Determination of the DNA Bend Angle Induced by the Restriction Endonuclease EcoRV in the Presence of Mg²⁺

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Timo Stöver, Eleonore Köhler, Ursula Fagin, Wolfgang Wende, Heiner Wolfs, and Alfred Pingoud

From the Zentrum Biochemie, Medizinische Hochschule Hannover, Konstanty-Gutschow-Strasse 8, D-300 Hannover, Germany

We have used the method of Zinkel and Crothers (Zinkel, S. S., and Crothers, D. M. (1990) Biopolymers 29, 29-38) to determine the degree of bending induced by the binding of the restriction endonuclease EcoRV to its recognition sequence (-GATATC-). A set of four calibration DNA fragments was constructed that contained zero, two, four, or six phased A-tracts in their centers and an EcoRV site at the 5'-end to account for the electrophoteric influence of the bound protein. The mobilities of these calibration molecules complexed with EcoRV were compared to that of a test DNA containing a central EcoRV site also complexed with EcoRV. The EcoRV-induced bend angle was found to be 44° ± 4°. These experiments were performed with a catalytically inactive EcoRV mutant that still binds DNA specifically in the presence of Mg²⁺. In the absence of Mg²⁺, which is necessary for specific binding, there is no difference in the mobilities of the fragments with a peripheral or a central EcoRV site complexed with EcoRV, indicating that nonspecific binding on average does not lead to measurable DNA bending.

“The distortion of DNA from the straight, regular (and essentially mythical) double-helical structure beloved of textbooks is a ubiquitous feature of protein-DNA complexes” (Travers, 1990). Curvature can be an intrinsic feature of the DNA itself (for recent reviews, see Crothers et al. (1990), Hagerman (1990, 1992), and Trifonov (1991)), either due to a specific sequence or as a consequence of supercoiling, but can also be caused by bending induced by protein binding. The bendability of a given DNA sequence presumably plays a substantial role in the DNA recognition by repressors, activators, and DNA-processing enzymes and in the packaging of DNA into nucleosomes (Travers, 1987, 1991).

Protein-induced DNA bending has been demonstrated to accompany many protein-DNA interactions. For the catabolite activator protein, for example, bending of the DNA has been shown both crystallographically in the protein-DNA complex (90°) (Weber and Stieitz, 1987) and in solution by gel shift experiments (100°) (Zinkel and Crothers, 1990). It has been proposed that a possible role of this bending is the formation of additional contacts of the DNA upstream of the catabolite activator protein binding sequence with the RNA polymerase (Zinkel and Crothers, 1991). A crystal structure of a 434-repressor fragment with a 20-mer reveals bending and overwinding of DNA in the central AT-rich region that is accompanied by a dramatic narrowing of the minor groove

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DNA Bending by EcoRV

A

418(Center)-Cassette
GATCGTACTGATACTGCTGAC
CATATGCTCTAGCAGCTGCTAG

418(End)-Cassette
GATCGTACTTACGGGCCTGAC
CATATGGCTGCTGCTAG

Bend I-Cassette
GATCGTTTTTTCATAAAA

Bend II-Cassette
GATCGTTTTTTCATAAAA

Bend III-Cassette
GATCGTTTTTTCATAAAA

B

5'-PCR-Primers

EcoRV
GATATCAGCTCATATGGCGTCTAGC

BamHI
GATCGTACTGATATCGCTGAC

pAT153
CTAGGACAGCCCTACATAGCTGGCTGATAGCTGCTAG

3'-PCR-Primers

CCGCCCCCTCGAACAACCGCGGT

Fig. 1. a, oligodeoxynucleotide cassettes. Shown are synthetic double-stranded oligodeoxynucleotide cassettes that were cloned into the BamHI site of pAT153 to yield, upon PCR, the different constructs: 418(center), 418(end), and Bends I–III. b, PCR primers. Shown are PCR primers that were used to obtain the five different constructs: 418(center), 418(end), and Bends I–III. Primers were chosen such that the length of all fragments was exactly the same and such that the A-tracts (for Bends I–III) and the EcoRV site (for construct 418(center)) were always perfectly centered. Primers for Bends II and III introduced a new EcoRV site at the 5'-end of the fragment; the primer for construct 418(center) deleted the natural EcoRV site of pAT153. The locations of the PCR primers with respect to the DNA to be amplified are indicated.

into the BamHI site of pAT153. The oligodeoxynucleotides had compatible BamHI overhangs, but did not restore the site, thus providing a selectable restriction marker. Oligodeoxynucleotide Bends I–III also had an NsiI site (Fig. 1A). After purification of ligated plasmids, positive clones were identified by restriction digest and verified by sequencing.

The plasmids then served as PCR templates for the production of DNA fragments of identical length (418 bp). PCR primers were chosen such that the bending A-tracts (Bends I–III), the EcoRV site (construct 418(center)), and the stuffer fragment (construct 418(end)) were always positioned at the exact center of the fragment and such that the length of the fragment was always exactly the same (Fig. 1B).

In the PCR reaction, an EcoRV site was introduced 5 base pairs from the 5'-end of both the bending standards and the fragment containing the stuffer sequence (construct 418(end)) with an appropriate PCR primer. The fragment containing the EcoRV site in the center (construct 418(center)) had no EcoRV site at the end. The different constructs are shown in Fig. 2.

Polymerase Chain Reaction—PCR was carried out in a TPS*5.4 thermocycler (version 3.2; Landgraf, Hannover, Germany). For radioactive labeling of the fragments during PCR, [α-32P]dATP (American sham, Braunschweig, Germany) was used. The following reaction conditions were chosen: a 50-μl volume of 4.21 μM template DNA, 0.4 μM concentration of each primer, 0.2 mM dNTP mixture, 5 μl (10×) of Taq buffer, 3 units of Taq polymerase (Amersham), and 15 μCi of [α-32P]dATP. Cycle 1 was for 30 s at 92 °C; cycle 2 for 90 s at 92 °C, 90 s at 54 °C, and 210 s at 72 °C; and cycle 3 for 360 s at 72 °C and 30 s at 0 °C. Cycles 1 and 3 were run once; cycle 2 was repeated 30 times.

Purification of PCR Products—PCR products were separated by gel electrophoresis on a 6% polyacrylamide gel and visualized by autoradiography. The DNA fragments of interest were excised and eluted with water. The purified fragments were used for gel electrophoretic mobility shift experiments.

Gel Electrophoretic Mobility Shift Experiments—Gel electrophoretic mobility shift experiments with EcoRV have been described in detail by Thielking et al. (1992). In the experiments reported here, the concentration of the DNA fragments was 1.25 nM, and the concentration of the His-tagged EcoRV mutant D90A (Selent et al., 1992; Wende et al., 1991) was 25 nM. Bands were visualized by autoradiography.

Measurement of Gel Electrophoretic Mobilities—The mobilities of the different DNA constructs and the protein-DNA complexes were measured from the autoradiographs of the gels. An R<sub>e</sub> value was computed for the uncomplexed DNA species and for the protein-DNA complexes. R<sub>e</sub> is the apparent length of the molecule, deter-
RESULTS

The DNA fragments 418(center) (with the EcoRV recognition sequence in the center), 418(end) (with the EcoRV recognition sequence 5 base pairs from the 5'-end), and a stuffer cassette in the center (Bend I (two A-tracts), Bend II (four A-tracts), and Bend III (six A-tracts)) were constructed as described under “Materials and Methods” (Fig. 2). Bends I–III serve as standards for the degree of DNA bending. A-trtracts phased at 10.5-bp intervals are especially suited as standards as their bending propensities have been studied extensively with different methods, i.e. electric dichroism (Levene et al., 1986), cyclization kinetics (Zahn and Blattner, 1987; Koo and Crothers, 1988; Koo et al., 1990), and crystallographic studies (Nelson et al., 1987). According to these independent methods, one A-tract induces a bending of 18°. Therefore, Bends I–III have been assigned 36°, 72°, and 108° of bending, respectively.

The A-tracts were placed in the center of the DNA molecule under study, and an EcoRV recognition site was placed 5 base pairs from the 5'-end to account for the contribution of bound protein. The DNA that served to determine the EcoRV-induced bending was a fragment of identical length with an EcoRV site instead of A-tracts in the center. Construct 418(end) (with the EcoRV sequence at the 5'-end) was chosen as a reference. Binding of EcoRV to the end of the DNA fragment should not influence the mobility, except for the increase in molecular weight of the complex, as the end-to-end distance of the DNA remains more or less the same. This approach is similar to the one described by Zinkel and Crothers (1990) for the catabolite activator protein.

Mobility of Different Bend Constructs on Polyacrylamide Gels.—To show the different mobilities of the five constructs in the absence of protein, they were separated by electrophoresis on both EDTA- and Mg²⁺-containing polyacrylamide gels. The position of the EcoRV site on the DNA fragment does not influence the mobility of the free DNA (data not shown). As shown in Fig. 3 (top), an increasing number of A-trtracts leads to an increase in gel retardation. The relative mobility of the different constructs is independent of the presence of Mg²⁺. It should be noted that Mg²⁺-containing gels need a longer run time to achieve the same separation of the different fragments, but the relative distance between the bands representing the different fragments remain the same.

Gel Electrophoretic Mobility Shift Assays—For the gel electrophoretic mobility shift assays, the EcoRV mutant D90A was chosen. This mutant still binds to DNA specifically in the presence of Mg²⁺, but does not catalyze the cleavage reaction. In the absence of Mg²⁺, however, the D90A mutant (Thielking et al., 1992), like the wild-type enzyme (Taylor et al., 1991), binds to DNA largely nonspecifically. In this study, the experiments were carried out with a His-tagged D90A mutant. It must be emphasized that NH₂-terminal His tags leave DNA binding and DNA cleavage activities of the wild-type EcoRV as well as the EcoRV mutants unaffected (Wende et al., 1991).³

In Fig. 3 (top), binding of D90A to the different DNA constructs in the absence of Mg²⁺ is shown. All constructs yield several band shifts, although only one EcoRV-binding site is present, indicating nonspecific binding (Thielking et al., 1992). Due to their intrinsic bends, Bends I–III show a different mobility compared to the DNA without A-trtracts both in the free and the enzyme-bound DNAs. Constructs 418(center) (lane b) and 418(end) (lane a) show exactly the same mobility, which is reduced to the same extent when the DNA is complexed with the D90A mutant (lanes g and f).

In Fig. 3 (bottom), a gel electrophoretic mobility shift assay of D90A with the different constructs in the presence of Mg²⁺ is shown. Upon specific binding as it occurs in the presence of Mg²⁺, only a single band shift occurs. The test bend (construct 418(center)) (lane g) now shows a markedly different mobility than construct 418(end) (lane f) due to the bending by EcoRV. In the gel electrophoretic mobility shift experiments conducted in the presence of Mg²⁺ (Fig. 3, bottom), but not, however, in its absence (Fig. 3, top), the main shifted band is accompanied by a minor band of higher mobility. We have observed this phenomenon before with the EcoRV mutant D90A (Thielking et al., 1992); we believe that the satellite band is due to a conformational isomer of EcoRV.

³ W. Wende, H. Maschke, J. Alves, and A. Pingoud, manuscript in preparation.
FIG. 3. Gel electrophoretic mobility shift experiments with D90A mutant and five different constructs. 1.25 nM $^{32}$P-labeled DNA constructs (418 bp long) were incubated with a 20-fold molar excess of D90A and separated by electrophoresis on a 6% polyacrylamide gel. The experiment was carried out in the absence (top) and presence (bottom) of Mg$^{2+}$. Lanes a-e show the free DNA fragments (constructs 418(end) and 418(center) and Bends I-III; lanes f-j show the same fragments when complexed with D90A. In the absence of Mg$^{2+}$ (top), multiple band shifts appear, corresponding to enzyme-DNA complexes of 1:1, 2:1, 3:1, etc. In the presence of Mg$^{2+}$ (bottom), only one band shift is observed, corresponding to the 1:1 complex. (For a discussion of the weak satellite bands in lanes f-j, see text.)

Two different methods of evaluation of gel electrophoretic mobility shift experiments were used, both leading to the same result. The method of Koo and Crothers (1988) was developed for ligated multimers of duplexed oligonucleotides containing A-tracts. For these molecules, the $R_L$ value is a quadratic function of the curvature of the molecule. In this study, however, the molecules contain long stretches of straight DNA; in fact, the A-tracts represent $\leq$14% of the DNA molecule. Therefore, we used, in addition to the method of Koo and Crothers, a direct procedure in which the $R_F$ value was plotted versus the degree of bending. By both evaluation procedures, a bending angle of $44^\circ \pm 4^\circ$ was determined for a specific EcoRV-DNA complex in the presence of Mg$^{2+}$ (Fig. 4, top and bottom). For comparison, the bending angle observed in the crystal structure (Winkler, 1992) is $\approx 55^\circ$.

*Estimate of Error*—To estimate the error in the determi-
nation of the bend angle, several factors have to be taken into account. 1) There is some degree of error in the accuracy of the measurement of gel mobilities. 2) Some uncertainty arises from the extrapolation of the test point from the curve. 3) The radii of the curvature of the EcoRV bend may be slightly different from those of an equivalent bend directed by A-tracts. On the basis of two independent experiments, we estimate the total error to be on the order of 4°, i.e., ~10%.

**DISCUSSION**

The crystal structure of a specific EcoRV-DNA complex has demonstrated that the DNA is highly distorted compared to regular B-DNA. Most conspicuous is the bending with a bend angle of ~55° (Winkler, 1992). It is tempting to assume that this distortion, which drives the DNA into a rather unfavorable conformation, is needed for specific binding and catalysis in as much as it maximizes protein-nucleic acid contacts, provides a binding site for the essential cofactor Mg\(^{2+}\) (Vermote et al., 1992; Vermote and Halford, 1992), increases the reactivity of the DNA, and positions catalytically relevant groups of the protein and the DNA (Jeltsch et al., 1992). On the other hand, the specific EcoRV-DNA complex analyzed crystallographically was obtained in the absence of Mg\(^{2+}\) to prevent cleavage, i.e., under conditions in which EcoRV binds all DNA sequences (specific and nonspecific) with similar affinity (Taylor et al., 1991). Only in the presence of Mg\(^{2+}\) does EcoRV show a strong preference in binding specific DNA sequences (Thielking et al., 1992). Hence, the question arises as to what extent the distortion of the DNA seen in the crystal structure is also present in solution, in particular, whether it is a characteristic feature of the recognition complex formed in the presence of Mg\(^{2+}\). We have chosen to study this problem with gel electrophoretic mobility shift experiments following methods similar to those described by Zinkel and Crothers (1990) for the determination of the DNA bending induced by the catabolite activator protein. To be able to carry out these experiments in the absence and presence of Mg\(^{2+}\), we have employed a mutant of EcoRV, D90A, which carries a substitution in the active site, is catalytically inactive, but retains its sequence-specific binding capacity (Selent et al., 1992). The experiments were performed with a NH\(_2\)-terminally His\(_{6}\)-tagged EcoRV mutant. As the NH\(_2\) terminus of EcoRV is far away from the DNA-binding site or the subunit-subunit interface, no interference of the His tag with the enzymatic function of EcoRV was expected. Indeed, a comparison of the DNA binding and DNA cleavage activities of wild-type EcoRV and His\(_{6}\)-EcoRV, as well as several mutants, without and with His\(_{6}\) tags, shows that the 6 extra His residues at the NH\(_{2}\) terminus have no effect on these activities. For the work described here it was important to demonstrate that D90A and His\(_{6}\)-D90A with the same concentration dependence produce multiple band shifts in the absence of Mg\(^{2+}\) and a single band shift in the presence of Mg\(^{2+}\) in a gel electrophoretic mobility shift assay with a 377-bp fragment (Thielking et al., 1992). 4

Our results show that in the absence of Mg\(^{2+}\), EcoRV does not lead to bending of the DNA as in the gel retardation assays, no differences were detected among the mobilities of the complexes of the D90A mutant with DNAs containing a centrally or a peripherally located EcoRV site. This finding suggests that in contrast to the crystal structure, there is no bending in the absence of the cofactor. However, from Fig. 3 (top) it is clear that binding is nonspecific in the absence of the cofactor Mg\(^{2+}\) because instead of just one band, multiple band shifts appear. This means that even in the 1:1 complex, the enzyme probably does not occupy the cognate site, but rather can be located at any site. As the 418-bp-long DNA
fragment employed in this study contains ~400 noncognate-binding sites and as the preference for the cognate site is low in the absence of Mg$^{2+}$, the proportion of complexes with the enzyme occupying the cognate site is too small to be seen in a gel shift assay. Therefore, with the approach chosen, nothing can be concluded for the DNA bending induced by EcoRV when occupying a cognate site in the absence of Mg$^{2+}$. It is clear, however, that averaged over time and space (i.e. when EcoRV associates to and dissociates from a noncognate site in order to associate with another noncognate site, only to dissociate again, etc.), no bending occurs.

In the presence of Mg$^{2+}$, when EcoRV forms a specific complex with DNA, DNA bending by $4^\circ$ occurs, as our gel electrophoretic mobility shift experiments demonstrate. This bend angle is similar to the one determined from the crystal structure for a specific complex formed in the absence of Mg$^{2+}$. The small difference in bend angles may be due to packing forces or may be a consequence of the presence or absence of Mg$^{2+}$. We conclude from the similarity of the two values that the specific EcoRV-DNA complex obtained in the absence of Mg$^{2+}$ has most of the features of a true recognition complex, which requires Mg$^{2+}$ bound to the active site. This conclusion is supported by the finding that Mg$^{2+}$ soaked into a co-crystal does not lead to major structural changes.5

We envisage the process by which EcoRV "finds" its recognition site on a macromolecular DNA substrate as random association-dissociation events, possibly facilitated by linear diffusion along the DNA. Conformational fluctuations of the protein and the DNA serve to probe for optimal interactions as they are formed when EcoRV occupies a cognate site. These lead to further conformational changes, including bending of the DNA, which in turn allows Mg$^{2+}$ to associate with the complex and to initiate catalysis.

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