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Reverse gyrase binding to DNA alters the double helix structure and produces single-strand cleavage in the absence of ATP

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Stoichiometric amounts of pure reverse gyrase, a type I topoisomerase from the archaebacterium *Sulfolobus acidocaldarius* were incubated at 75°C with circular DNA containing a single-chain scission. After covalent closure by a thermophilic ligase and removal of bound protein molecules, negatively supercoiled DNA was produced. This finding, obtained in the absence of ATP, contrasts with the ATP-dependent positive supercoiling catalyzed by reverse gyrase and is interpreted as the result of enzyme binding to DNA at high temperature. Another consequence of reverse gyrase stoichiometric binding to DNA is the formation of a cleavable complex which results in the production of single-strand breaks in the presence of detergent. Like eubacterial type I topoisomerase (protein ω), reverse gyrase is tightly attached to the 5′ termini of the cleaved DNA. In the light of these results, a comparison is tentatively made between reverse gyrase and the eubacterial type I (ω) and type II (gyrase) topoisomerases.

Key words: archaebacteria/gyration/positive supercoiling/topoisomerase I

Introduction

Since the discovery of DNA gyrase (Gellert *et al.*, 1976), a number of studies have been undertaken to explain the mechanism by which this topoisomerase was able to introduce negative supercoils into a relaxed DNA in an ATP-driven reaction (Liu and Wang, 1978; Brown and Cozzarelli, 1979; Gellert *et al.*, 1980; Wang, 1987). During the same period, the prominent biological role of DNA negative supercoiling and the ways by which this supercoiling is maintained in a cell were partly elucidated in eubacteria (Drlica, 1984).

According to these data, the discovery, in 1984, of a positive supercoiling activity in the thermophilic archaebacterium *Sulfolobus acidocaldarius* (Kikuchi and Asai, 1984) was totally unexpected. The novel enzyme responsible for this activity and called 'reverse gyrase' appeared to be a type I topoisomerase: indeed, it was able to introduce positive supercoils by steps of one in a closed circular DNA, in an ATP-dependent process (Forterre *et al.*, 1985; Nakasu and Kikuchi, 1985). In contrast, the enzyme relaxes a supercoiled DNA quite inefficiently in the absence of ATP (M.Nadal *et al.*, unpublished data). Reverse gyrase, as is the case for other type I topoisomerases from various organisms, represents the major topoisomerase activity in *Sulfolobus* cells. It is not restricted to the genus *Sulfolobus*: an enzyme with nearly identical properties has been recently isolated from an anaerobic archaebacterium *Desulfurococcus amylolyticus*, which belongs to a group distant from *Sulfolobus* in taxonomic terms (Collin *et al.*, 1988; Slesarev, 1988). Furthermore, positive supercoiling also takes place *in vivo*, in a virus-like particle present in *Sulfolobus* (Nadal *et al.*, 1986). These results naturally put into question the relationships between reverse gyrase and other known topoisomerases and the mechanisms by which reverse gyrase is able to perform this new type of topoisomerization.

In previous work, we purified reverse gyrase to homogeneity and determined its physicochemical properties (Nadal *et al.*, 1988). The pure enzyme is made of a single polypeptide of ~128 kd. It is able to sustain positive supercoiling of DNA in the presence of ATP, without addition of any protein cofactor: this eliminates the trivial hypothesis of the combined action of a classical topoisomerase and a DNA-binding protein. As for gyrase, the reaction is catalytic and, in the presence of polyethylene glycol, it is highly processive: in these conditions, each reverse gyrase molecule was able to catalyze ~50 cycles of positive supercoiling per minute at 75°C (Forterre *et al.*, 1985). This value is close to the turnover found for DNA gyrase (Sugino and Cozzarelli, 1980; Maxwell and Gellert, 1984).

In an attempt to elucidate the mechanism of reverse gyration, we have investigated the effects of reverse gyrase stoichiometric binding on double-stranded DNA. We report here that this binding alters the DNA structure in two ways: (i) reduction of the linking number in a circular duplex after covalent closure by a ligase; (ii) singe-stranded DNA cleavage.

Results

Negative supercoiling upon covalent closure of DNA in the presence of bound reverse gyrase

This type of experiment is based on the simple observation that, once ligated, the linking number of the DNA product is an invariant and may be accurately measured by agarose gel electrophoresis (Depew and Wang, 1975). We prepared pBR 322 DNA molecules containing one single-strand break per circle. This DNA was incubated for 10 min at 75°C with various concentrations of reverse gyrase and quickly sealed by the thermophilic DNA ligase HB8 (see Materials and methods). In order to look only at the binding of reverse gyrase to DNA, ATP was omitted from the reaction mixture, the salt concentration was raised to 60 mM, and the time of incubation with ligase was reduced to 5 min. In these conditions, practically no topological conversion could occur

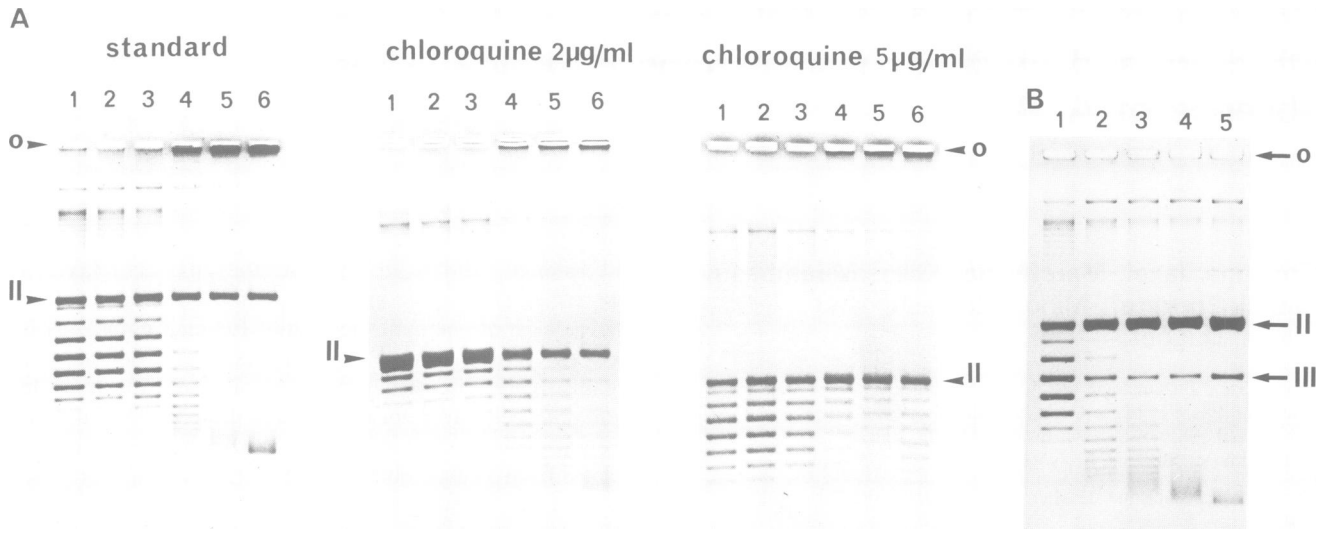

Fig. 1. Negative supercoiling of the DNA after reverse gyrase binding and covalent closure by ligase at high temperature. The reactions were carried out as described under Materials and methods. After extraction with 25 µl phenol/chloroform, then with 25 µl chloroform, and addition of 1% SDS, 0.85 mg/ml bromophenol blue, and 15% sucrose, each sample was divided into three aliquots. These were loaded on three different 1.3% agarose gels with various chloroquine concentrations and electrophoresed at 1.5 V/cm for 20 h at 5°C in a buffer containing 40 mM Tris-acetate (pH 7.8), 1 mM EDTA. (**A**) Without proteinase K treatment. **Left panel**, standard gel; **central panel**, 2 µg/ml choroquine; **right panel**, 5 µg/ml chloroquine. **Lanes 1–6**, incubation with increasing number of reverse gyrase molecules per DNA circle: 0 (**lanes 1**), 3 (**lanes 2**), 6 (**lanes 3**), 12 (**lanes 4**), 18 (**lanes 5**) and 24 (**lanes 6**). The positions of the origin of migration (o) and of form II DNA are indicated by arrows. (**B**) With proteinase K treatment. Incubation with 0 (**lane 1**), 9 (**lane 2**), 15 (**lane 3**), 21 (**lane 4**) and 36 (**lane 5**) reverse gyrase molecules per circle. The positions of the origin of migration (o), form II and form III linear DNAs are indicated.

after closure by ligase (M.Nadal, unpublished data). In addition, the possible interference of ligase with the reverse gyrase–DNA complex was reduced by using extremely diluted ligase. Finally, we have checked that the integrity of form II DNA was not affected by 15 min incubation at 75°C in our conditions, and that reverse gyrase remained >85% active after 15 min at 79°C in the absence of DNA (M.Nadal and P.Mollat, unpublished data).

The results of such an experiment are shown in Figure 1: closure in the absence of reverse gyrase produced a distribution of negative topoisomers (lane 1, left panel) expected from the different physicochemical conditions between the reaction mixture (75°C, 6 mM $MgCl_2$) and the electrophoresis (5°C, no $MgCl_2$) (M.Duguet, unpublished results, see also Figure 3). Binding of reverse gyrase to DNA results in a shift in this distribution after ligation and removal of proteins by SDS. The DNA substrate was progressively supercoiled, as a function of reverse gyrase added (lanes 2–6, left panel). When a wide range of reverse gyrase-to-DNA ratio was used (Figure 1B), the superhelical density increased to high values (lane 5). The sign of this supercoiling was determined by analysis in agarose gels containing chloroquine. With 2 µg/ml chloroquine, the mobility of the various topoisomers was reduced, indicating that these were all negatively supercoiled (1A, central panel). This was confirmed with 5 µg/ml chloroquine (1A, right panel): under these conditions, the control without reverse gyrase was positively supercoiled (lane 1); its mobility decreased with increasing concentrations of reverse gyrase (lanes 1–4), then again increased (lanes 5 and 6). The appearance of negative topoisomers in lanes 5 and 6 was clearly visible: these topoisomers are shifted with respect to those of lanes 1–4. The conclusion of this experiment is that the linking number

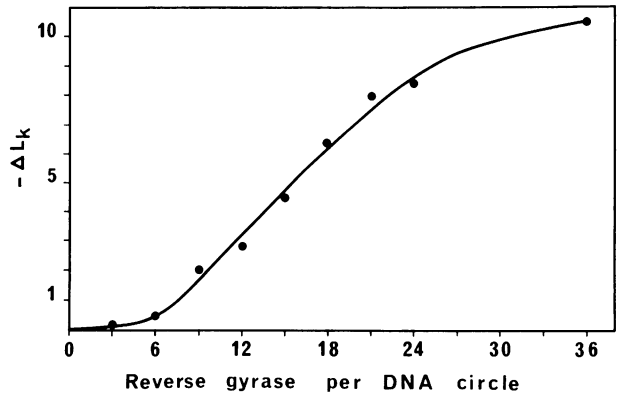

Fig. 2. Variation in the DNA linking number as a function of reverse gyrase molecules per circle. The electrophoretic data of Figure 1 were used to quantitate the change in the linking number observed in the presence of various concentrations of reverse gyrase. Chloroquine gels were used when necessary to resolve the different topoisomers of a given distribution. For each reverse gyrase-to-DNA ratio, the position of the center of each Guassian distribution of topoisomers was determined from the densitometric profile of the gels by using Depew and Wang's treatment (1975). The linking number difference (ΔLk), measured by subtracting the position of the intial distribution (in the absence of reverse gyrase) from the position of each distribution determined as above, was plotted against the number of reverse gyrase molecules per DNA circle. The lag in the response observed at low protein ratio may reflect the fact that only a fraction of the enzyme is able to bind in these conditions.

of the DNA was reduced after binding of reverse gyrase and closure.

In a series of experiments the change in the linking number of the DNA was measured for each topoisomer distribution and plotted against the ratio of total reverse gyrase molecules

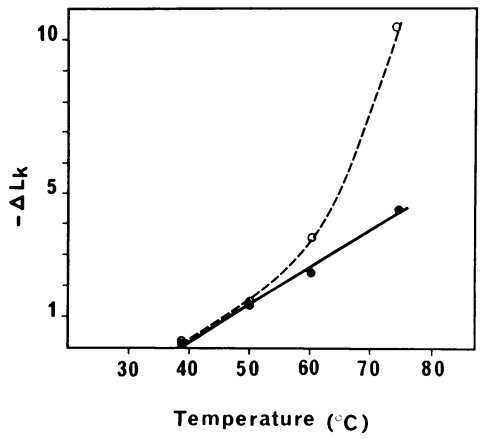

Fig. 3. Effect of temperature on the change in linking number promoted by reverse gyrase. pBR322 form II was incubated as described in Figure 1 with reverse gyrase (ratio of 18 molecules per DNA circle) at various temperatures and sealed by ligase. The variation in the linking number of the DNA was measured by using the distribution obtained at 37.6°C in the absence of reverse gyrase as a reference (ΔLk = 0), and plotted against temperature: —— without reverse gyrase; - - - - - with reverse gyrase.

per DNA input (Figure 2). However, we noted that upon increase of reverse gyrase-to-DNA ratio, a large part of the DNA (up to 50%) failed to penetrate into the gel (Figure 1, lanes 4–6 on all three panels), although the samples were treated by SDS. As is shown in the second part of the Results, this DNA is linked by a covalent bond to reverse gyrase molecules and is not productive in terms of supercoiling, since it cannot be further ligated. If we assume that the molar reverse gyrase-to-DNA ratio in the 'productive' complex is identical to the input reverse gyrase-to-DNA ratio, a linking number difference (ΔLk) of −0.5 per reverse gyrase molecule can be deduced from the slope of the linear part of the curve in Figure 2 (see Discussion).

These experiments allowed us to titrate the enzyme bound to the DNA from the measure of ΔLk and to investigate the effect of temperature on this binding. In a control experiment (Figure 3), the decrease in the twist of the DNA alone appeared linear with temperature between 37 and 75°C. Up to 50°C, addition of reverse gyrase did not affect this process, while at higher temperature the linking number of the DNA was clearly decreased. These results suggest that the lack of enzymatic activity previously observed below 50°C (Mirambeau *et al.*, 1984) was likely due to the lack of reverse gyrase binding to DNA. This was confirmed by the following experiment: reverse gyrase and DNA were first incubated at 75°C, then quickly cooled to 23°C and the DNA was sealed by ligase at various times after the temperature shift. In this case, the linking number difference dropped rapidly, suggesting that the complex was readily dissociated at 23°C (data not shown).

DNA cleavage and covalent binding of reverse gyrase to the 5′ ends

As already shown in the above experiments, an important portion of the complex between reverse gyrase and DNA was resistant to SDS treatment (Figure 1A). However, when the samples were incubated with proteinase K before electrophoresis, all the DNA penetrated into the gel (Figure 1B), even at a high protein-to-DNA ratio (lane 5). Form II was

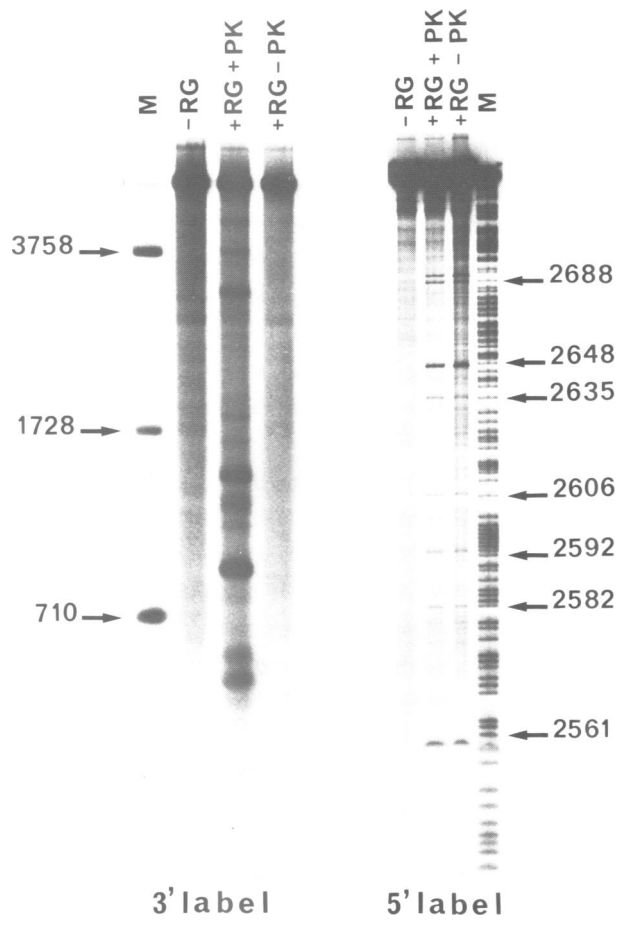

Fig. 4. Cleavage of SV40 DNA by reverse gyrase: electrophoretic analysis of the products. A restriction fragment of SV40 DNA, labeled either at the 3′ or at the 5′ end on only one of the two strands (see Materials and methods) was pre-incubated for 15 min at 75°C in the following mixture (20 μl): 50 mM Tris–HCl, pH 7.8, 10 mM NaCl, 10 mM $MgCl_2$, 0.5 mM DTT and 30 μg/ml bovine serum albumin. Pure reverse gyrase (1 pmol, 120 ng) was added and the mixture was further incubated for 1.5 min at 75°C. The reaction was stopped by addition of 1% SDS (final concentration) and 0.5 mg/ml proteinase K. After 30 min incubation at 50°C, the products were extracted twice with phenol/chloroform, denatured in 16 mM NaOH, heated and submitted to electrophoresis. **3′ label:** agarose gel electrophoresis. A *BanI–HpaII* fragment (5191 bp, 0.01 pmol) of SV40 was used. The incubation products were denatured by heating at 85°C in 15 mM NaOH, 0.3 mM EDTA, 0.8% Ficoll, 0.01% Bromocresol green, and submitted to 1% agarose gel electrophoresis in Tris–borate–EDTA buffer (see Materials and methods) for 15 h at 2 V/cm. **Lane M**: DNA markers; the size in bp is indicated. **Lane −RG**: incubation in the absence of reverse gyrase. **Lane +RG +PK**: incubation with reverse gyrase followed by proteinase K treatment. **Lane +RG −PK**: incubation with reverse gyrase, without proteinase K treatment. **5′ label**: polyacrylamide gel electrophoresis. A *BclI–BamHI* fragment of SV40 (237 bp, 0.06 pmol) was used. The incubation products were precipitated by ethanol after addition of 0.3 M sodium acetate, 1 mM EDTA, and 50 μg/ml tRNA. The pellet was dried and taken in 3 μl of the Maxam–Gilbert solution (80% formamide, 10 mM NaOH, 1 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue). Electrophoresis was performed in 6% acrylamide, 7 M urea at 3000 V for 70 min (55°C). **Lane M**: ladder of purines. The arrows refer to the position in the SV40 nucleotide sequence.

considerably increased, while form III, which was not detected without proteinase K treatment, became clearly visible (compare lane 5 of Figure 1B with lane 6 of Figure 1A). These results suggest that, under conditions

prevailing for other topoisomerases, a part of the DNA was cleaved and remained covalently linked to reverse gyrase. It was only released upon proteinase K treatment and was not circularized during incubation with ligase. The majority of this cleavage occurred as single-strand breaks (form II), but at high protein-to-DNA ratio a significant amount of form III was also produced (see Discussion).

In order to determine which end (5′ or 3′) of the DNA was bound to reverse gyrase, we prepared a fragment of DNA labeled at only one of the two 3′ ends (see Materials and methods). This fragment was incubated with excess reverse gyrase (RG-to-DNA ratio of 100) for 1.5 min at 75°C in the absence of ATP, and the products were analyzed by gel electrophoresis (Figure 4, left). The labeled fragments produced by cleavage occurred only after treatment with proteinase K. In contrast, when the DNA was labeled on one of the 5′ ends (RG-to-DNA ratio of 18), the labeled fragments were revealed even when the sample was not treated with proteinase K (Figure 4, right). These results indicate that the 5′ ends of the breaks were tightly bound to the enzyme, while the 3′ ends were free. In a control experiment with eukaryotic topoisomerase I, labeled fragments appeared only upon proteinase K treatment when the 5′ end was labeled, and without proteinase K treatment when the label was on the 3′ end (data not shown).

Discussion

Reverse gyrase provides a unique example of a type I topoisomerase able to perform a reaction of gyration. Moreover, it is also a unique case of a topoisomerase I which is dependent on ATP. The results described in this paper indicate that, despite these quite unusual properties, the mechanism of reverse gyrase involves some features which are shared by other topoisomerases: (i) formation of transient single-strand breaks which allow strand passage and rejoining; (ii) alterations of the DNA structure which allow gyration.

Comparison with classical topoisomerase I

Like eubacterial topoisomerase I (protein ω) (Depew *et al.*, 1978), reverse gyrase increases the linking number of a circular DNA and is linked to the 5′ end of the transient single-strand break. In contrast, the eukaryotic enzyme is linked to the 3′ end of the DNA and can either increase or decrease the linking number (Champoux, 1978). Based on these properties, reverse gyrase appears closer to ω than to eukaryotic topoisomerase I.

However, several discrepancies arise between reverse gyrase and ω. First, addition of ATP promotes the catalytic activity of reverse gyrase, when it is not required for ω. Consequently, formation of a cleavable complex with reverse gyrase is enhanced in non-catalytic conditions, i.e. in the absence of ATP. Second, trapping of reverse gyrase in such a complex takes place in the presence of magnesium, while magnesium strongly reduces this trapping in the case of ω. Third, a negatively supercoiled DNA substrate is not required for trapping reverse gyrase in a cleavable complex with double-stranded DNA. However, it is not excluded that the enhanced affinity of protein ω for a negatively supercoiled substrate (Tse and Wang, 1980) is shared by reverse gyrase: for instance, base pair opening (i.e. production of single-stranded regions) is favored by the high temperature required. This is compatible: (i) with the appearance of linear DNA when reverse gyrase is trapped in a complex with nicked circular DNA, and (ii) with the optimal processivity of both enzymes in the absence of monovalent cation. However, we cannot exclude the possibility that reverse gyrase could perform transient single-strand breaks in a stable duplex as does the eukaryotic topoisomerase I (Champoux, 1976), although the salt requirements are different.

Comparison with eubacterial gyrase

Although it is a type I topoisomerase, reverse gyrase is the second example of a topoisomerase able to catalyze supercoiling in a circular DNA. There is apparently a remarkable symmetry in the mode of action of these enzymes: gyrase catalyzes ATP-dependent negative supercoiling, and its stoichiometric binding to DNA (in the absence of ATP) promotes positive supercoiling. Conversely, reverse gyrase catalyzes ATP-dependent positive supercoiling and its stoichiometric binding to DNA (in the absence of ATP) promotes negative supercoiling. Thus, these two enzymes could apparently perform DNA gyration in a similar fashion: an initial modification of DNA structure is further offset by an opposite distortion which drives the reaction (Liu and Wang, 1978).

The increase in linking number produced by gyrase binding (+1 turn) was interpreted as a right-handed wrapping of duplex DNA around gyrase molecules. Furthermore, it was suggested that the sign inversion of this wrapping in the presence of ATP, coupled with double-strand break and rejoining, generates a cycle of negative supercoiling ($\Delta Lk = -2$).

This mechanistic pathway, however, does not fit well with the present data on reverse gyrase. First, reverse gyration must be compatible with changes in the DNA linking number by increments of 1. Second, reverse gyrase is a relatively small (120−130 kd) protein, apparently without quaternary structure when compared to gyrase, a tetramer of ~400 kd.

The decrease in linking number observed after reverse gyrase binding gives an 'unwinding value' of −0.5 turn per enzyme molecule present in the incubation mixture. However, as in the case of gyrase (Liu and Wang, 1978), the actual proportion of bound enzyme molecules is not known: the above value is therefore a minimum estimate. The structural change of DNA that produces this 'unwinding' remains to be determined. Whatever it is−base pair opening, partial wrapping, bending or simply untwisting of the DNA−this structural change likely initiates the reaction. Then, ATP binding may produce a distorsion that allows strand break and passage, leading to an elementary cycle of supercoiling.

Clearly, further work is needed to derive a precise model for positive supercoiling. This task will be of wide interest to elucidate the mechanistic models of eubacterial and eukaryotic topoisomerases which are still not completely clarified. Finally, it should be stressed that, according to recent data (Wu *et al.*, 1988), positively supercoiled DNA may be produced in eubacteria and in eukaryotes in the course of transcription.

Materials and methods

Chemicals and enzymes

NAD^+ and ethidium bromide were purchased from Boehringer (Mannheim). Chloroquine, dithiothreitol, EDTA and bovine serum albumin were

from Sigma Chemical Co. SDS was from Serva, sucrose from BDH, and agarose A37 NA from IBF (France). Proteinase K and other chemicals were from Merck (Darmstadt).

DNA substrates

pBR322 DNA form I was prepared as previously described (Duguet *et al.*, 1983). A form II containing one single-strand scission per molecule was prepared by incubation of form I with pancreatic DNase in the presence of saturating concentrations of ethidium bromide (Barzilai, 1973).

Preparation of labeled DNA fragments. SV40 DNA was digested by *Ban*I to produce linear DNA with 5′ protruding ends. This DNA was labeled on its 3′ ends by incubation with Klenow polymerase in the presence of [α-^{32}P]dGTP and further digested by *Hpa*II to produce a 5191 bp fragment labeled on only one end. To produce a fragment of 237 bp labeled on one 5′ end, SV40 DNA was first cleaved by *Bam*HI, dephosphorylated, treated with polynucleotide kinase in the presence of [γ-^{32}P]ATP, digested by *Bcl*I, and finally electroeluted.

Enzymes

Reverse gyrase was purified to homogeneity from *S.acidocaldarius* by using a six-step purification procedure, as previously described (Nadal *et al.*, 1988). Purified reverse gyrase was dialyzed and concentrated to 330 μg/ml. It was stable for several months at 4°C.

DNA ligase from *Thermus thermophilus* was purified to near homogeneity as described (Takahashi *et al.*, 1984). The enzyme uses NAD as a cofactor, is active between 15 and 85°C and very low amounts (i.e. 10^{-5} units/μg DNA) are sufficient to seal a nick. To ensure a quick closure of the DNA, an excess ligase activity ($2 \times 10^{-3}-10^{-2}$ U/μg DNA) was used in the incubations with reverse gyrase. This amount of ligase was still very low in terms of protein concentration.

Binding of reverse gyrase to DNA and covalent closure by ligase

Reaction mixtures (22 μl each in siliconized tubes) contained: 32 mM Tris–HCl, pH 7.8, 6 mM $MgCl_2$, 30 mM KCl, 25 mM NaCl, 5 mM sodium phosphate, 0.85 mM NAD, 6 mM DTT, 0.05 mM EDTA, 30 μg/ml bovine serum albumin, 584 ng of pBR 322 form II DNA with one single-strand break per circle and various amounts of pure reverse gyrase. Two microliters of the proper dilution of the *T.thermophilus* DNA ligase in 10 mM Tris–HCl, pH 7.4, 100 mM KCl, 20% glycerol were introduced, prior to incubation at 75°C, as a drop on the inner wall of each siliconized tube placed horizontally, but not mixed with the reaction medium. After 10 min incubation at 75°C, each tube was quickly agitated to mix the ligase drop with the other components and further incubated for 5 min at 75°C. The tubes were quickly cooled on ice, centrifuged for 30 s and EDTA was added to a final concentration of 50 mM. In some cases, SDS was added to 1% and the mixtures were incubated for 30 min at 50°C with 0.5 mg/ml proteinase K.

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