fragment from the plasmid. All plasmids were cloned into DH5α *Escherichia coli* cells. The Miniprep Purification Kit (Qiagen, Valencia, CA) was used to extract plasmid DNA from the cells.

To obtain gapped-DNA fragments, the circular plasmids were first treated with the N.BbvC IA nicking enzyme (New England Biolabs (NEB), Beverly, MA) at 37°C for 3 h. After cleanup by QIAquick PCR Purification Kit (Qiagen), the nicked plasmids were treated with the N.BstNB I enzyme (NEB) at 55°C for 3 h. The double-nicked plasmids were purified twice by the same QIAquick Kit to remove the enzyme, salts, and the short oligonucleotide from the gap. The standard protocol using a microcentrifuge, provided by Qiagen, was applied. The gapped plasmids were digested by *Hind*III enzyme to produce gapped-DNA fragments of 196–203 bp in length.

Fragments with two or four A_5-tracts

The segments with A-tracts were incorporated into the plasmid with 200 bp *Hind*III insert. The 42-bp segment of the plasmid located between *Xho*I sites was replaced by a segment of the same length containing two or four in-phase A_5-tracts. For the case of four A-tracts, the plasmids with both orientations of the 42-bp segment were obtained. The new plasmids were cloned into DH5α *E. coli* cells.

Radioactive labeling

The *Hind*III fragments of 196–203 bp were end labeled together with the rest of the plasmid by ^{32}P in the exchange reaction. The mixture, containing 0.2 pmol of each fragment, 7 μl of [γ-^{32}P]ATP (10 mCi/ml; PerkinElmer, Wellesley, MA), and five units of T4 polynucleotide kinase (NEB), was incubated in 16 μl of kinase buffer at 37°C for 40 min. After labeling, the *Hind*III and the kinase were heat inactivated at 65°C for 20 min.

Ligation time course

Ligations were performed in 100 μl of the T4 DNA ligation buffer (NEB) at 22°C. The concentration of the DNA fragments was 0.5–10 nM, depending on the expected value of the *j*-factor. The concentration of T4 DNA ligase (NEB) in the ligation mixture was 0.01–0.1 units/μl. At specific time intervals, portions of the ligation mixture were withdrawn from the reaction solution and quenched with EDTA. The mole ratio of Mg^{2+} to EDTA was 1:10. Unincorporated radioactive label in each ligation sample was removed by centrifugation in a Sephadex G-50 minicolumn (Biomax, Odenton, MD).

Gel electrophoresis and data analysis

The ligation products were separated in 2.2% MetaPhor agarose (Cambrex Bio Science, Rockland, ME) gels. Under continuous circulation and cooling of TBE electrophoresis buffer, the gels were run at room temperature at 4.5 V/cm, for 8 h. After electrophoresis, the gels were equilibrated in ethanol/glycerol solution, dried between cellophane sheets in gel drying frame (Owl Separation Systems, Portsmouth, NH), and quantitated using Storm PhosphorImager and ImageQuant software (Amersham Bioscience, Piscataway, NJ).

Calculation of *j*-factor

An algorithm based on a set of conditional probabilities was used for the *j*-factor calculation (Podtelezhnikov et al., 2000). The corresponding program, jfm2full.c, and a sample data file, jfm2full.data, are available at http://crab.chem.nyu.edu/jfactor/index.html. The program makes it possible to perform fast and accurate calculations of the *j*-factor values for a chain consisting of segments of equal lengths. The program assumes that the bending, torsional rigidities and the minimal energy orientation of adjacent segments are specified for each segment independently. We found that for our fragments the calculated values of *j*-factor do not depend on the segment length if one segment corresponds to <10 bp. Throughout all the calculations presented in this study, one straight segment of the chain corresponded to 2 bp. The gap of 4 nt was modeled by three or four segments of the same length, 0.68 nm. We tested that only the overall bending rigidity of the gap, but not the precise length of the single-stranded region corresponding to the gap, affects the *j*-factor values for our DNA fragments. The values of persistence length, helical repeat, and bending rigidity of the double helix were equal to 48.5 nm, 10.49 bp/(helix turn), and 2.4×10^{-19} erg $\times$ cm, respectively. The bending rigidity of the chain segment corresponding to the gap was found by fitting the experimental results

FIGURE 2 Sequences of DNA fragments used in the study. (*A*) 200-bp-long fragment. The recognition sites for different endonucleases are underlined. The arrows show positions of nicks produced by nicking enzymes. The shadowed region is removed from the fragment resulting in a gap. (*B*) 42-bp segments with two and four A_5-tracts, which were used to replace the segment of the same length located between *Xho*I sites in panel *A*.

(see Fig. 5). The value of the torsional rigidity of the segment corresponding to the gap was chosen to be 1000 times smaller than the value for the other segments to provide the uniform distribution of the torsional orientations of the fragment ends.

RESULTS

Preparation of gapped-DNA fragments

Discovery and artificial design of endonucleases that introduce sequence-specific nicks made it possible to sequence-specifically introduce a gap into the double helix (Higgins et al., 2001; Protozanova et al., 2004; Wang and Hays, 2001; Xu et al., 2001). We designed and cloned into a plasmid DNA a 200-bp-long insert, which contains recognition sites for two nicking endonucleases, N.BbvC IA and N.BstNB I, in the vicinity of one end of the insert. To obtain the gapped fragment, the plasmid was sequentially treated with the nicking enzymes, after which a spin column was applied to remove the short single-stranded oligonucleotide between the nicks. The fragment was cut out from the plasmid by the *Hind*III restriction enzyme just before the radioactive labeling and ligation. There was no need to isolate the fragment from the rest of the plasmid to measure the fragment *j*-factor. Actually, the presence of a large amount of the bulk DNA in the equimolar amount facilitates the determination of the fragment concentration.

We used polyacrylamide gel electrophoresis to analyze the procedure of creating the gap. We found, in agreement with the results reported previously (Mills et al., 1994; Protozanova et al., 2004), that a single nick entails a minor change in the mobility of the fragment (Fig. 3, *lanes 2* and *3*). The second nick releases a tetranucleotide that is in thermodynamic equilibrium with respect to its binding to the gapped DNA. When gel electrophoresis starts, the tetranucleotide irreversibly dissociates, and as a result the gapped fragment moves through the gel. The gapped fragment has a lower mobility than the same fragment without a gap (Fig. 3, *lanes 3* and *4*). Still, the addition of DNA ligase to the double-nicked sample before gel electrophoresis partially restores the intact fragment (Fig. 3, *lane 5*). Therefore, we used a column purification to remove the tetranucleotide from the sample. Ligation performed after the column purification did not result in any traces of intact DNA (Fig. 3, *lane 6*).

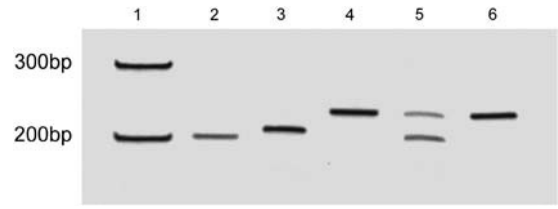

FIGURE 3 Analysis, in 8% nondenaturing PAGE, of creating a gap in the 200-bp-long DNA fragment shown in Fig. 2. Lane 1, DNA size marker; lane 2, a plasmid carrying the fragment digested by *Hind*III; lane 3, the same as in lane 2 but also treated by the nicking enzyme N.BbvC IA; lane 4, the same as in lane 3 but additionally treated by the N.BstNB I nicking enzyme; lane 5, the plasmid treated by the nicking enzymes, then by ligase followed by the *Hind*III digestion; and lane 6, the same as in lane 5 but with purification from the tetranucleotide before the treatment by ligase.

Conformational properties of the gapped DNA

To obtain the *j*-factor value of a fragment, we measured the ratio of the amounts of circular fragments, $C(t)$, and linear and circular dimers of the fragments, $D(t)$, formed during the early stage of fragment ligation (Taylor and Hagerman, 1990; Vologodskaia and Vologodskii, 2002):

$$j = 2M_0 \lim_{t\to 0} C(t)/D(t), \qquad (1)$$

where M_0 is the initial concentration of the fragment and t is the reaction time. Since it was shown that the ratio does not change over a wide range of the ligase concentration (Taylor and Hagerman, 1990), we can choose this concentration in such a way that the timescale of the reaction is in a convenient range. The circular monomers of the fragments and linear and circular dimers are then separated by gel electrophoresis to measure their relative amounts. To perform extrapolation of $C(t)/D(t)$ to zero reaction time, one needs to measure the ratio for a few values of t. Fig. 4 shows a typical result of such an experiment.

We assumed that a 4-nt-long gap is sufficient to completely eliminate the oscillations of the *j*-factor with the fragment length. This would mean that the bending rigidity of the gap does not depend on its torsional deformation and all torsional orientations of the gap ends are equally probable. To test this assumption, we determined *j*-factors for DNA fragments of different lengths with the same gap. The sequence of all fragments was identical except for single-nucleotide deletions/insertions in the left quarter of the fragment. Fig. 5 shows the results of these measurements. No *j*-factor oscillations are observed for the gapped fragments. We tested that the measured *j*-factor value for the gapped fragments does not change upon a 10-fold increase of the ligase concentration, from 0.002 to 0.02 units/μl. For comparison, Fig. 5 shows the *j*-factor values for the same fragments in cases of intact strands. Oscillations with a large amplitude are observed in this case.

To obtain conformational parameters of DNA fragments from the measured values of *j*-factors, one needs to fit the measurements by the theoretical values. Conformational properties of regular, intrinsically straight DNA fragments 200 bp in length or longer are well described by the wormlike chain model of the double helix (Vologodskaia and Vologodskii, 2002). Shimada and Yamakawa (1984) obtained a convenient analytical expression for *j*-factors of the model. We used their equation to fit the data for *j*-factors of fragments without gaps (see Fig. 5). The same results for *j*-factors are obtained in computer simulations that use a discrete wormlike chain model. A DNA molecule is modeled in this case as a chain consisting of N rigid segments. The discrete model can

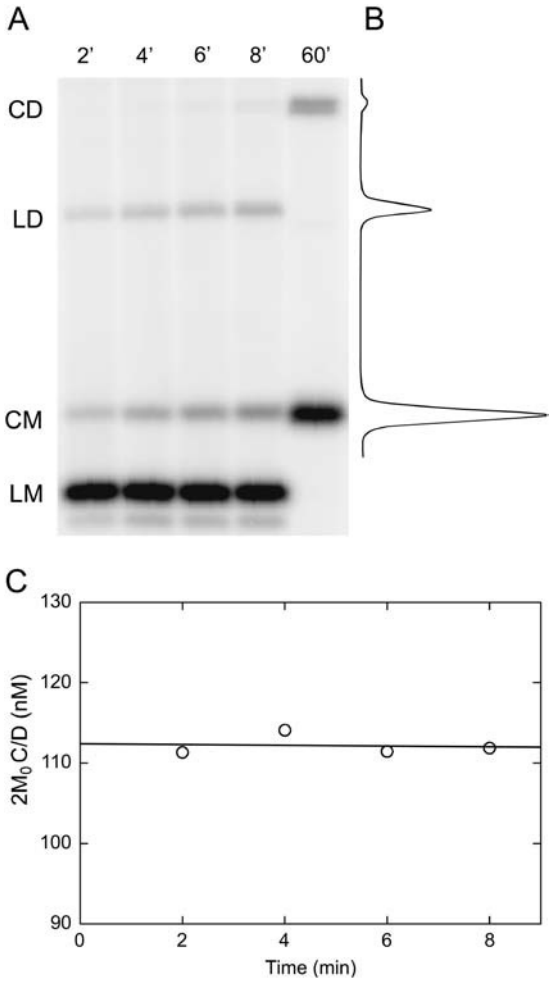

FIGURE 4 *j*-factor determination from the ligation time course. (*A*) Typical result of agarose gel electrophoresis shows separated bands of the linear monomers (*LM*), the reaction substrate, linear dimers (*LD*), circular monomers (*CM*), and circular dimers (*CD*). (*B*) PhosphorImager scan of the bands for $t = 8'$. (*C*) The ratio $2M_0C(t)/D(t)$ is extrapolated to zero ligation time to obtain the *j*-factor value. Both linear and circular dimers were included in $D(t)$. The data are for a 200-bp-long fragment carrying four phased A_5-tracts.

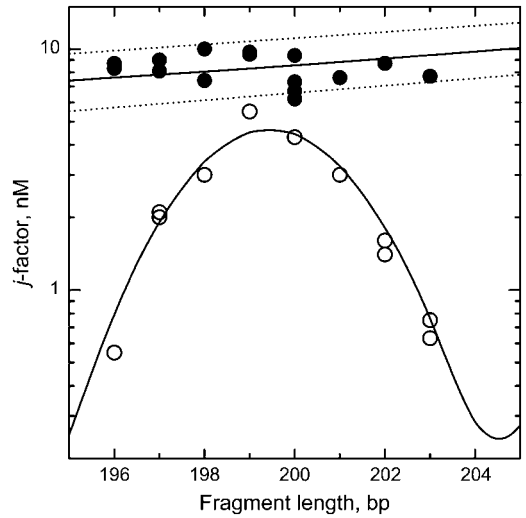

FIGURE 5 Dependence of *j*-factors on the length of DNA fragments. The *j*-factor values were measured for intact DNA fragments (○) and for same fragments with a 4-nt-long gap (●). The sequence of the fragments is shown in Fig. 2 except for a few point mutations/deletions that were introduced to change the fragment length. The straight line corresponds to the theoretical *j*-factor values calculated for the fragments with gaps. The gap bending rigidity, g_{gap}, was adjusted to yield the best fit between the theoretical curve and experiment (*solid line*). To illustrate the sensitivity of the theoretical dependence to g_{gap}, we performed the computations for two more values of g_{gap}(*dotted lines*), $1.5g^0_{gap}$ and $g^0_{gap}/1.5$, where g^0_{gap} is the best-fit value. The computation assumes that all torsional orientations of the fragment ends are equally probable (see Materials and Methods for details). The experimental data for intact fragments (*without gaps*) were approximated by the theoretical dependence (Shimada and Yamakawa, 1984) by adjusting the values of three parameters, a, γ, and C. The best fit (*solid line*) corresponds to $a =$ 48.5 nm, $\gamma = 10.49$ bp/(helix turn), and $C = 2.4 \times 10^{-19}$ erg $\times$ cm.

be easily generalized to account for irregularities in the double helix, like intrinsic bends and variations of the bending and torsional rigidities along the molecule.

Within the framework of the generalized discrete wormlike chain, the DNA molecule is presented as a chain of N straight segments of equal lengths. The segment orientations are specified by orthogonal triplets of unit vectors attached to the beginning of each vector. The minimal energy orientation of the triplets i and $i + 1$ are specified by three Euler angles, $\left(\psi^0_i, \sigma^0_i, \theta^0_i\right)$, where σ^0_i defines the angle of the equilibrium bend, and the sum $\psi^0_i + \theta^0_i$ defines the equilibrium twist. If $(\Delta\psi_i, \Delta\sigma_i, \Delta\theta_i)$ define the deflection of the actual orientation of the triplet $i + 1$ relative to its own minimal energy orientation, the elastic energy associated with the junction between segments i and $i + 1$ is

$$E_i = \frac{g_i}{2}(\Delta\sigma_i)^2 + \frac{C_i}{2}(\Delta\psi_i + \Delta\theta_i)^2, \qquad (2)$$

where g_i and C_i are the bending and torsional rigidities of segment i. The total energy of the chain is the sum over all $N - 1$ junctions. Each segment here represents a few basepairs of the double helix. Its length, l, has to be chosen so that a conformational property of interest does not change by further reduction of l (see Vologodskii and Frank-Kamenetskii, 1992, for details). For any particular value of l, the bending constants g_i are redefined to keep the DNA persistence length value unchanged (Frank-Kamenetskii et al., 1985). Clearly, one or more segments of the chain can model a single-stranded region of DNA. Such a model was described at length by Podtelezhnikov et al. (2000) who also suggested an efficient algorithm for the *j*-factor calculations for small DNA fragments.

The values of the DNA persistence length, a, the torsional rigidity of the double helix, C, and the DNA helical repeat, γ, were obtained by fitting the experimental data (see Fig. 5). They are in full agreement with numerous previous results (reviewed in Hagerman, 1988; see also Taylor and Hagerman, 1990; Vologodskaia and Vologodskii, 2002): $a = 48.5$ nm, $C = 2.4 \times 10^{-19}$ erg $\times$ cm, $\gamma = 10.49$ bp/turn. We used these

values of the double helix parameters when we fit the experimental values of *j*-factors for the gapped fragments. Since no oscillations of *j*-factor were observed for the gapped fragments (see Fig. 5), we concluded that all torsional orientations of the fragment ends were equally probable. Therefore, the fitting included only one parameter, the bending rigidity of the gap. We found the best fit when we assumed the bending rigidity of the single-stranded DNA, normalized per a nucleotide, to be ~13 times smaller than the corresponding value for the double helix, normalized per a basepair. The computed values of *j*-factors for the gapped fragments are shown in Fig. 5. The above value of the bending rigidity of the gap was used for all other computations of *j*-factor for the gapped-DNA fragments throughout this work.

Use of gapped fragments in a DNA cyclization approach

The results shown in Fig. 5 demonstrate that the gapped-DNA fragments can be very convenient in the measurements of DNA intrinsic or induced bends, since the gap makes all torsional orientations of the fragment ends equivalent. To show the sensitivity and accuracy of the approach, we applied it to DNA fragments with intrinsic bends originating from A-tracts. These bends have been well studied by various techniques. It has been shown that the A_6-tract causes a bend of 18° (Crothers et al., 1992; MacDonald et al., 2001), whereas the A_4-tract causes only a bend of 9° (Barbic et al., 2003). Thus, DNA fragments with A-tracts provide a good testing system for our approach.

We prepared 200-bp-long gapped-DNA fragments containing two or four A_5-tracts by replacing the fragment part between two *Xho*I sites (see Materials and Methods and Fig. 7). The middle of the A-tract block and the gap were separated by ~100 bp in these fragments, so in the circular form of the fragment, the distance from the gap to the middle of the A-tract was identical in clockwise and counterclockwise directions. The distances between adjacent A-tracts were 10–11 bp, to match the DNA helical repeat of 10.5 bp. Fig. 6 shows the *j*-factor values experimentally determined for these fragments together with the computed values of the *j*-factor. To confirm that the bending rigidity of the gap does not depend on the direction of the bending, we performed experiments for two different orientations of the segment with four A_5-tracts inside the 200-bp fragment. In the computations, we used the value of bending rigidity of single-stranded DNA obtained above; thus, there were no adjustable parameters in the analysis. We performed the computation assuming different values of the bend angle associated with each A_5-tract, 13°, 15°, and 17° (see Fig. 6). Clearly, comparison between the experimental and theoretical data allows us to distinguish between those three values of the bend angle, demonstrating the remarkable sensitivity of the approach. The theoretical result for the bend angle of 15° gives the best fit with the experimental data. Although there is

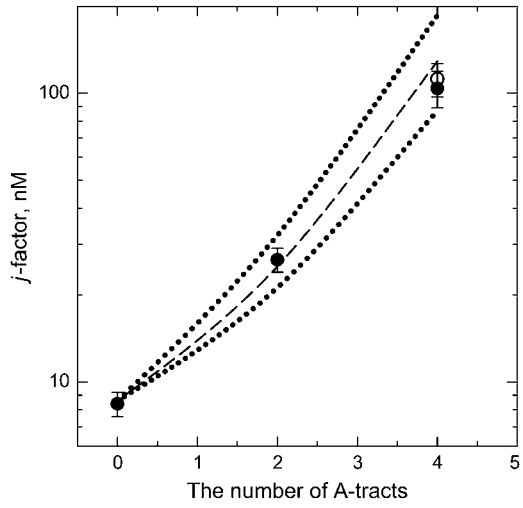

FIGURE 6 The *j*-factor values for 200-bp-long fragments carrying A_5-tracts. The experimental data obtained for gapped-DNA fragments with zero, two, and four phased A_5-tracts (●, ○) are shown together with the theoretical *j*-factor values. Solid and open circles correspond to two different orientations of the segment with four A_5-tracts in the 200-bp fragment. The theoretical values were calculated for the bend angle of 13° (*lower dotted line*), 15° (*dashed line*), and 17° (*upper dotted line*) per A-tract.

no direct data for the bend angle associated with the A_5-track in the literature, the values of the electrophoretic mobility suggest that the angle for A_5 is closer to that for A_6 than for A_4 (Koo et al., 1986). Thus, the obtained value of 15° for A_5 meets well the expectations based on the known data.

Sensitivity and accuracy of the approach

We calculated the expected values of *j*-factor for the gapped fragment consisting of the 200 bp duplex as a function of the induced bend angle. We assumed that three equal bends are located between two *Xho*I sites, so that adjacent bends are separated by 21 bp. Thus, the gap and the bends were symmetrically located in the circular form of the fragment, as diagrammed in Fig. 1. The calculations show that the *j*-factor value changes by nearly 300 times when the angle increases from 0° to 150° (Fig. 7 *A*), demonstrating the remarkable sensitivity of the approach. We quantitatively estimated the accuracy of the angle determination by this method. The estimation is based on the data in Fig. 7 *A* and on the assumption that the relative error of *j*-factor measurements is 15%, a value definitely achievable experimentally. Fig. 7 *B* shows the estimated relative error of the obtained angle as a function of the angle value. One can see from the plot that for the range of angles between 45° and 135° the expected relative error should be close to 5%.

In general, the bend can be localized at a particular point of the fragment or can be distributed along a certain part of it. We analyzed, by computer simulations, how the distribution of the bend along the DNA fragment affects the *j*-factor value. The computations showed that the distribution of the

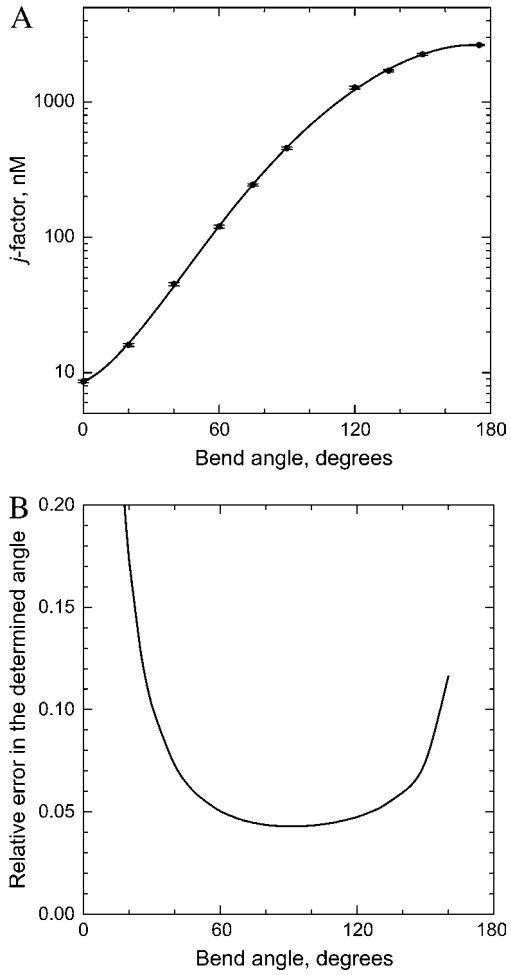

FIGURE 7 Dependence of the *j*-factor value and the relative error of the bend angle determination on the bend angle. The data are calculated for a 200-bp-long fragment carrying a 4-nt-long gap on the basis of the theoretical model with the value of the gap rigidity constant determined from data in Fig. 5. (*A*) Theoretical dependence of the *j*-factor on the bend angle. (*B*) The relative error of the angle determination that corresponds to 15% error in the *j*-factor measurements.

bend angle has a nearly negligible effect on the *j*-factor values if the bend angle is smaller than 150° (data not shown). Thus, the dependence of the *j*-factor on the bend angle shown in Fig. 7 *A* can be considered as a universal one for a 200-bp-long fragment with the 4-nt-long gap, under the condition that in circular form of the fragment an induced or intrinsic bend is symmetrically located relative to the gap.

DISCUSSION

We used cyclization of short DNA fragments to study conformational properties of the 4-nt-long DNA gap. We have shown that the gap makes all torsional orientations of DNA ends equally probable. We have also found that the bending rigidity of the gapped fragments does not depend on the gap torsional deformation (see Fig. 5) or on the bend direction. The latter conclusion is supported by the fact that we have obtained the same values of the *j*-factor for the gapped fragments with two different orientations of the intrinsic bend associated with phased A_5-tracts (Fig. 6). The same conclusion about isotropic bending rigidity of gaps of three or more nucleotides in length was drawn earlier in a study of the electrophoretic mobility of gapped-DNA fragments (Mills et al., 1994). It should be noted, however, that single-nucleotide gaps do not have isotropic bending rigidity (Guo and Tullius, 2003).

We have found that the gap bending rigidity, calculated per nucleotide, is 13 times smaller than the bending rigidity of the double-stranded DNA, calculated per basepair. Our data do not allow us to determine internucleotide spacing, h, in the single-stranded region. If we assume that $h = 0.6$ nm (Mills et al., 1999), the above value of the gap bending rigidity corresponds to 6.5 nm for the persistence length of single-stranded DNA, a_{ss}. This value of a_{ss} is significantly larger than previous estimations for single-stranded DNA molecules in the absence of the single-stranded stacking, which are in the range of 1–3 nm (Achter and Felsenfeld, 1971; Inners and Felsenfeld, 1970; Mills et al., 1999; Murphy et al., 2004; Rivetti et al., 1998; Smith et al., 1971). However, stacking interaction, which is well pronounced in oligo(dA) at room temperature but negligible in oligo(dT), increases a_{ss} greatly (Eisenberg and Felsenfeld, 1967; Mills et al., 1999). Probably the most accurate estimation of a_{ss} for oligo(dA), $a_{ss} = 7.8$ nm, was obtained in the transient electric birefringence study (Mills et al., 1999). Thus, the flexibility of our gapped segment, GCAG, is closer to one of oligo(dA) than of oligo(dT).

It should also be noted that the flexibility of a gap is affected by electrostatic repulsion between surrounding double-stranded segments. The effect should make large bends at the gap, which are important for the cyclization of short fragments, less probable. This means that the value of bending rigidity of a gap obtained in the cyclization studies can be higher than the value determined by other approaches. To test that the electrostatic repulsion between the double-stranded segments contributes to the gap stiffness, we repeated the ligation experiment for the 200-bp-long gapped fragment in a buffer containing 1 mM of $MgCl_2$ (the standard buffer contains 10 mM of magnesium ions). We found the *j*-factor value to be 6.3 nM in this buffer (data not shown), notably lower than the value in the standard buffer, 8.6 nM. This reduction of the *j*-factor value corresponds to ~30% increase of the gap bending rigidity. The persistence length of double-stranded DNA is not affected by this change of magnesium concentration (Taylor and Hagerman, 1990). It is most probably also the case for the stiffness of the short single-stranded gap. On the other hand, we know that such a change of the reaction buffer changes the electrostatic repulsion between segments of double-stranded DNA (Rybenkov et al., 1997). Thus, the result supports our assumption that electrostatic repulsion between double-stranded segments surrounding the gap contributes to the gap stiffness.

Using this well-characterized gap, we have developed and validated a convenient and accurate approach to estimate bend angles in short DNA fragments. The approach consists of measurement of the *j*-factor value for a short DNA fragment (200-bp-long in our case) with a gap (4-nt-long in our case) located near one of the fragment's termini in the linear form. The bend can be intrinsic (caused by a specific DNA sequence) or induced by protein (or other ligand) binding.

A uniform distribution of torsional orientations of the fragment ends means that one does not need to run cyclization experiments for different fragment lengths to account for their torsional phasing. This makes our approach very convenient. It is also important that the approach does not require a complicated theoretical analysis. If one used our 200-bp gapped-DNA fragment as a standard base and only replaced its central part, the bend angle could be determined from the calibration curve shown in Fig. 7 *A*. For a fragment with a different length of the duplex or with a different sequence/length of the gap, the calibration curve should be recalculated. There are two parameters which determine the *j*-factor value of unbent gapped DNA: the duplex DNA bending rigidity (measured in terms of DNA persistence length *a*) and the gap bending rigidity. The value of *a* for duplex DNA is well known and equals 48 ± 1 nm for DNA with a typical sequence in a buffer with a few mM of magnesium ions (Taylor and Hagerman, 1990; Vologodskaia and Vologodskii, 2002). Thus, to calculate the calibration curve for a different gapped fragment, one has only to adjust the gap bending rigidity parameter. The parameter can be found by fitting the calculated and measured *j*-factor values for the unbent gapped fragment. A corresponding computer program is available at the authors' web site (see Materials and Methods). It should be noted that *j*-factors for DNA fragments of ~200 bp in length can be reliably calculated within framework of the wormlike chain model (Podtelezhnikov et al., 2000; Shimada and Yamakawa, 1984). An unexpectedly high cyclization efficiency reported recently for DNA fragments of ~100 bp in length (Cloutier and Widom, 2004) does not affect any data reported here.

The calibration curve for the bend angle determination, like the one in Fig. 7 *A*, makes it possible to determine the angle with very high accuracy. For a wide range of bend angles, we expect the relative error in the bend angle determination by our approach to be within 5% (see Fig. 7 *B*). We have validated our approach by determining the bend angles for two and four phased A_5-tracts. As a result, the bend introduced by one A_5-tract has been accurately estimated as being 15° (see Fig. 6). This figure agrees well with the data in literature. Note that the measured effect of the *j*-factor increase can be caused, for our gapped-DNA fragment, by directional bends or by increasing the local flexibility of the double helix. Our approach does not allow one to distinguish between these two possibilities. In many cases, however, our knowledge about a DNA-protein complex allows us to assume that the protein binding should not cause a notable increase of the DNA flexibility. In other cases, additional approaches such as electron or atomic-force microscopy (see Kuhn et al., 2004, for example) are required to discriminate between those two possibilities. Of course, there may be situations when both effects contribute to the observed *j*-factor value.

Our cyclization-based analysis of DNA bends is very simple from a technical point of view. This is true, in particular, because there is no need to separate the fragment from the rest of the vector plasmid (see Results). Sometimes, however, the presence of the equal molar amount of much longer DNA can be an obstacle for the binding between the protein and the fragment. In such a case, one needs to separate the fragment from the rest of the plasmid. We found that a simple way to solve this problem is to amplify the fragment, with short additional ends carrying the restriction sites, by PCR. We verified that the fragments obtained by such a way give the same values of *j*-factors (data not shown).

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