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DNA gyrase can supercoil DNA circles as small as 174 base pairs

Andrew D.Bates and Anthony Maxwell

Department of Biochemistry, University of Leicester, University Road, Leicester, LE1 7RH, UK

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DNA gyrase introduces negative supercoils into closed-circular DNA using the free energy of ATP hydrolysis. Consideration of steric and thermodynamic aspects of the supercoiling reaction indicates that there should be a lower limit to the size of DNA circle which can be supercoiled by gyrase. We have investigated the supercoiling reaction of circles from 116–427 base pairs (bp) in size and have determined that gyrase can supercoil certain relaxed isomers of circles as small as 174 bp, dependent on the final superhelix density of the supercoiled product. Furthermore, this limiting superhelical density (−0.11) is the same as that determined for the supercoiling of plasmid pBR322. We also find that although circles in the range 116–152 bp cannot be supercoiled, they can nevertheless be relaxed by gyrase when positively supercoiled. These data suggest that the conformational changes associated with the supercoiling reaction can be carried out by gyrase in a circle as small as 116 bp. We discuss these results with respect to the thermodynamics of DNA supercoiling and steric aspects of the gyrase mechanism.

Key words: DNA gyrase/supercoiling/*lox*P–cre/DNA small circles

Introduction

DNA topoisomerases are enzymes which can catalyse changes in the linking number of DNA (Gellert, 1981; Wang, 1985). All topoisomerases can relax negatively supercoiled DNA, but only bacterial DNA gyrase can introduce negative supercoils into a DNA substrate, in a reaction coupled to the hydrolysis of ATP. *Escherichia coli* DNA gyrase is composed of two distinct subunits, A and B, of mol. wt 97 000 and 90 000 respectively (Swanberg and Wang, 1987; Yamagishi *et al.*, 1986); the active enzyme comprises an A_2B_2 tetramer. In addition to the DNA supercoiling reaction, gyrase can also catalyse the relaxation of both positively and negatively supercoiled DNA (the former only in the presence of nucleotide), the unknotting of knotted DNA and the catenation and decatenation of duplex circles (Maxwell and Gellert, 1986).

Although the details of the DNA supercoiling reaction mechanism are incompletely understood, the principal steps have been identified (see Maxwell and Gellert, 1986 for review). The enzyme binds to DNA and a segment of ~120 bp is wrapped around the enzyme in a single positive superhelical turn. Cleavage of the DNA occurs in both strands near the centre of the wrapped segment with the formation of transient covalent bonds between the 5′-phosphoryl groups and tyrosines at position 122 of the A subunits (Horowitz and Wang, 1987). Another piece of DNA is then translocated through this double-stranded break, thus reducing the linking number of the DNA by 2. Catalytic supercoiling requires the hydrolysis of ATP.

The DNA substrates used in studies of DNA supercoiling by gyrase are generally circular molecules of several thousand base pairs in length. However, ATPase and binding measurements have shown that gyrase can interact with linear DNA molecules that are much shorter (<200 bp; Maxwell and Gellert, 1984). We have extended these observations by examining the gyrase supercoiling reaction using small circular DNA substrates. It can be reasoned that there must be a lower limiting size for such a substrate below which the gyrase supercoiling reaction will no longer take place. Beneath this threshold, one of two conditions will obtain; the introduction of two negative supercoils into the substrate (i.e. one turnover of the enzyme) would produce a superhelical density (SHD) greater than that thermodynamically achievable by gyrase (a **thermodynamic** limit), or the length of substrate DNA not bound in the gyrase–DNA complex will be insufficient to allow the required translocations to take place (a **steric** limit).

Results

A closed circular DNA relaxed by the action of a topoisomerase, or formed by ligation of a linear or nicked precursor, consists of a Gaussian distribution of topoisomers having individual linking numbers (*Lk* or α) differing by one, about a mean linking number corresponding, to a first approximation, to the twist of the equivalent open circular DNA under the same conditions (Tw^o or α^o; Horowitz and Wang, 1984; Wang, 1986). The equivalence of average linking number and Tw^o assumes that the average writhe of the open circular DNA is zero. This may not be the case if the DNA has a permanent curve or is anisotropically bendable; however in the absence of data pertaining to this issue, we will make this assumption. In the case of a large circle, such as pBR322 (4362 bp), the distribution consists of several topoisomers with relatively similar energies; the most abundant isomer can for most purposes be regarded as being fully relaxed and its linking number defined as Lk^o. In the case of the small circles described here, the distribution consists of only one or two isomers (e.g. Figures 2 and 3). Where two visible isomers occur, they will each be separated from the hypothetically relaxed state (corresponding to Tw^o) by a large energy (Horowitz and Wang, 1984); the definition of Lk^o as above is meaningless, and the isomers may more usefully be regarded as having a positive and negative SHD calculated as $(Lk - Tw^\circ)/Tw^\circ$.

We have prepared small double-stranded DNA circles ranging in size from 116 to 427 bp from *in vitro* constructs utilizing the *lox*P–cre recombination system of bacterio-

phage P1 (Figure 1), in a method developed by Hoess *et al.* (1985). The recombination protein cre acts on plasmid pRH42, which contains two *lox*P sites in direct repeat, to circularize the DNA between the sites, producing a large and a small DNA circle. Deletion of DNA between the sites and recloning yields a family of plasmids with varying *lox*P−*lox*P separation which may be used to prepare microgram quantities of small DNA circles. Relaxed distributions of the small circles were prepared, and the products of their reaction with *E.coli* DNA gyrase were analysed on polyacrylamide gels. Gels were run in the presence of ethidium bromide, which positively writhes the DNA and enhances the resolution of the topoisomers; the isomers of lower linking number migrate closer to the top of the gel. A sample gel illustrating the supercoiling of 174 and 196 bp circles is shown in Figure 2. Where two topoisomers are present in the relaxed distribution, as in Figure 2, each isomer may be considered separately as a substrate for supercoiling by gyrase. In the case of the 174 bp circle, for example, isomer $Lk = 17$ is a substrate whereas $Lk = 16$ is not. The only new product from the reaction is the isomer $Lk = 15$, formed by one turnover of gyrase on $Lk = 17$, a linking number change of -2. The conversion of the $Lk = 18$ isomer of a 196 bp circle to the $Lk = 16$ isomer is also illustrated. Supercoiling reactions have been carried out on several small circles prepared by *lox*P−cre recombination (Table I).

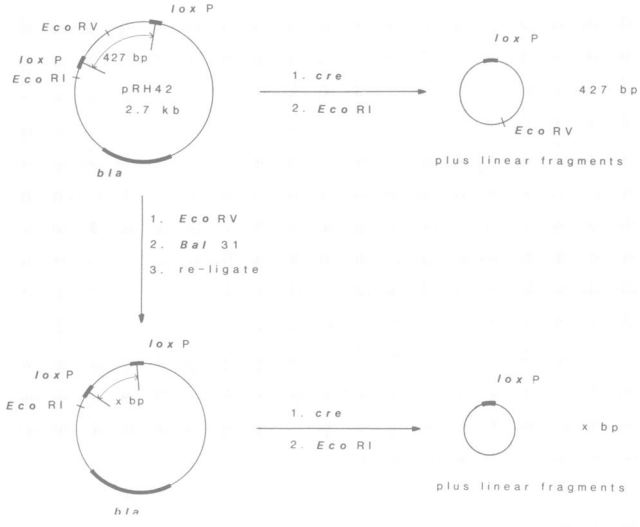

Fig. 1. Preparation of small DNA circles using the *lox*P−cre recombination system.

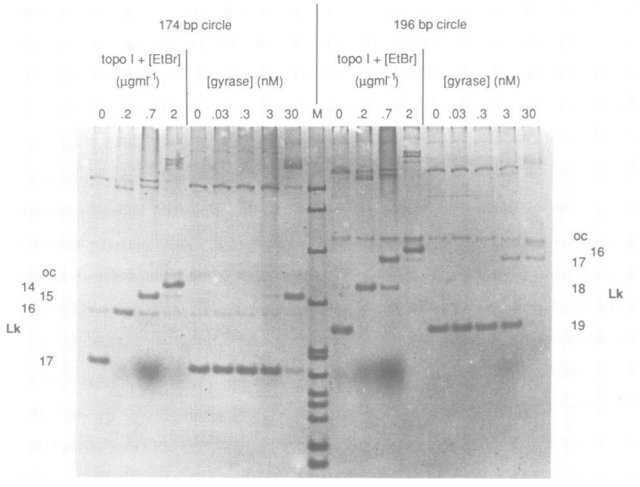

Fig. 2. Supercoiling of 174 and 196 bp circles by DNA gyrase. Negatively supercoiled topoisomers were generated by reaction with topoisomerase I in the presence of the ethidium bromide concentrations indicated (topo I + [EtBr]). Relaxed circles were incubated with DNA gyrase at the concentrations shown. *Lk* indicates the linking numbers of individual topoisomers and track M contains an *Msp*I digest of pBR322. Bands in the upper part of the gel correspond to oligomeric circular species of the same sequences.

In addition to circles prepared by the *lox*P−cre method, we have also investigated a 189 and a 378 bp circle prepared by ligation of a 189 bp linear fragment of an entirely unrelated sequence. Figure 3 shows the gyrase supercoiling reaction of the 189 bp circle; the single relaxed topoisomer of $Lk = 18$ is converted to $Lk = 16$.

Table I gives the SHD of the actual or potential products of a gyrase supercoiling reaction for all the relaxed isomers of the small circles studied. In fact, the final SHD is not necessarily a good measure of the free energy of a particular reaction, since the supercoiling free energy of the substrate should also be taken into account. This free energy is, however, difficult to determine, especially for positively supercoiled substrates (see Discussion), and hence for comparative purposes we have used SHD, a simpler and calculable parameter. The values of Tw^o for the relaxed

Table I. DNA gyrase supercoiling reactions of small circular DNA substrates

Circle size (bp)	Tw^o	*Lk*	SHD of product	Supercoiled ?
116	11.0	11	−0.19	−
128	12.2	12	−0.17	−
142	13.5	13	−0.18	−
		14	−0.11	−
152	14.5	14	−0.17	−
		15	−0.10	−
174	16.6	16	−0.16	−
		17	−0.09	+
189[a]	18.0	18	−0.11	+
196	18.7	18	−0.14	−
		19	−0.09	+
205	19.5	19	−0.13	−
		20	−0.08	+
207[a,b]	19.7	20	−0.09	+
211[a,b]	20.1	20	−0.10	+
217	20.7	20	−0.13	−
		21	−0.08	+
269	25.6	25	−0.10	+
		26	−0.06	+
378[a]	36.0	36	−0.06	+
427	40.7	40	−0.06	+
		41	−0.04	+

[a]DNA prepared by ligation, not by *lox*P−cre reaction.
[b]Data from Horowitz (1986).
Values of Tw^o were calculated as described in the text and the linking numbers of the relaxed topoisomers (*Lk*) assigned accordingly. The superhelical density (SHD) of the potential supercoiled product from one turnover of DNA gyrase was calculated for each topoisomer as $(Lk - Tw^o)/Tw^o$. The final column shows whether a particular isomer was a substrate for the gyrase supercoiling reaction. Results from Horowitz (1986) are included for comparison.

distributions have been calculated as N/h^o, where N is the size of circle in bp and h^o is the helical repeat of the Tw^o state, i.e. the number of bp comprising one complete turn of the helix. The parameter h^o has been taken to be 10.5 bp/turn, the accepted value for mixed sequence B-DNA. This value is a consensus of those determined previously for B-DNA, by DNase I digestion (Rhodes and Klug, 1980), topoisomer band shift (Peck and Wang, 1981) and from the ratios of topoisomers in relaxed distributions of small circles (Shore and Baldwin, 1983; Horowitz and Wang, 1984). Data on the ratio of topoisomers of the 152 bp circle in this study are consistent with this value (not shown). The linking numbers and superhelical densities in Table I were determined on the basis of the Tw^o values calculated as above. We also include in the Table the results of similar experiments on a 207 and a 211 bp circle, carried out by D.S.Horowitz (1986). These results and those of the 189 and 378 bp circles argue against any sequence dependence in the data in Table I.

For DNA circles ≥ 174 bp, supercoiling of a particular topoisomer by gyrase occurs in the cases where the SHD of the product is numerically ≤ -0.11 and does not occur where that SHD is numerically ≥ -0.13 (Table I). It has previously been found that the maximum SHD achievable by DNA gyrase is ~ -0.1 (Gellert *et al.*, 1976). For the purposes of comparison with the data in Table I, we have redetermined this limit for pBR322 under the supercoiling reaction conditions described in this paper. We found that the most supercoiled topoisomers of pBR322 had an SHD of -0.11 (data not shown). This is in good agreement with the value above; in addition, a limit of -0.11 has been recently determined by Westerhoff *et al.* (1988). It is reasonable to assume that this limit will hold for other DNA substrates of this type.

The viability of the *E.coli* DNA gyrase supercoiling reaction of small circles is thus correlated with the final SHD of the product circle and the threshold value is apparently the same as that for a large DNA circle. Although the substrate supercoiling energy should be taken into account, as described above, this conclusion is particularly clear from consideration of the 189 bp circle, a size which is an exact multiple of the 10.5 bp helical repeat, and which thus consists of one perfectly relaxed isomer which has no supercoiling energy. This isomer is a substrate for the supercoiling reaction, and the product has an SHD of exactly -0.11.

In the case of circles of 152 bp and smaller, none of the relaxed isomers are substrates in the supercoiling reaction (Table I). It is therefore possible that the steric limit described above has been reached for circles of these sizes. We have investigated this possibility in two ways using the 152 bp circle. DNA gyrase is known to form stable complexes with short linear DNA fragments, which may be visualized as bands on polyacrylamide gels retarded with respect to the free DNA position (Maxwell and Gellert, 1984). Such a gel retardation experiment (Figure 4) shows that DNA gyrase binds to the relaxed 152 bp circle at least as well as to the linearized circle and a control 207 bp fragment known to form a stable complex with gyrase (Rau *et al.*, 1987).

We have also attempted DNA supercoiling of the 152 bp circle by gyrase in the presence of ethidium bromide. Under these conditions, ethidium bromide will positively writhe the DNA and hence reduce any thermodynamic constraint on the supercoiling reaction. Figure 5 shows the results of such an experiment. In the absence of ethidium bromide, no change in linking number of the substrate is observed, but with added ethidium (0.53 μg/ml) the isomer $Lk = 15$ is converted to $Lk = 13$, and with an increased ethidium concentration, isomer $Lk = 14$ is converted to $Lk = 12$. It should be pointed out that this reaction is formally the ATP-dependent relaxation of positive supercoils, rather than the introduction of negative supercoils; nevertheless it is probable that the mechanisms are essentially equivalent. In analogous experiments to that shown in Figure 5, we have carried out the ethidium bromide-dependent reaction on 142, 128 and 116 bp circles, and have found that gyrase can also effect linking number changes in circles of these sizes (data not shown). For example, we found that in the presence of ethidium bromide, the $Lk = 11$ isomer of the 116 bp circle could be converted to $Lk = 9$. We have not found a DNA

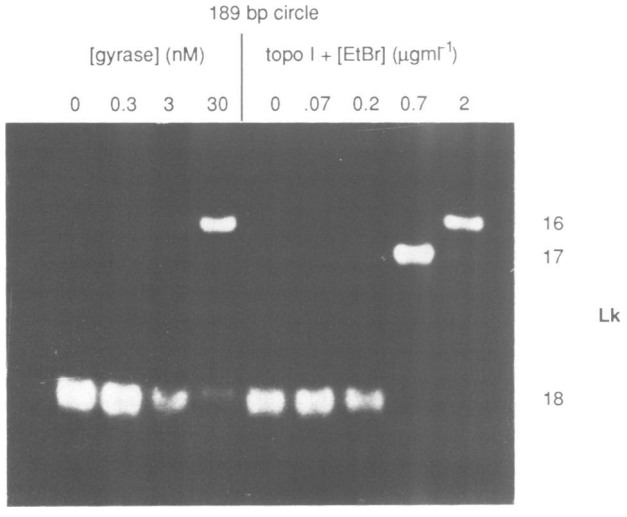

Fig. 3. Supercoiling of 189 bp circle by DNA gyrase. For details see the legend to Figure 2.

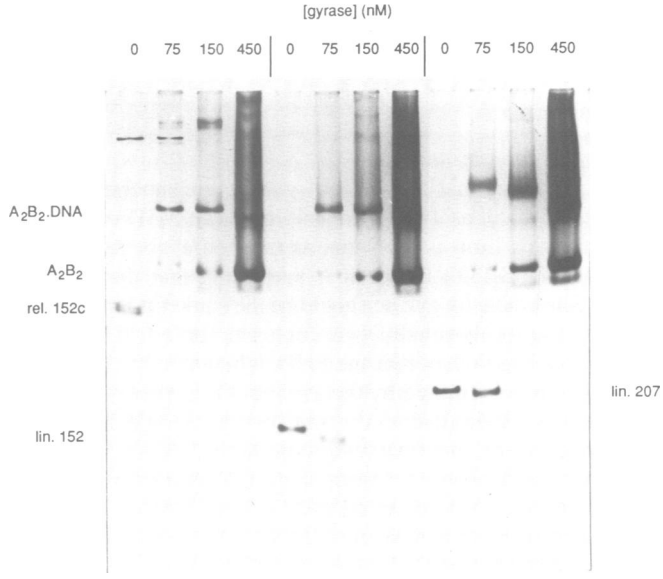

Fig. 4. Binding of 152 bp circle to DNA gyrase. A gel retardation assay showing the binding of gyrase to relaxed 152 bp circle (rel. 152), linear 152 bp fragment (lin. 152) and linear 207 bp fragment (lin. 207). Bands corresponding to free DNA gyrase (A_2B_2) and gyrase–DNA complex (A_2B_2.DNA) are indicated.

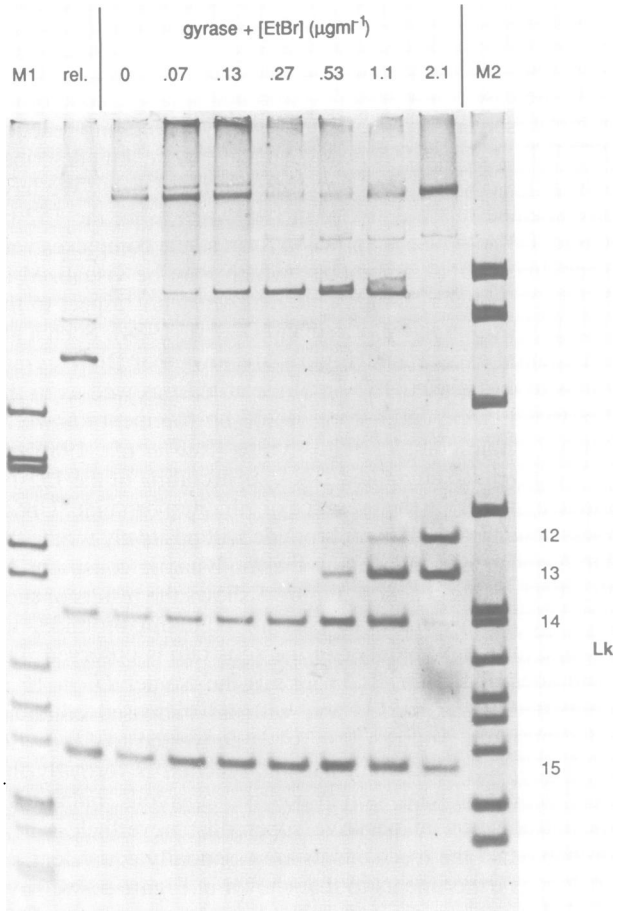

Fig. 5. Reaction of 152 bp circle with DNA gyrase. Relaxed 152 bp circle was incubated with DNA gyrase (30 nM) in the presence of the ethidium bromide concentrations indicated. The track marked rel. contains untreated relaxed 152 bp circle and tracks M1 and M2 contain respectively *Hha*I and *Msp*I digests of pBR322.

circle whose linking number cannot be changed by gyrase in the ethidium bromide-dependent reaction.

Discussion

Thermodynamic aspects

We have found that *E.coli* DNA gyrase can supercoil DNA circles as small as 174 bp in size, up to an SHD of −0.11 and that this limit is the same as that found for the supercoiling of pBR322 DNA. It is surprising that the limit of SHD achievable by gyrase should be the same for large DNA circles and for the small DNA circles in Table I. It has been suggested previously that the SHD achievable by gyrase in a large circle is limited by the available free energy of ATP hydrolysis. Specifically, the free energy required for the introduction of the final two superhelical turns has been equated to the energy liberated by the hydrolysis of two molecules of ATP (Maxwell and Gellert, 1986). Using the data of Horowitz and Wang (1984), this free energy may be calculated for pBR322 to be 114 kJ/mol, compared with the ΔG for the hydrolysis of two ATPs of ~ -120 kJ/mol (based on Simmons and Hill, 1976), i.e. there is an approximate equivalence between these two reactions in energetic terms.

It has previously been shown that the free energy of supercoiling is proportionally greater for a given SHD for DNA circles of <1000 bp than for those >2000 bp (Shore and Baldwin, 1983; Horowitz and Wang, 1984). This can be attributed to the greater unfavourability of writhing in small DNA circles with respect to large circles. Writhing of the DNA helix axis is an important component of negative supercoiling in large circles, but in small DNA circles, reductions in linking number are thought to be largely partitioned into untwisting of the helix (Shore and Baldwin, 1983; Horowitz and Wang, 1984). The variation of supercoiling free energy with length of DNA circle has been determined down to circle sizes of 245 bp (Shore and Baldwin, 1983) and 210 bp (Horowitz and Wang, 1984) from the ratios of topoisomers formed by the ligation of linear molecules. Using data for 210 bp circles we can calculate that the free energy change for the gyrase reaction we have observed in a 189 bp circle is at least 204 kJ/mol (the product isomer in this case has an SHD of exactly −0.11 and the substrate is completely relaxed). This value is almost twice that for the analogous step in pBR322. On the basis of these free energy calculations, we would therefore expect the SHD limit in small DNA circles to be lower than that for pBR322. The fact that they are apparently the same has several possible explanations.

One explanation for this energetic problem is that the enzyme may be acting stoichiometrically in reactions with small circles, to produce a single turnover of the substrate. If the enzyme does not dissociate from the DNA before disruption of the complex with denaturant, the overall free energy for the supercoiling reaction need not determine the viability of the reaction. However, control experiments on the 174 bp circle using less enzyme and longer reaction times than those described in Figure 2 indicate that the enzyme is indeed catalyzing the reaction. A reaction containing 4.7 nM 174 bp $Lk = 17$ isomer and 550 pM gyrase shows $>50\%$ conversion to $Lk = 15$ isomer in 22 h. The turnover is thus at least 1000 times slower than the initial rate of reaction using relaxed pBR322 (data not shown). It should be pointed out, however, that the rate of supercoiling of pBR322 is much slower near to the supercoiling limit and it is possible that the rate becomes similar to that occurring in the reactions of the small circles.

If the thermodynamic analysis above is correct, and gyrase can carry out a supercoiling reaction requiring an energy input of 204 kJ/mol, this implies that more than two ATP molecules can be hydrolysed during one turnover of the enzyme. A corollary of this is that the supercoiling reaction of pBR322 must be limited by factors other than the thermodynamics of the system, since the calculated free energy for the final step of supercoiling is <204 kJ/mol. For example, the limit of gyrase supercoiling in pBR322 may occur when the rate of the ATP-independent relaxation reaction equals that of the supercoiling reaction. Alternatively, it is possible that highly negatively supercoiled pBR322 is unable to form a complex with DNA gyrase which is competent for supercoiling.

An alternative explanation, however, is that the available data for the free energy of supercoiling in small circles does not pertain to the experiments described here. There are several potential problems in the application of free energies of supercoiling as determined by Shore and Baldwin (1983) and Horowitz and Wang (1984) to the present situation. Firstly, the method of calculation based on the ratios of topoisomers in relaxed distributions, makes the implicit assumption that the free energy is equal for positive and

negative supercoiling. In the case of large DNA circles, the symmetry of the Gaussian distribution of relaxed topoisomers indicates the equivalence of the free energies on either side of the mean linking number. Indeed it is to be expected that the ΔG for positive and negative supercoiling will be the same at low specific linking difference in large circles, if the change in linking number is largely partitioned into writhing of the DNA. In the case of small DNA circles, however, where at most two isomers will be visible in the relaxed distribution (e.g. Figures 2 and 3), the symmetry or otherwise of the distribution is not apparent. Furthermore, if the linking number changes are largely partitioned into twist (Horowitz and Wang, 1984), then it is to be expected that the free energy of positive supercoiling will be somewhat greater than that of negative supercoiling. For these reasons, the free energy determinations for small circles must be regarded as potentially inaccurate.

Secondly, the SHDs of the product circles in the gyrase reactions described here (up to −0.11) are much greater than those used in the free energy studies (up to −0.03; Shore and Baldwin, 1983; Horowitz and Wang, 1984), such that the quadratic dependence between the free energy of supercoiling and linking difference may no longer apply. Indeed the observation of significant structural changes in small circles at high SHDs (e.g. Nordheim and Meese, 1988) may indicate departures from this quadratic relationship. These considerations bring into question the energetic paradox described above. It is clear that further analysis of the free energy relationships of small DNA circles is required.

We have however shown that DNA gyrase cannot carry out supercoiling reactions on isomers from the relaxed distributions of circles of 152 bp and smaller; the failure of isomers of these circles to be supercoiled is likely to be a thermodynamic effect (see below).

Steric aspects

We have found firstly that gyrase can carry out a supercoiling reaction on a substrate as small as 174 bp and secondly, by performing similar experiments in the presence of ethidium bromide, that gyrase can relax positive supercoils in circles as small as 116 bp. We have not to date prepared a circle smaller than 116 bp and indeed the only known smaller DNA circle is one of 105 bp, prepared in extremely small quantities by ligation of double-stranded oligonucleotides designed to contain a permanent bend, and which is thus composed of a rather artificial sequence (Ulanovsky *et al.*, 1986).

DNA gyrase is known from a variety of studies, e.g. nuclease protection and related experiments (see Maxwell and Gellert, 1986 for review) to wrap a segment of DNA of 120−150 bp around itself in a positive superhelical sense. It has been suggested previously (Gellert, 1981) that the section of DNA passed through the double-stranded break in the wrapped segment in the course of the supercoiling reaction must be close to or within the wrap, in order to determine the directionality of the supercoiling reaction. Alternative models of the supercoiling reaction (e.g. Wang *et al.*, 1981), have suggested that a distal segment of DNA is translocated through the DNA break. Given that the supercoiling of large and small DNA circles by gyrase are mechanistically equivalent, an assumption which seems reasonable in view of their similar properties, the experiments described in this paper directly address these questions, and can distinguish between these alternative models.

The relaxation of positive supercoils by gyrase is a nucleotide-dependent reaction (Gellert, 1981), and seems mechanistically to be analogous to the introduction of negative supercoils into a relaxed substrate, albeit an energetically favourable rather than unfavourable reaction. We therefore interpret the ability of gyrase to reduce the linking number of topoisomers of 116−152 bp circles only in the presence of ethidium bromide (which introduces positive writhes into closed circular DNA by decreasing the twist) to mean that the negative supercoiling reaction in these circle sizes is prevented by thermodynamic factors, and that the conformational changes associated with the supercoiling reaction can be performed by gyrase in a circle as small as 116 bp. These results strongly suggest that the actual length of the wrapped segment in the gyrase−DNA complex is closer to 120 than 150 bp, since a 116 bp circle is presumably able to form such a complex at the start of the reaction. Indeed, data derived from transient electric dichroism and DNase I footprinting experiments suggest a value of between 100 and 120 bp (Rau *et al.*, 1987). In addition, the data described in this paper suggest that the translocated DNA can indeed comprise part of the initially wrapped segment, and that this wrap is not maintained throughout the supercoiling cycle. By implication, therefore, gyrase must deform the DNA into a circle of ~60 bp in size in order to achieve strand crossing during the supercoiling reaction of a 116 bp circle. Clearly the mechanistic details of this process remain to be resolved.

Materials and methods

DNA

All plasmids were purified using the alkaline lysis procedure of Birnboim and Doly (1979) and two cycles of caesium chloride centrifugation.

Small circles were prepared from a family of *lox*P-containing plasmids derived from plasmid pRH42 (Abremski *et al.*, 1983). Plasmid pRH42 contains two *lox*P sites in direct repeat, with a centre-to-centre distance of 427 bp and a unique *Eco*RV restriction site between the *lox*P sites. Digestion of pRH42 (1 μg) with *Eco*RV followed by treatment with *Bal*31 exonuclease (0.01 U, 15−45 min, 30°C), religation with T4 DNA ligase and transformation into strain RH800 (Hoess and Fan, 1975) yields a set of plasmids with varying separation between the *lox*P sites (Hoess *et al.*, 1985). The exact separations of the sites were determined by direct plasmid sequencing (Chen and Seeburg, 1985). The plasmids pAB97, 102, 112, 113 and 253 have *lox*P−*lox*P distances of 205, 217, 269, 196 and 174 bp respectively. Plasmids pRH42−40, −407, −614 and −686 (Hoess *et al.*, 1985) have separations of 152, 142, 128 and 116 bp respectively.

The plasmid pRM216, containing three repeated copies of a 189 bp *Bam*HI λ_{TO} terminator fragment, was prepared from strain RM216 (gift of R.Menzel). A 207 bp fragment, derived from the *Xenopus* 5S rRNA gene, was prepared as described previously (Rau *et al.*, 1987).

Proteins

Cre recombination protein from bacteriophage P1 was purified from the overproducing strain KA1333 according to the procedure described in Abremski and Hoess (1984), but omitting the final gel filtration step. The protein was stored in the phosphocellulose elution buffer [20 mM Tris−HCl (pH 7.5), 600 mM NaCl, 1 mM EDTA, 10% (w/v) glycerol] as aliquots at −70°C and was used directly in recombination reactions.

Chicken erythrocyte DNA topoisomerase I was prepared as described by Trask and Muller (1984), except that the final phenyl−Sepharose column step was omitted. Topoisomerase I was stored as aliquots in 50 mM Tris−HCl (pH 7.5), 50 mM sodium bisulphite, 1 mM EDTA, 0.5 mM PMSF, 10% (w/v) glycerol at −70°C. One unit of topoisomerase I relaxes 300 ng DNA in a 30 μl reaction volume in 1 h at 25°C.

E.coli DNA gyrase A and B subunits were prepared acording to the method of Mizuuchi *et al.* (1984). Equimolar quantities of each subunit were reconstituted to the A_2B_2 active enzyme by incubation for 30 min at 25°C in 50 mM Tris−HCl (pH 7.5), 100 mM KCl, 10% (w/v) glycerol, 5 mM DTT, 1 mM EDTA and stored as aliquots at −70°C.

Other enzymes used in this study were obtained from the sources indicated:

*Eco*RI, *Eco*RV (S.E.Halford); *Bam*HI, *Msp*I, T4 polynucleotide kinase, *Bal*31 exonuclease (BRL); T4 DNA ligase, calf intestinal alkaline phosphatase (Boehringer) and *Xmn*I (New England Biolabs).

loxP – cre recombination reactions

A *lox*P-containing plasmid (200 μg) was incubated with cre protein (115 μg) in 50 mM Tris–HCl (pH 7.5), 33 mM NaCl, 5 mM spermidine, 50 μg/ml bovine serum albumin (BSA) for 30 min at 37°C followed by 5 min at 70°C to inactivate the cre (Abremski and Hoess, 1984). The solution was then made 10 mM in $MgCl_2$ and cleaved with *Eco*RI to linearize the large product circle and unreacted plasmid. The DNA was purified by phenol extraction and ethanol precipitation. The small circles were used either directly (i.e. with contaminating large linear DNA fragments) or after purification by caesium chloride centrifugation from preparations on 10 times the above scale.

189 bp circularization

The 189 bp *Bam*HI fragment from pRM216 was purified by electroelution from an agarose gel, dephosphorylated with alkaline phosphatase and labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The labelled fragment was ligated at a concentration of 100 ng/ml in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP, 100 μg/ml BSA and 4 U/ml T4 DNA ligase for 1 h at 25°C. The DNA was purified by phenol extraction and ethanol precipitation.

Topoisomerase I relaxation reactions

Relaxed distributions of topoisomers were prepared by incubation of purified small circles (~5 μg/ml, in 20 mM Tris–HCl (pH 8.0), 200 mM NaCl, 0.25 mM EDTA, 5% (w/v) glycerol, 100 μg/ml BSA and 3 U/μl chicken erythrocyte topoisomerase I (Camerini-Otero and Felsenfeld, 1977). Negatively supercoiled topoisomers were prepared by reaction of small circles (~0.3 μg/ml) with topoisomerase I as above in the presence of the concentrations of ethidium bromide indicated.

DNA gyrase reactions

Relaxed distributions of small circles (~2 nM of *lox*P–cre, 1 nM of 189 bp) were reacted with DNA gyrase at the concentrations indicated in 35 mM Tris–HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 9 μg/ml tRNA, 5 mM DTT, 1.7 mM ATP, 100 μg/ml BSA and 6.5% (w/v) glycerol at 25°C for 1 h. Samples were phenol/chloroform extracted, ethanol precipitated and analysed on 5% polyacrylamide gels (acrylamide: bis; 19:1) in 50 mM Tris–phosphate, 1 mM EDTA (pH 7.2) containing 0.3–1 μg/ml ethidium bromide and silver stained or autoradiographed (189 bp). Gyrase reactions were also carried out under the above conditions with the inclusion of ethidium bromide at the concentrations indicated.

DNA gyrase supercoiling reactions of pBR322 were carried out under the conditions described above. The SHD of the most supercoiled isomer was determined according to the method of Keller (1975).

Gel retardation

Samples of purified relaxed 152 bp circle (~6 nM), linear 152 bp fragment (~6 nM) and linear 207 bp fragment (~36 nM) were incubated with the concentrations of gyrase shown in 50 mM Tris–HCl (pH 7.5), 55 mM KCl, 4 mM $MgCl_2$, 5 mM DTT, 5% (w/v) glycerol for 1 h at 25°C and electrophoresed through a 5% polyacrylamide gel (acrylamide: bis; 29:1) in 90 mM Tris–borate (pH 7.0), 10 mM $MgCl_2$, and silver stained.

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