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Protein-induced unwinding of DNA: measurement by gel electrophoresis of complexes with DNA minicircles. Application to restriction endonuclease EcoRI, catabolite gene activator protein and lac repressor

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Abstract

An electrophoretic procedure for the measurement of the helix unwinding induced by a sequence-specific protein is described. The method, which was applied here to EcoR I, CAP and lac repressor, involved the migration of the complexes with positively and negatively supercoiled DNA minicircles carrying a single protein binding site.

Mobility shifts of complexes relative to naked DNAs appeared to be a result of i) the unwinding; of ii) an increase in the molecular frictional coefficient, which led to a retardation; of iii) bending, in the particular case of CAP, which induced an acceleration; and of iv) looping, in the case of lac repressor, which also resulted in an acceleration.

Under conditions where the migration of the naked topoisomers was V-like (topoisomer mobility showed the same linear increase with both negative and positive supercoilings; Zivanovic et al. (1986) J. Mol. Biol., *192,* 645-660), the protein unwinding contribution to mobility was assumed to be identical to that experimentally observed in the case of a thermal unwinding: all negatively supercoiled topoisomers were retarded and all positively supercoiled topoisomers were accelerated to the same extent. In contrast, the mobility contribution of the frictional term, as well as those of bending and looping, appeared to vary strongly with the magnitude of the supercoiling, but only weakly with its polarity. As a consequence, these latter contributions may approximately cancel when one is measuring the difference between the shifts observed for two comigrating, negatively and positively supercoiled, topoisomers, allowing the unwinding to be calculated. While estimates obtained for EcoR I, 23 ± 3 °, and CAP, about 29 °, were in good agreement with previous measurements using topoisomerase I, the value found for lac repressor, 13 to 16°, was significantly smaller.

Introduction

The local helix unwinding, U, induced by the binding of a sequence-specific protein has so far been measured through relaxation with topoisomerase I (or nick closure with ligase) of a complex of the protein with a plasmid DNA molecule usually containing several protein binding sites in order to increase the accuracy. This relaxation (or closure), when compared to that of the naked plasmid, allowed the determination of U as the increment in the linking number of the most probable configuration of the DNA. This method has initially been applied to *E. coli* RNA polymerase by Saucier & Wang (1), and was subsequently used with lac repressor (2), restriction endonuclease Eco R I (3), catabolite gene activator protein (CAP) (4), *Xenopus* transcription factor IIIA (5), and again *E. coli* RNA polymerase (6). Another method, which has been of more limited use, takes advantage of the property of a protein with an ability to unwind the DNA to exhibit a higher affinity for DNA of larger negative supercoiling. Quantification of this effect, together with the knowledge of the free energy of superhelix formation, has permitted an estimation of the unwinding angle of lac repressor (7) and of *E. coli* RNA polymerase (6).

This paper describes a new method for the measurement of U, which involves the electrophoretic migration of complexes of the proteins with positively and negatively supercoiled DNA minicircles containing a single protein binding site. The method is applied below to EcoR I, CAP and lac repressor.

Materials and Methods

a) Proteins.

Experiments shown in this paper used EcoR I from Boehringer. EcoR I from Promega Biotec was however also used with similar results. CAP (0.9 mg and 20 nmoles dimers/ml) was purified as previously described (8). Lac repressor (7.7 mg and 51 nmoles tetramers / ml) (a gift of Dr. J. G. Brahms) was purified from the *E. coli* overproducing strain BMH 7414 according to ref. 9. The isopropyl β-D-thiogalactoside (IPTG) binding essay (10) showed a protein activity of 95-100 %. Molecular weights of the active proteins are 62,000 and 45,000 for EcoR I and CAP in their dimeric forms (11-14), respectively, and 154,000 for lac repressor in its tetrameric form (15), as compared with 460,000 for a ~700 bp DNA ring. Isoelectric points are 6.3 (16), 9.2 (17) and 5.6 (18), respectively, implying that both EcoR I and lac repressor are negatively charged under conditions of electrophoresis of protein-DNA complexes (see buffers pH below), while CAP is positively charged.

b) DNAs.

665 and 669 bp fragments were purified from Sau3A digests of plasmids pBR322 (19) and pBR fill H, respectively. pBR fill H was obtained from pBR322 by Hind III cleavage, filling-in of 3' recessed ends using the Klenow fragment of *E. coli* DNA polymerase I and the four deoxynucleoside triphosphates according to standard procedures, circularization using T4 DNA ligase, followed by transformation of *E. coli* cells. Both fragments contained a single EcoR I site, whose position relative to Sau 3A and insertion Hind III sites is shown in fig. 6 (bottom).

The 646 bp fragment originates from an Alu I digest of plasmid p256-II 4. This plasmid was constructed by inserting a tandem repeat of a 5S RNA gene-containing 256 bp fragment obtained from a partial EcoR I digestion of plasmid pLV405-10 (this plasmid contains 10 tandem repeats of the 256 bp fragment; ref. 20) into the EcoR I site of plasmid pARA1. pARA1 was constructed from pBR322 (21): the EcoR I site at position 4360 (19) was destroyed by filling-in, the Ava I site at position 1424 was duplicated, and a EcoR I site was introduced in between. The 648 bp fragment is identical to the 646 bp fragment except for two additional base pairs (CC:GG) flanking the EcoR I site. The 648 bp fragment was purified from an Alu I digest of plasmid Ra1. This plasmid was obtained by inserting a subfragment of the above 256 bp fragment into the EcoR I site of pARA1. This subfragment was prepared as follows: the 256 bp fragment (with EcoR I ends) was first digested with Nci I to generate a 195 bp EcoR I-Nci I fragment, and the ends of this latter fragment were trimmed with S1. 8 bp long EcoR I linkers were then ligated to these ends, and the mixture digested to completion with EcoR I. As shown by the sequencing of the corresponding region of plasmid Ra1 (22), S1 had removed only the 4 overhanging single stranded nucleotides of the primitive EcoR I end in the 195 bp fragment but no nucleotide in double stranded form, so that linker ligation resulted in a partial

duplication of the EcoR I site and in the 2 bp addition reported above. Both 646 and 648 bp fragments contain a single EcoR I site.

The 731 bp fragment was purified from a Sau 3A digest of plasmid pBB3. This plasmid was obtained from plasmid pBB2 by the insertion at the BstE II site of a 53 bp fragment containing the CAP binding sequence. pBB2 is a derivative of pBR322 which contains a 11 bp BstE II linker in the filled-in Cla I site. The 53 bp insert was obtained from an uneven-ended Hha I-BstN I DNA fragment consisting of strands of 41 and 44 nucleotides (8) by filling-in of the 3'-recessed BstN I end using the Klenow fragment of *E. coli* DNA polymerase I and trimming of the 3' protruding Hha I end with T4 DNA polymerase, ligation of 11 bp BstE II linkers, followed by digestion with BstE II. Because Cla I and EcoR I sites are close to each other in pBR322 (19), the 731 bp fragment actually contains the above 665 bp fragment.

The 722 bp fragment originates from a FnuD II digest of plasmid pKK233-2 (a prokaryotic expression vector from Pharmacia) and contains a single natural lac operator and no pseudo-operators.

c) Topoisomers.

Topoisomer preparation procedures have previously been described (23). Briefly, fragments were dephosphorylated with alkaline phosphatase, labeled with [γ- ^{32}P] ATP using T4 polynucleotide kinase, and circularized at low DNA concentration (about 0.3 µg/ml) with T4 DNA ligase in the presence of netropsin (positively supercoiled topoisomers) or ethidium bromide (negatively supercoiled topoisomers). In the cases of flush-ended 646, 648 and 722 bp fragments, however, the circularization was poor. In contrast, their polymerization, as performed by ligation at higher DNA concentrations (10 µg/ml), was satisfactory. Ligation products were subsequently digested with EcoR I. The (unique) site of this secondary cleavage was asymmetrically located in the original fragments so that only tandem repeats gave back fragments of starting sizes, while non-tandem repeats generated fragments of both smaller and larger sizes. Fragments were then purified, labeled and circularized as indicated above. Topoisomers were individually purified by preparative electrophoreses in 4 % polyacrylamide (acrylamide to bisacrylamide = 20:1, w/w) gels in the presence or absence of ethidium bromide as reported (23).

d) DNA-protein complexes.

EcoR I complexes were formed in the absence of Mg^{2+} (3) by incubation of the individually purified, appropriately mixed, labeled topoisomers (in trace amounts) with various concentrations of the protein in 10 mM Tris-HCl, pH 7.5, 80 mM NaCl and 5 mM 2-mercaptoethanol, together with 70 ng/ml of the corresponding unlabeled, linear, fragment. 10 µg/ml of a 1374 bp fragment originating from a Sau3A digest of pBR322 (19), and containing no EcoR I site, was added as a carrier, as well as 5 µg/ml of negatively supercoiled topoisomers (average superhelical density ≈ -0.05) of the same fragment prepared by circularization with T4 DNA ligase in the presence of ethidium bromide. [This latter DNA was found to be necessary in order to get sharp bands in the gels, possibly because it traps proteins which are known to contaminate the present enzyme preparations and which may bind aspecifically to supercoiled DNA.] Lac repressor complexes were made under conditions similar to those used above for the EcoR I complexes except for the following: NaCl concentration was reduced to 50 mM; the concentration of the unlabeled,

linear, fragment was 250 ng (0.52 pmole) / ml; and concentrations of both linear and negatively supercoiled 1374 bp fragment were 2.5 µg/ml. CAP complexes were generated in the buffer used with lac repressor supplemented with 10 µM cAMP, in the presence of 320 ng (0.67 pmole) / ml of the unlabeled, linear, fragment. No extraneous carrier DNA was used.

Serial dilutions of the three proteins added to the above incubation mixtures were made in 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5, 50 mM KCl, 1 mM dithiothreitol, 200 µg/ml BSA and 50 % glycerol. EcoR I and lac repressor dilutions were found to be stable for at least several weeks at -20 °C, while CAP dilutions had to be made fresh each time. All incubations were at room temperature for 30 min. One and two hr incubations were also performed without apparent modification in the proportions of complexes over naked DNAs (as visualized by gel electrophoresis; see below), suggesting the equilibria were reached with the three proteins in less than 30 min under the present conditions. After incubations were completed, the 10 µl fractions were diluted to 25 µl by addition of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, 10 µM cAMP (for CAP), 2.5 mM 2-mercaptoethanol, 0.1 mg/ml BSA, 20 % sucrose (for EcoR I) or 10 % glycerol (for lac repressor and CAP), and 0.02 % xylene cyanol, and were immediately loaded in the gel.

Control experiments (not shown) were also performed by incubating under the above conditions each of the three proteins with topoisomers lacking the corresponding interaction sites. No specific nor nonspecific complexes appeared to form at the protein concentrations used (see the figure legends), as indicated by the absence of shift or even blurring of the bands in the gels.

e) Gel electrophoreses.

Protein-DNA complexes were electrophoresed using a Pharmacia GE2/4LS apparatus in which the plastic coil had been replaced with a longer glass coil of larger inside diameter so as to regulate the gel temperature within ± 0.1 °C. Two buffers were used: 20 mM sodium acetate, 2 mM EDTA and 40 mM Tris-acetate, pH 7.8 (Tris-acetate buffer) or 10 mM Tris-HCl and 1 mM EDTA, pH 7.5 (TE buffer). In the case of EcoR I, the Tris-acetate buffer led to sharper bands, and only experiments in this buffer are reported. With CAP, in contrast, bands were sharper in the TE buffer, while both buffers gave bands of similar quality with lac repressor. The buffer was recirculated between the two reservoirs. Gels (0.12 cm x 17 cm x 20 cm) were preelectrophoresed for 1 to 2 hrs and electrophoresed at 250 volts (Tris-acetate) or 450 volts (TE) for 7 to 8.5 hrs at 25 and 37 °C, or 12 hrs at 15 °C. Gels were dried before autoradiography.

f) V-like migration of DNA minicircles, and the effect of a thermal unwinding of the double helix.

The electrophoretic V-like migration, which is observed when the ring size and the gel concentration are properly adjusted to each other, has been documented in two recent articles from this laboratory (23, 24). Fig. 1 a shows typical Vs obtained with 665 bp topoisomers electrophoresed in two identical gels (not shown) at 25 and 45 °C, respectively. Mobilities of the topoisomers relative to the linear fragment were plotted as a function of $\Delta\alpha$ [$\Delta\alpha = \alpha - \alpha(0)$], their linking number difference *relative to topoisomer 0* (topoisomer 0 is by convention the least migrating topoisomer in the 25 °C gel). The $\Delta\alpha$ value at the apex of the V, $\Delta\alpha_V$ (ΔLk_V in refs 23 and 24), corresponds to the most probable configuration of the ring. Its

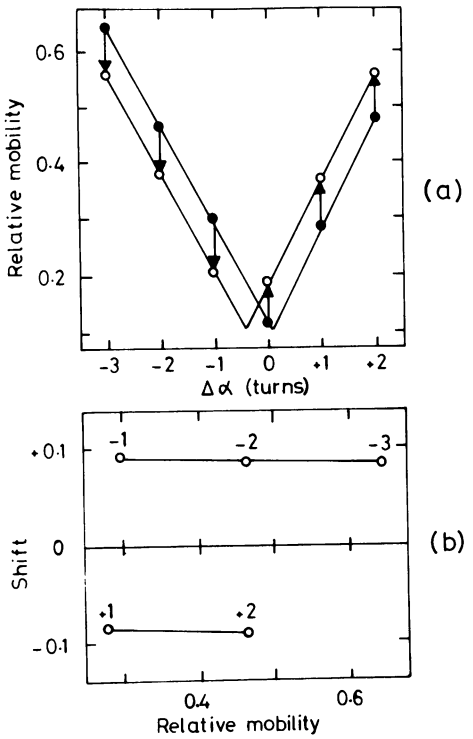

Figure 1 : V-like migration of 665 bp topoisomers, and the effect of a DNA thermal unwinding. a) Ratios between distances migrated from the start by the topoisomers and by the linear fragment in two gels (not shown) identical to that described in legend to fig. 2 and run at 25 (●) and 45 °C (O), respectively, were plotted as functions of linking number differences relative to topoisomer 0, $\Delta\alpha$. Vs were constructed as previously described (23). Values of $\Delta\alpha$ at the apexes, as measured using a least squares procedure, were $\Delta\alpha_V$ = +0.054 and -0.442 turn at 25 and 45 °C, respectively, leading to topoisomer 0 supercoilings τ = -0.054 and +0.442 turn (see eq. 1 in Materials and Methods). Arrows indicate the shifts of the topoisomers upon the temperature increase. The figure was adapted from fig. 2 in ref. 23. b) Shifts measured in (a) (in unit of relative mobility) were plotted as functions of topoisomer (numbers) relative mobilities at 25 °C, and straight lines were drawn through data points of negatively and positively supercoiled topoisomers, respectively. Data for topoisomer 0 was not included (see text).

linking number, $\alpha°$, is then equal to $\alpha(0) + \Delta\alpha_V$. Topoisomer linking number differences *relative to the most probable configuration*, τ (25), are in turn given by the equation:

$$\tau = \alpha - \alpha° = \Delta\alpha - \Delta\alpha_V \qquad (1)$$

τ is also termed the constraint (26), the number of titratable superhelical turns (27), or the supercoiling . $\Delta\alpha_V$ at 25 °C, as measured in fig. 1, is equal to +0.054 turn (see the figure legend). Topoisomer 0 is therefore slightly negatively supercoiled (τ = -0.054 turn), this negative supercoiling increasing for

topoisomers -1 to -3; topoisomers +1 and +2, in contrast, are positively supercoiled ($\tau > 0$). Upon increasing the gel temperature to 45 °C, the double helix unwinds by U ($U > 0$) and τ, as shown in eq. (1), increases by $\Delta\tau = -\Delta\alpha°$ ($\Delta\alpha° < 0$). As a consequence, and as shown in fig. 1 a, positively supercoiled topoisomers are accelerated and negatively supercoiled topoisomers (except topoisomer 0) are retarded to approximately the same extent (arrows). Topoisomer 0 indeed shows an acceleration, however smaller than that of topoisomers +1 and +2, due to a change in its supercoiling polarity from negative to positive. This results in a displacement of the whole V parallel to the $\Delta\alpha$ axis toward negative $\Delta\alpha$ values. The new $\Delta\alpha_V$ value is -0.442 turn (see the figure legend), which gives $\Delta\alpha° = -0.442 - 0.054 = -0.496$ turn = -U. [From this, a rate of thermal unwinding, U x 360/665/20 = 0.013 °/°C/bp, could be derived, which is identical to the rates obtained from other methods (28, 29).]

When U is small, the apex displacement becomes difficult to measure. An alternative procedure may then be used which exploits the shifts, S, of the topoisomers (except topoisomer 0; see above). S is taken as positive for a retardation and negative for an acceleration, and is described by the equations

$$S^- = +S_U \qquad (2)$$

$$S^+ = -S_U \qquad (3)$$

in which S_U ($S_U > 0$) is the unwinding (and here the only) contribution to S, and the - and + superscripts refer to negatively and positively supercoiled topoisomers, respectively. S^- and S^+ were plotted in fig. 1 b as functions of topoisomer relative mobilities at the lower temperature. As expected, the lines joining the data points for negatively and positively supercoiled topoisomers, respectively, are approximately horizontal and symmetrically located relative to the abscissa. Their distance

$$S^- - S^+ = 2S_U \qquad (4)$$

then gives $S_U = 0.09$. Knowing that the average difference in the relative mobilities of adjacent topoisomers ($\Delta\alpha = 1$ turn) is equal to 0.18, S_U can subsequently be converted into U = -0.5 turn.

Results and Discussion

a) Binding of EcoR I.

Fig. 2 shows the pattern obtained when topoisomers of two 665 and 669 bp fragments, which have identical sequences except for their 4 bp difference in length (see Materials and Methods), and which contain a single EcoR I site, are electrophoresed after incubation in the absence of Mg^{2+} with increasing amounts of the enzyme (lanes 0_-, 1_- and 2_- contain a mixture of topoisomers 0, -1, -2 and -3; and lanes 0_+, 1_+, 2_+ and 3_+, a mixture of topoisomers 0, +1 and +2). Most topoisomers appear to be shifted upward upon binding of the protein, although to variable extents. Positively supercoiled topoisomers (+1 and +2) are only slightly shifted, while the shift is larger for negatively supercoiled topoisomers (-1, -2 and -3), and actually increases with their supercoiling. Two topoisomers, 665 bp topoisomer 0 and 669 bp topoisomer +1, appear not to be affected.

These shifts were plotted in fig. 3 a as functions of naked topoisomer mobilities. Neglecting the intrinsic influence of the 4 bp (0.6 %) difference in length between the two fragments on these shifts, data for both 665 and 669 bp topoisomers can be superimposed in the same curve. As shown in the figure,

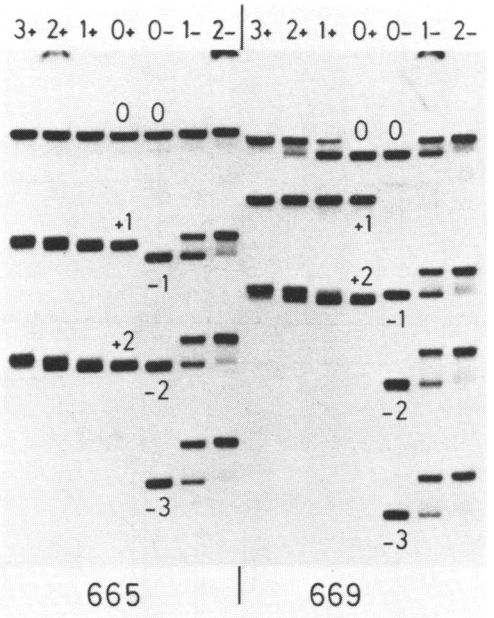

Figure 2 : Binding of EcoR I to 665 and 669 bp topoisomers. Fragments originate from Sau 3A digests of plasmids pBR322 (19) and pBR fill H (see Materials and Methods), respectively, are of identical sequences except for their 4 bp difference in length, and contain a single EcoR I binding site. Topoisomers (numbers) were prepared, purified, appropriately mixed, incubated with 0 (lanes 0_- and 0_+), 75 (1_-), 200 (2_-), 50 (1_+), 150 (2_+) and 300 (3_+) U EcoR I/ml, diluted and electrophoresed in a 4 % polyacrylamide (acrylamide/bisacrylamide = 30/1; w/w) gel in Tris-acetate buffer at 25 °C as described in Materials and Methods. The radioactivity at the top of the gel was added after the electrophoresis was completed and before drying to show the start. An autoradiogram is shown.

data points for topoisomers of supercoiling of equal polarity are generally aligned, although their scattering is larger in the case of positively supercoiled topoisomers which encompass a more reduced domain of mobilities (but see corresponding data at 37 °C in fig. 3 b). The two straight dotted lines so obtained are no longer horizontal as in fig. 1, but have positive slopes. This suggests that a positive retardation term

$$S_R = a \,.\, Mob. + b \qquad (5)$$

adds up to S_U, the thermal unwinding in eqs (2) and (3) (see Materials and Methods) and now the protein unwinding contribution to the shift, to give the total observed shifts, S. In this equation, Mob. is the topoisomer mobility, and a and b are constants; constant a is positive. One has

$$S^- = S_R^- + S_U \qquad (6)$$

$$S^+ = S_R^+ - S_U \qquad (7)$$

in which the - and + superscripts refer to negatively and positively supercoiled topoisomers, respectively. As eq. (2), eq. (6) applies only to topoisomers whose supercoiling is sufficiently negative not to become positive after the unwinding, justifying the exclusion of 665 bp topoisomer 0 from fig. 3 a (see the figure

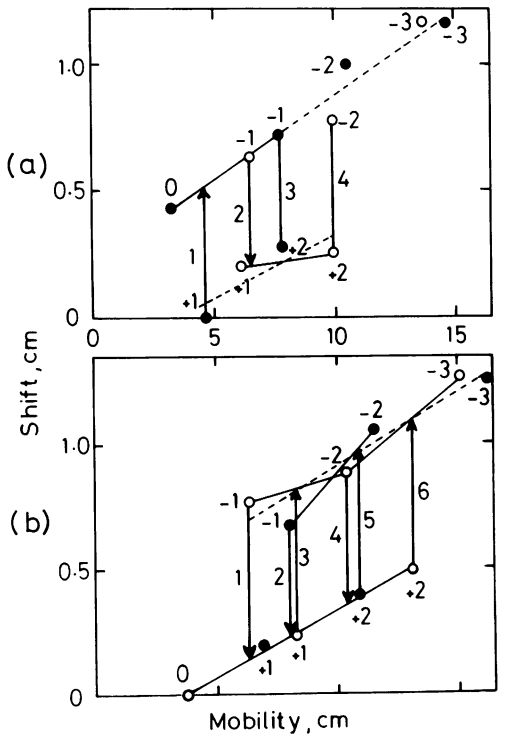

Figure 3 : Shift-vs-mobility curves of EcoR I complexes with 665 (O) and 669 bp (●) topoisomers. a) Mobility differences between naked topoisomers (numbers) and their complexes were measured in the gel shown in fig. 2 and were plotted as functions of mobilities of naked DNAs. 665 bp topoisomer 0 was not taken into account because its negative supercoiling measured from the V (not shown) (τ = -0.05 turn; see eq. 1 in Materials and Methods) is too small (see Results), in contrast to 669 bp topoisomer 0 (τ = -0.32 turn). Vertical bars indicate couples of homologous negatively and positively supercoiled, comigrating, topoisomers from which the unwinding was calculated (see Results). When no comigrating topoisomer exists, a virtual topoisomer (arrow) was used whose mobility was determined by linear interpolation (solid lines) between the two homologous proximal topoisomers. Unwinding values were 25.6 ° (arrow number 1), 20.7 ° (2), 20.7 ° (3) and 25.6 ° (4). Dotted lines were drawn through data points of negatively and positively supercoiled topoisomers, respectively, using a least squares procedure. b) Same as in (a), except that data were derived from a gel run at 37 °C (not shown), otherwise identical to that displayed in fig. 2. Data from 669 bp topoisomer 0 (τ = -0.03 turn) was now ignored while that from 665 bp topoisomer 0 (τ = + 0.2 turn) was taken into account. U values were 25.1 ° (arrow number 1), 17.3 ° (2), 23.9 ° (3), 20.6 ° (4), 23.9 ° (5) and 24.7 ° (6).

legend). [Because its supercoiling polarity may be uncertain, topoisomer 0 will as a rule not be taken into account when located in the vicinity of the apex of the V; see below.]

The approximate parallelism observed between the dotted lines in fig. 3 a then requires constant a in eq. (5) not to depend significantly on supercoiling polarity. Moreover, the observation that these lines

extrapolate to approximately equal and opposite shift values at zero mobility (not shown) suggests that constant b is close to zero. It follows that S_R^- may be approximately equal to S_R^+ in eqs (6) and (7).

The reason for this may be found in the origin of S_R. S_R is in principle the sum of two components i) A charge modification term, which results in an acceleration or a retardation of the complexes depending on the protein net charge, negative or positive, respectively (30); EcoR I being negatively charged (see Materials and Methods), this contribution is an acceleration; and ii) A frictional term, which leads to a retardation. The observation that S_R corresponds to a retardation indicates that the electrostatic term is small relative to the frictional term, and therefore that the increase in S_R with mobility (see eq. 5) reflects that of the frictional term. Such dependence of the frictional term on mobility, *i. e.* on supercoiling, was indeed expected because supercoiling compacts the topoisomers, while leaving the conformation of the bound protein presumably unaffected.

If the electrostatic term obviously does not depend on supercoiling polarity, the frictional term may *a priori* depend on it through a differential orientation of the protein relative to the DNA. With a protein bound on one side of the double helix, the retardation may be different depending upon whether the protein is facing inside or outside. Such orientation should in turn vary with the topoisomer and the gel temperature. The reason for the apparent failure of S_R to significantly depend on supercoiling polarity may therefore be that this orientation is fixed, or that its effect on the mobility of the complexes is small.

Eqs (6) and (7) may then be subtracted from each other to give

$$S^- - S^+ = 2S_U$$

which is eq. (4) in Materials and Methods. This equation, when applied to the two couples of comigrating 665 bp topoisomers -2 and +2, and 669 bp topoisomers -1 and +2 (vertical bars numbers 4 and 3 in fig. 3 a), leads to S_U = 0.26 and 0.21 cm, respectively. With an average distance of 3.66 cm between adjacent topoisomers measured on the two Vs (not shown), one obtains an unwinding U = 0.26/3.66 = 0.071 turn and 0.21/3.66 = 0.057 turn, i. e. 25.6 and 20.7 °. In fact, eq. (4) can also be applied to the virtual negatively supercoiled 669 bp topoisomer comigrating with 669 bp topoisomer +1. The mobility of this virtual topoisomer can be estimated by linear interpolation between the shifts of 669 bp topoisomers 0 and -1 (see arrow number 1 in fig. 3 a; this interpolation is figured by the solid line joining the corresponding data points). One obtains U = 25.6 °. U = 20.7 ° can similarly be derived from 665 bp topoisomer -1 and a linear interpolation between 665 bp topoisomers +1 and +2 (arrow number 2 in fig. 3a). One finally obtains U = 23.2 ± 3 ° (mean ± standard deviation for 4 measurements).

EcoR I complexes with the same topoisomers were also electrophoresed at 37 °C (gel not shown). The corresponding shift-vs-mobility plot is presented in fig. 3 b. Data points for positively supercoiled topoisomers are now well aligned, while those for negatively supercoiled topoisomers appear to be more scattered than in fig. 3 a. Six values of U were derived (arrows in fig. 3 b). They were comprised between 17.3 and 25.1 ° (see legend to fig. 3 b), had a mean of 22.6 ° and a standard deviation of 3 °, very similar to the values derived above at 25°C.

A possible dependence of the unwinding on the sequence environment of the recognition sequence was also tested by using topoisomers of two more fragments, 646 and 648 bp in length, the longer being derived from the shorter by a 2 bp addition in the vicinity of the EcoR I site (see Materials and Methods). A unique value of U = 21.1 ° was derived at 25 °C, while two identical values of 22.6 ° were calculated at 37 °C, in close agreement with the figures measured above from 665 and 669 bp fragments. Four estimates of U (22.0 and 26.2 ° at 25 °C, and 18.6 and 21.9 ° at 37 °C) were also obtained from a 731 bp fragment which is used below with CAP and which contains the 665 bp fragment (see Materials and Methods).

Taking all results into account, one gets U = 22.5 ± 2.5 ° (mean ± standard deviation for 17 measurements). This value is actually similar to that found by the relaxation method (see Introduction) (25 ± 5 °; ref. 3), and from the X-ray structure of co-crystalline complexes of the enzyme with oligonucleotides containing its recognition sequence (25 °; ref. 31).

b) Binding of CAP.

Fig. 4 a shows the results obtained upon gel electrophoresis at 25 °C of 731 bp DNA minicircles carrying a single binding site for CAP after incubation with increasing amounts of the protein. In contrast to EcoR I, CAP appears to cause a large downward shift to topoisomer 0 (lanes 1, 2, 3 and 4). This shift decreases upon increasing the supercoiling, to virtually cancel for topoisomers -1 and +2, and reverses its direction for topoisomers -2 and -3, which are retarded.

Such downward shift cannot be due to a charge effect since the protein is positively charged (see Materials and Methods), which should result in a retardation (see above). This is rather likely to be the consequence of the large bending inflicted on the DNA by CAP (33, 34). Bending is indeed expected to lead to a condensation of the circular DNA molecule and therefore to an increase in its mobility, this effect being predominant for topoisomer 0 because it has the most expanded configuration in the first place. It is noteworthy that such acceleration is not in contradiction with the retardation shown by a bent linear DNA fragment (34, 35). This is clear if one remembers that retardation caused by bending is always much smaller than that observed when the fragment is circularized (without supercoiling). Upon supercoiling, bending should become less efficient to increase the mobility since the starting molecule is already more condensed. In contrast, S_R is expected to increase with supercoiling (see above), and, together with S_U, may compensate (topoisomers -1 and +2) or overcompensate (topoisomers -2 and -3) this acceleration.

Shifts were plotted as functions of mobilities in fig. 4 b. Figure 4 c displays similar results obtained upon increasing the gel temperature to 37 °C (gel not shown). In both cases, topoisomer -3 deviates from the alignment of the other negatively supercoiled topoisomers, and for this reason will be ignored. As expected, the plots show, when compared to those obtained with EcoR I (fig. 3), a large displacement toward negative values of the ordinate. This displacement can be taken into account by adding a negative acceleration term, S_A, in above equations (5) and (6), to give

$$S^- = S_R^- + S_A^- + S_U \qquad (8)$$

$$S^+ = S_R^+ + S_A^+ - S_U \qquad (9)$$

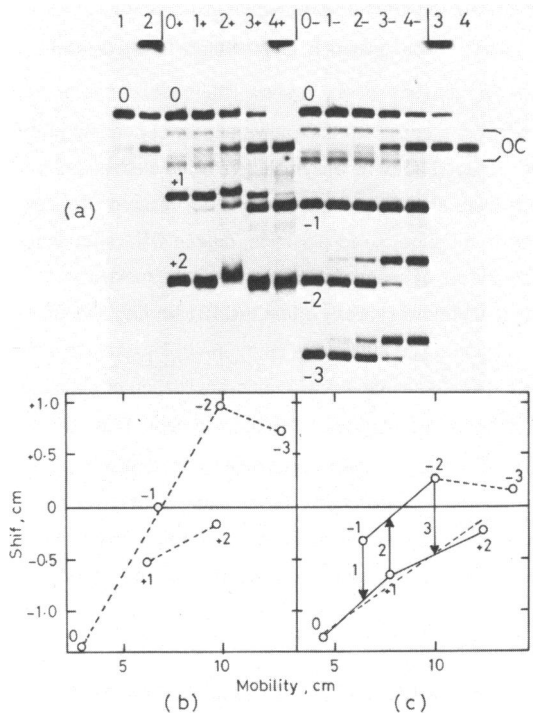

Figure 4 : Binding of CAP to 731 bp topoisomers. The fragment was purified from a Sau3A digest of plasmid pBB3, and contains a single binding site for CAP (see Materials and Methods). a) Complexes were formed as described in Materials and Methods with 0 (lanes 0_- and 0_+), 2 (1_-), 4 (1, 1_+, 2_-), 6 (2, 3_-, 2_+), 12 (3, 4_-, 3_+) and 24 (4, 4_+) pmoles CAP / ml. These concentrations correspond to molar ratios of CAP over the unlabeled linear 731 bp fragment of 0, 3, 6, 9, 18 and 36, respectively. Electrophoresis was performed in a 4 % polyacrylamide (acrylamide/bisacrylamide = 30/1; w/w) gel at 25 °C in TE buffer (see Materials and Methods) supplemented with 10 µM cAMP. An autoradiogram is shown. OC: open circular DNA. The significant amount of open circular DNA observed in starting topoisomers (lanes 0_- and 0_+) results from radioactive decay. The generation of two main bands by this process, a feature of unknown origin, has previously been noted (32). b) Shifts and mobilities were measured in (a). By convention, the shift is negative when complexes migrate faster than naked DNAs (see Materials and Methods). The data point for topoisomer 0 is shown, although its negative supercoiling (τ = -0.05 turn) is small. Its large downward shift is indeed expected to result mostly from bending rather than unwinding (see text). c) Same as (b) except that data were taken from a gel run at 37 °C (not shown), otherwise identical to that displayed in (a). Topoisomer 0 had here a supercoiling of about +0.2 turn. U values were 27 ° (arrow number 1), 26 ° (2) and 33 ° (3).

If S_R is again a linear function of mobility (see eq.5), then S_A should also vary linearly with supercoiling (up to topoisomer -2 for "negative" topoisomers) since the combination of the two terms does. This leads to $S_A = a \cdot Mob. + b$, with constant b being negative. It would then suffice, for the shift-vs-mobility segments to be nearly parallel as observed in fig. 4 c (but not in fig. 4 b; see below), that both constants a in S_A and

S_R (see eq. 5) be only weakly dependent upon supercoiling polarity. This formal reasoning however cannot tell whether this is also true for constant b, and therefore for S_A as a whole.

In fact, there are specific reasons for S_A to show little dependence on supercoiling polarity. These reasons become clear when one considers the known effects of bending on the orientation of a circular DNA molecule. First, as deduced from the enzymatic preferred cleavage of one side of the double helix, sequence-directed bends were shown to rotationaly orient a relaxed DNA minicircle (36). Second, in a complementary observation on supercoiled plasmids, curved DNA sequences were found by electron microscopy to occupy the apexes of supertwisted domains in the molecules (37). The large bending of the DNA induced by CAP may therefore similarly orient the 731 bp ring, whether relaxed or supercoiled. In other words, the protein around which the DNA is bent (38-40) should face inside in the relaxed complex. The bend should then be distorted symmetrically upon application of opposite and equal supercoilings, leading to similar S_A values for the corresponding topoisomers. This prediction is in keeping with the observation that a bent DNA sequence inserted in a plasmid increased the absolute values of the writhes of negatively and positively supercoiled DNAs by similar amounts (41). A computer simulation of the effect of a 90° wedge also showed the same increase in the absolute writhing of 600 bp DNA minicircles, regardless of the supercoiling polarity; moreover, the effect of this bend was found to decrease with increasing supercoiling, as suggested above (M. Le Bret; unpublished).

S_U may then be again obtained from the approximately parallel shift-vs-mobility segments in fig. 4 c through the difference between eqs (8) and (9). This leads to three estimates of U, 27, 26 and 33 °, of mean 29 ° (arrows in figure 4 c). This value is actually consistent with that derived using the relaxation method (0.1 ± 0.06 turn; ref. 4).

As shown by the lack of parallelism between segments of the shift-vs-mobility plots in fig. 4 b, the orientation and the distorsion of the bend may not always be symmetrical. If the bend is not in a single geometrical plane and makes, for example, an arc of a superhelix, its distorsion may indeed be facilitated in one direction relative to the other. This however does not suffice to explain the temperature effect, which may rather be due to sequence-directed bends (see below) competing somewhat with the CAP-induced bend in orienting the molecule. A slight shift in the balance between the two kinds of bends may then result from a change in their relative orientation as a consequence of the thermal alteration in the twist of the double helix.

c) Binding of lac repressor.

Fig. 5 a displays the electrophoretic migration at 15 °C of 722 bp topoisomers after their incubation with increasing amounts of repressor. The DNA carries a single operator (see Materials and Methods). Interestingly, topoisomers +1 and -1 show shifts which are smaller than those of neighboring topoisomers 0, +2 and -2. As a result, the corresponding shift-vs-mobility plot (fig. 5 b) exhibits a breakage in the "positive" curve at the position of topoisomer +1. Moreover, the segments joining data points of topoisomers +1 and +2, and -1 and -2, respectively, are parallel and both extrapolate to negative values of the shift at zero mobility, as previously observed with CAP (fig. 4). By analogy with the case of CAP, this

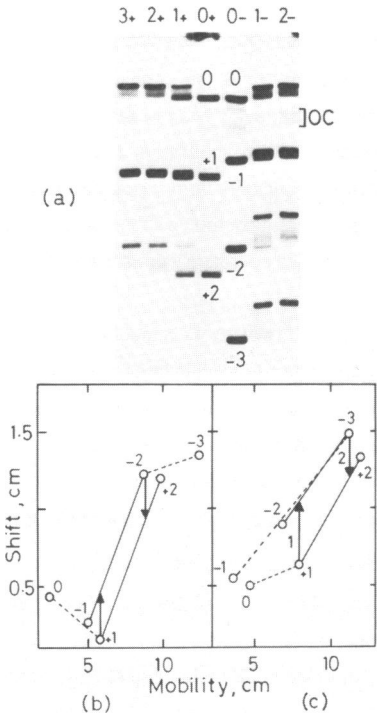

Figure 5 : Binding of lac repressor to 722 bp topoisomers. The fragment originates from a FnuD II digest of plasmid pKK 233-2 and contains a single natural lac operator (see Materials and Methods). a) Complexes were formed as described in Materials and Methods using 0 (lanes 0_-, 0_+), 1.8 (1_-, 1_+), 5 (2_-, 2_+) and 15 (3_+) pmoles repressor / ml, corresponding to molar ratios over the linear fragment of 0, 3.5, 9.6, and 29, respectively. Electrophoresis was performed in a 4 % polyacrylamide (acrylamide / bisacrylamide = 30/1; w/w) gel at 15 °C in Tris-acetate buffer. An autoradiogram is shown. OC: open circular DNA. b) Data were taken from the gel shown in (a). Supercoiling of topoisomer 0 was τ = +0.15 turn. Values of U were both equal to 16 ° (arrows). c) Data from a gel run at 37 °C, otherwise identical to that displayed in (a). Supercoiling of topoisomer 0 was τ = +0.6 turn. Unwinding estimates were 20 ° (arrow number 1) and 13 ° (2).

suggests that complexes, starting with topoisomers +1 and -1, also undergo an acceleration. This acceleration is however different from that of CAP complexes which was maximal for topoisomer 0 (fig. 4), consistent with the inability of lac repressor to bend the DNA (ref. 34; see also below).

Upon electrophoresis of the same complexes at 37 °C (not shown), shifts appear to normalize to a large extent: the breakage in the "positive" curve is reduced (fig. 5 c), and data points of topoisomers -1, -2 and -3 are now close to a straight line which extrapolates to a positive value of the shift at zero mobility, a feature already shown by the EcoR I shift-vs-mobility plots (fig. 3). Inasmuch as such "standard" behavior justifies the measurement of U, two values, 20 and 13 °, were derived, of mean equal to 16 ° (arrows in fig. 5 c).

Two identical values of U equal to 16 ° were also tentatively measured from the parallel segments in fig. 5 b (arrows). The identity of these values with the mean value just obtained clearly raises the possibility that the acceleration shown by complexes at 15 °C (see above) might not depend much on supercoiling polarity. In fact, such electrophoretic behavior closely resembles that observed with lac repressor complexes on DNA minicircles carrying two operators (42). There, the acceleration was directly demonstrated by electron microscopy to originate from a loop occurring through the interaction of the two operators with a single repressor tetramer. In contrast with the present results, however, this looping could also form in topoisomer 0, providing the two operators were separated by an integral number of turns of the double helix (42).

This therefore suggests that loops form at 15 °C in topoisomers +1 and -1 between the operator and other weaker binding sites which are known to exist in large number in any DNA sequence (43), and that the role of supercoiling is mainly to decrease the unfavorable energy of the process. In this respect, negative and positive supercoilings should indeed behave the same. Looping is likely to occur in topoisomers +2, -2 and -3 as well. These topoisomers are however more condensed than topoisomers +1 and -1 in the first place, so that, as reasoned above in the case of bending, the loop acceleration effect, S_A, should be smaller. S_R, in contrast, is expected to increase rapidly with supercoiling in the case of this massive protein (see Materials and Methods), explaining the large overall retardation observed for the corresponding complexes (fig. 5 a).

Electrophoreses were also performed in TE buffer (see Materials and Methods) at 25 and 37 °C. Four values of the unwinding were measured, between 10 and 18 °, of mean 13 °. Interestingly, a breakage in the "positive" curve at the position of topoisomer +1 was also observed at 25 °C, but not at 37 °C (not shown).

The mean figures obtained, U = 13 and 16 °, are significantly smaller than the 40 °, and 54 ± 4 °, values respectively obtained using the supercoiling-dependence-of-affinity procedure (see Introduction) (ref. 7; as corrected in ref. 25) and the relaxation method (2). In fact, there are now compelling reasons to believe that these latter values were overestimates. The plasmids used by these authors indeed contained more than one operator, making the occurrence of loops very likely. Loops were indeed found to increase the affinity of lac repressor to supercoiled DNA (44-46) and to constrain superhelical turns which could no longer be relaxed with the topoisomerase (42). Moreover, the present figures do appear more consistent with the observation that, upon topoisomerase relaxation of complexes of lac repressor with DNA minicircles containing a single operator, no difference could be found in the resulting topoisomer distribution relative to the relaxation of the naked DNA (42).

d) Sequence-directed and protein-induced bends.

Because DNA in the co-crystals of EcoR I with oligonucleotides containing its recognition sequence is kinked (12, 31), it was interesting to investigate whether the protein would bend the DNA in solution. Fig. 6 (top) displays the gel migration of circularly permuted linear 665 bp fragments after incubation with increasing concentrations of EcoR I. The figure shows significant variations in the mobility of both complexes and naked fragments. The Dde I fragment appears to be the fastest one, followed by the

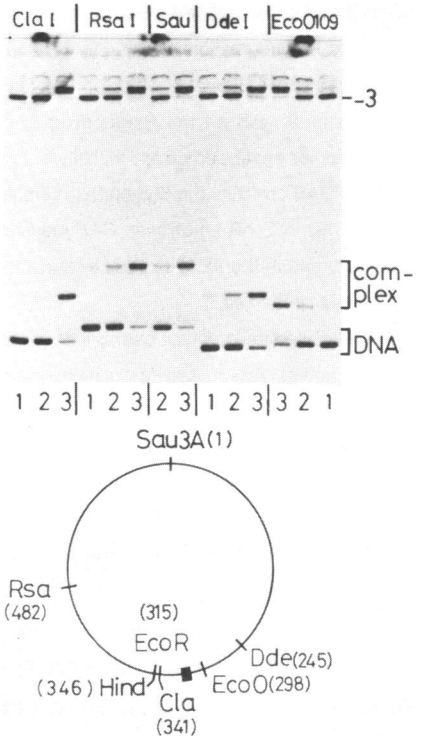

Figure 6 : Sequence-directed and EcoR I-induced bends in the 665 bp fragment. Complexes were formed between the protein and circularly permuted linear 665 bp fragments (obtained by digestion of topoisomer -3 with the indicated endonucleases), as described in Materials and Methods for complexes with supercoiled DNAs. The uncleaved topoisomer was added in the incubation mixtures as a control. EcoR I concentrations were 32 (lane 1), 80 (2) and 200 (3) U/ml. Electrophoresis was in a 6 % polyacrylamide (acrylamide / bisacrylamide = 30/1; w/w) gel in Tris-acetate buffer at 25 °C. An autoradiogram is displayed, together with the restriction map of the circularized fragment. The map also shows the positions of the EcoR I binding site (EcoR) and of the Hind III site (Hind) where the 4 bp were inserted to give the 669 bp fragment.

EcoO109 fragment. This suggests the presence of a sequence-directed bend in between the Dde I and EcoO109 sites, closer to the Dde I site (see restriction map in fig. 6, bottom). The mobility of the Cla I fragment is also large, suggesting that a bend is also present in between the Cla I and EcoO109 sites, consistent with the known curvature of the EcoR I recognition sequence (47). Most complexes follow the same variation of mobility as naked fragments, except complexes on EcoO109 and Dde I fragments whose variation is reversed. Maximal mobilities are now reached for complexes on EcoO109 and Cla I fragments, in keeping with the location of the EcoR I site in between these two sites (fig. 6, bottom).

It may therefore be concluded that EcoR I induces a significant bending also in solution, which possibly reinforces the preexisting one. This bend does not necessarily generate a compaction of the

circular molecule. This will occur only for a proper orientation of the bend. Another orientation may be neutral or even lead to an expansion. This orientation will actually depend on the balance between the two kinds of bends, sequence-directed and EcoR I-induced, which appear to be of similar magnitude. The lack of dependence of U on gel temperature and on the fragment used (see above), however, strongly suggests the effect of such compaction or expansion on the mobility of EcoR I complexes is negligible.

The 731 bp fragment used with CAP contains the 665 bp fragment (see Materials and Methods), and is therefore likely to be bent. In contrast to EcoR I, however, CAP induces a much larger bend than those directed by the fragment sequences, and is therefore expected to essentially impose its own orientation and to always compact the molecule, as observed.

An experiment similar to that displayed in fig. 6 was performed with repressor complexes on circularly permuted 722 bp fragments (not shown). Again, a differential migration of the naked fragments was observed, but, in contrast to the case of EcoR I, migration of the complexes closely parallels that of the naked DNAs, confirming the inability of lac repressor to make a bend (34).

Conclusion

An electrophoretic method for the measurement of the DNA local unwinding, U, induced by a protein has been described. The procedure involves the determination of the shifts in mobility shown by DNA minicircles upon protein binding. Under the conditions used, the migration of the naked topoisomers was V-like, and the contribution of the unwinding to the shifts, S_U, was assumed to be as observed for a thermal unwinding. Thermal unwinding was found to retard all negatively supercoiled topoisomers and accelerate all positively supercoiled topoisomers to the same extent, leading to shift-vs-mobility plots showing two parallel and horizontal straight lines (fig. 1). U could then be obtained from the difference in their ordinates (see Materials and Methods).

The proteins examined here were found to disturb this simple pattern in different ways. However, the shift-vs-mobility curves, or at least segments of them, usually stayed parallel to each other. The aim of the preceding sections was to understand the basis for the specific effect of each protein and from that, to show that the above simple calculation of U remained essentially valid.

We feel confident that the present method may lead to fairly accurate estimates of the unwinding, as long as shift-vs-mobility plots show parallel segments. At the same time the method offers the unique advantage, through direct visualization of the complexes, of demonstrating other specific actions of the protein on the DNA, such as bending and looping. The method should therefore be a useful complement to the physico-chemical techniques already available for the study of DNA-protein interactions.

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