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High positive supercoiling *in vitro* catalyzed by an ATP and polyethylene glycol-stimulated topoisomerase from *Sulfolobus acidocaldarius*

Patrick Forterre[1], Gilles Mirambeau[2], Christine Jaxel[2,3], Marc Nadal[2] and Michel Duguet[2]

Institut J. Monod, Université Paris VII, 2, place Jussieu, 75230 Paris Cedex 05, and [2]Laboratoire d'Enzymologie des Acides Nucleiques, Université Pierre et Marie Curie, UA 554 CNRS, 96, Bd. Raspail, 75006 Paris, France

[1]Present address: Groupe de Biologie et Génétique Moléculaires, Institut de Recherches Scientifiques sur le Cancer, BP no. 8, 94802 Villejuif Cedex, France

[3]Present address: CNRS-CERCOA, 2-8 rue Henry Dunant, 94320 Thiais, France

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A topoisomerase, able to introduce positive supercoils in a closed circular DNA, has been isolated from the archaebacterium *Sulfolobus acidocaldarius*. This enzyme, fully active at 75°C, performed *in vitro* positive supercoiling either from negatively supercoiled, or from relaxed DNA in a catalytic reaction. In the presence of polyethylene glycol (PEG 6000), this reaction became very fast and highly processive, and the product was positively supercoiled DNA with a high superhelical density (form I+). Very low (5–10 μM) ATP concentrations were sufficient to support full supercoiling; the non-hydrolyzable analogue adenosine-5′-0-(3-thiotriphosphate) also sustained the production of positive supercoils, but to a lesser extent, suggesting that ATP hydrolysis was necessary for efficient activity. Nevertheless, low residual positive supercoiling occurred, even in the absence of ATP, when the substrate was negatively supercoiled. Finally, the different ATP-driven topoisomerizations observed, i.e., relaxation of negative supercoils and positive supercoiling, in all cases increased the linking number of DNA in steps of 1, suggesting the action of a type I, rather than a type II topoisomerase.
Key words: Archaebacteria/gyration in reverse/polyethylene glycol/topoisomerase/supercoiling

Introduction

DNA topoisomerases are enzymes which change the topological state of DNA (for a review, see Cozzarelli, 1980; Gellert, 1981; Drlica, 1984). They have been classified into two types (I and II) depending on the type of transient break (single or double strand) they introduce into DNA. In eubacteria, a major role of these enzymes is to control the superhelicity of DNA, which in turn regulates gene expression (Pruss *et al.*, 1982; Di Nardo *et al.*, 1982; Fischer, 1984). This control results from a balance between type II topoisomerase (gyrase) that creates negative superhelicity, and type I topoisomerase (ω protein) that removes negative supercoils. Recent reports suggest that gene expression in eukaryotic cells could also be controlled by the level of DNA superhelicity (Harland *et al.*, 1983; Ryoji and Worcel, 1984; Glikin *et al.*, 1984). However, type I and type II enzymes are not antagonist in these cells: both enzymes relax DNA, and type II enzyme, although evolutionarily related to bacterial gyrase cannot produce negatively supercoiled DNA from relaxed DNA (Miller *et al.*, 1981; Shelton *et al.*, 1983; Villeponteau *et al.*, 1984; Goto *et al.*, 1984). Surprisingly, ATP is required by eukaryotic type II topoisomerase to relax DNA, an energetically favorable reaction.

We have begun a search for DNA topoisomerases in archaebacteria, a group of organisms as distantly related to other bacteria (eubacteria) as to eukaryotes (Woese, 1981; Zillig *et al.*, 1984). Our aim is to elucidate the evolutionary pathway of topoisomerases and topoisomerisation mechanisms. We have previously described an ATP-dependent relaxing activity in the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius* (Mirambeau *et al.*, 1984). A survey for DNA topoisomerases activities in this organism has also been undertaken by Kikuchi and Asai (1984). They reported two remarkable features; first, in contrast to the situation in eukaryotes and eubacteria, type II topoisomerases prevailed and could be fractionated into three distinct enzymatic preparations and second, one of these type II enzymes was able to introduce positive superhelical turns into a DNA that was initially negatively supercoiled. These authors called the activity 'reverse gyrase'. Compared with classical gyration, reverse gyration was incomplete with a maximum of eight positive superhelical turns introduced in pBR322 DNA.

In our screening for *S. acidocaldarius* topoisomerases, we have also detected a 'reverse gyrase' activity. We report here that addition of polyethylene glycol allows the reaction to proceed up to positive form I (complete reaction) in a highly processive and catalytic fashion. The enzyme can use either a relaxed DNA or a negatively supercoiled DNA as a substrate. Recently, we have found (Prunell, Goulet and Forterre, unpublished results, and this work) that in our hands, the enzyme performs relaxation and positive supercoiling in the presence of ATP by changing the linking number in steps of 1. This and other results presented here suggest that positive supercoiling is due to a type I enzyme rather than a type II. We discuss the possible origins for discrepancies between our results and those of Kikuchi and Asai (1984).

Results

Isolation of a 'positive supercoiling' activity

We have previously reported the isolation of a thermophilic ATP-dependent DNA relaxing activity from the archaebacterium *S. acidocaldarius* (Mirambeau *et al.*, 1984). This activity was initially detected in the 0.8 M ammonium chloride wash of a polymin P precipitate from the crude lysate. An endonuclease prevented the detection of topoisomerase in the polymin P supernatant and in the first wash (0.2 M NH_4Cl). We have further found that both the supernatant and the first wash contained a large part of the relaxing activity that can be separated from endonuclease on phosphocellulose. We have therefore devised a new purification procedure in which the supernatant and the 1.2 M NH_4Cl wash of the polymin P precipitation were pooled and chromatographed on a phosphocellulose column with a 0.2–0.8 M linear phosphate gradient.

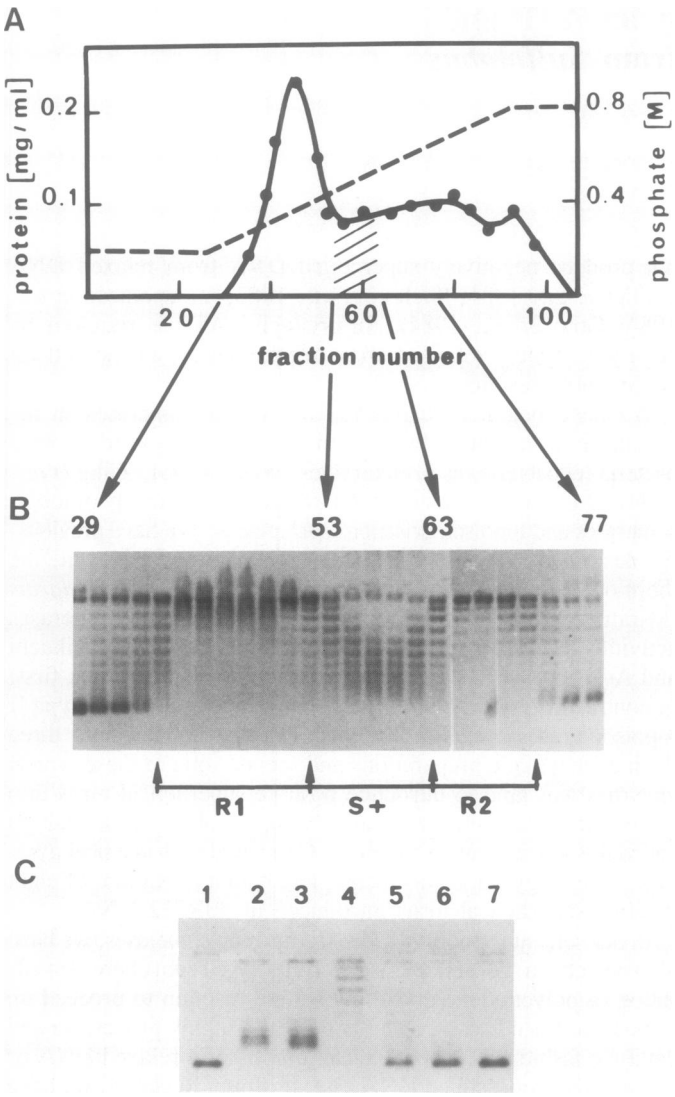

Fig. 1. Elution of topoisomerase activity from phosphocellulose column. **(A)** Elution profile of proteins. 80% of the loaded proteins did not bind phosphocellulose.-●- protein concentration; --- phosphate gradient; ▨ pooled topoisomerase fractions. **(B)** Fractions eluted from the column were assayed for relaxation of negatively supercoiled pBR322 DNA, as described in Materials and methods. 0.4 μg DNA incubated with 2 μl fractions 29,31,33 . . . to 77 was analysed in agarose gel. The fast migrating band in fractions 29 and 77 is pBR322 form I (unreacted). Three distinct regions, R1 (fractions 37–49), S^+ (fractions 51–63), R2 (fractions 65–71) are visible on the gel. Fractions 37 (left of R1) and 73 (right of R2) are composed of negatively supercoiled topoisomers; fractions 51–63 are composed of positively supercoiled topoisomers. **(C)** Fraction 59 (center of region S^+) was diluted and assayed as in **B**. **Lane 1**: pBR322 form I control; **lanes 2–7:** incubation of the DNA with, respectively, 3; 2; 1; 0.5; 0.25; 0.125 μl fraction 59.

Figure 1B shows the relaxing activity of the fractions eluted from this column. Relaxation was tested in the presence of ATP at 70°C in a mixture containing 10 mM $MgCl_2$, 150 mM KCl, and negative pBR322 form I as a substrate. Two peaks of relaxation (R1 and R2) were apparent. Analysis of the reaction products on a chloroquine-containing gel suggested that topoisomers in the region between peaks R1 and R2 (region S^+) were positively supercoiled: chloroquine decreases the twist between the two strands of the double helix and consequently increases the writhing in a closed circular DNA duplex. A chloroquine concentration of 3 μg/ml moderately decreased the mobility of intermediate negatively supercoiled pBR322 topoisomers and increased the mobility of positive supercoils. When adding such a drug concentration into the gel, the mobility of topoisomers increases in region S^+, whilst it decreased before peak R1 and after peak R2 (data not shown). Analysis of the DNA species on a two dimensional gel (Wang *et al.*, 1983) confirmed that topoisomers in this region were indeed positively supercoiled (Figure 2, panel E). The possibility that these species were not supercoiled, but knotted DNA was ruled out, since they were relaxed by treatment with eukaryotic type I topoisomerase or low quantitites of DNase I (not shown). The identity of the DNA produced by incubation with fractions in region S^+ was also substantiated by the observation that upon dilution of the fractions, relaxation first increased, and then decreased (Figure 1C). This result suggests that a single enzyme could perform both positive supercoiling and relaxation of the DNA in the presence of ATP. Indeed, both reactions represent the same type of topological conversion, i.e., an increase in the linking number of the DNA.

To purify further the activity of positive supercoiling, we have pooled the fractions in region S^+ (0.4–0.5 M potassium phosphate). This eliminated most of the proteins retained on phosphocellulose and eluted at lower ionic strength (Figure 1A). Pooled fractions were absorbed on an FPLC column (sulfopropyl type) and proteins eluted by a KCl gradient (0–0.6 M): a peak of positively supercoiled DNA flanked by two regions of relaxation was again observed. Upon 5-fold dilution, this peak was converted to a peak of relaxation (not shown). At this stage of purification, only four polypeptides were detectable in the most active fractions on an SDS polyacrylamide gel, after silver staining (apparent mol. wts. 132 000; 56 000; 42 000 and 30 000). These polypeptides were not in stoichiometric amounts, and two of them (56 000 and 30 000) were absent in other fractions containing positive supercoiling activity and isolated during the purification of *Sulfolobus* DNA polymerase. The activity sedimented at 7.5S in a glycerol gradient and therefore could correspond to the 132 000 mol. wt. polypeptide.

Optimal conditions for positive supercoiling

We have previously shown that ATP-dependent relaxation catalyzed by *Sulfolobus* extracts was efficient only when the temperature was raised above 60°C (Mirambeau *et al.*, 1984). Above 80°C, some nicked (Form II) DNA was generated, and we have therefore chosen 75°C as the optimal assay temperature. In the same way, ATP-dependent positive supercoiling was optimal at high temperature (75°C) in the presence of 5–10 mM magnesium (Figure 3A) and 20–60 mM potassium (not shown). In these temperature and salt conditions, the reaction was fast and processive. Kinetic analysis showed the direct appearance of positive supercoils (demonstrated by analysis in chloroquine gels) after only 1 min incubation (Figure 3B, lane 2), although a large part of the DNA substrate remained negatively supercoiled (Figure 3B, lanes 2–4). Further incubation converted the entire substrate to positive supercoils (lanes 5,6), but did not increase greatly the superhelical density. This contrasts with the conditions (70°C, 10 mM $MgCl_2$, 150 mM KCl), used for the detection of activities during fractionation (Figure 1B and C) in which the reaction was distributive: all the intermediates between form I (fraction 29) and positive supercoils (fraction 59) are visible on Figure 1B. The average positive superhelicity was ~10 superhelical turns in processive conditions when observed in a gel run at 25°C (Figure 3A, lanes 6,7), which means that an average of 15–17 positive turns were formed in the reaction

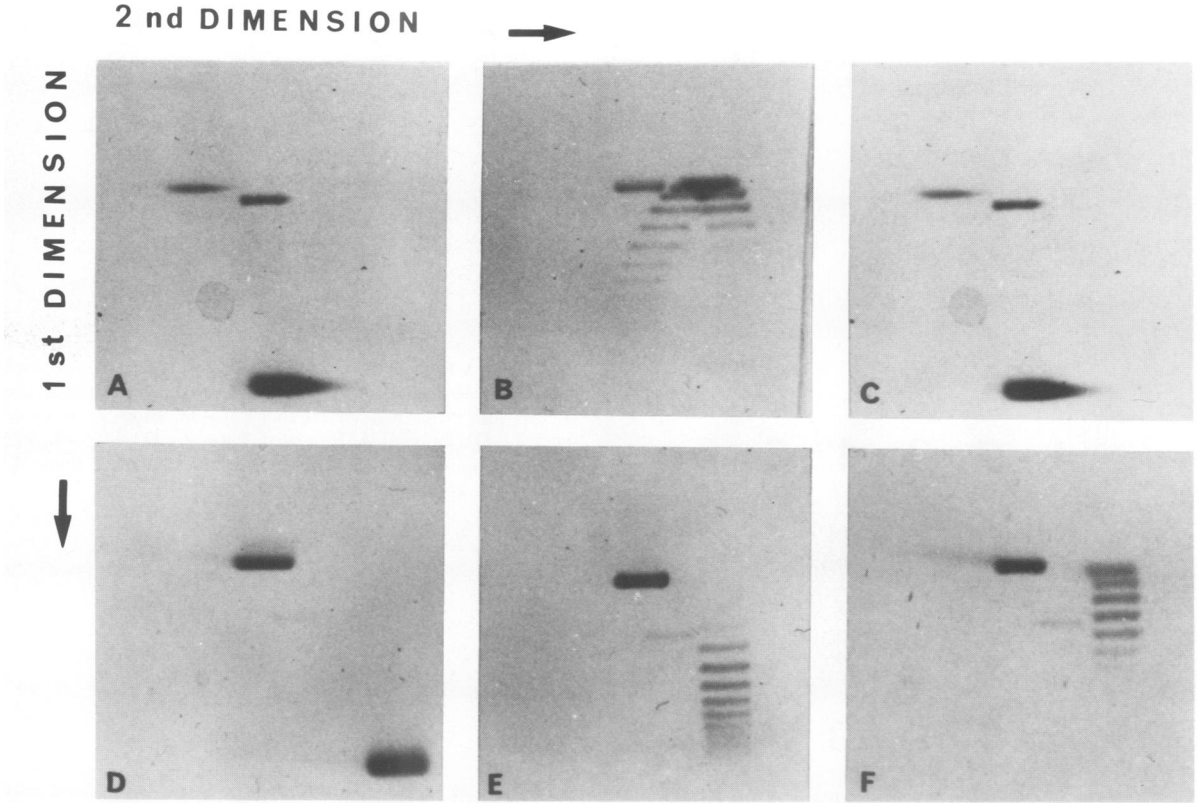

Fig. 2. Two dimensional mini-gel analysis of the topological conversions catalysed by *Sulfolobus* topoisomerase. The analysis was performed as described in Materials and methods. **Panels A** and **C** are pBR322 (0.25 μg) control. The three visible bands are, respectively: negatively supercoiled DNA (lower band), form II (upper band), negatively supercoiled dimer (leftmost band). In the conditions used, negative form I migrated slightly faster than form II in the second dimension. **Panel B** is relaxed pBR322 control: the left part of the arch contains negatively supercoiled DNA; the right part contains positively supercoiled DNA. **Panel D**, pBR322 DNA (0.25 μg) after incubation with 2 μl (0.2 μg) *Sulfolobus* topoisomerase (FPLC pool) in the presence of 10% PEG for 30 min at 75°C (see Materials and methods). **Panel E,** same as **D**, but in the absence of PEG. **Panel F,** same as **E**, but ATP omitted. Upper band in **D, E, F,** is form II.

mixture at 75°C (if one assumes a linear change in the twist of the DNA with temperature up to 75°C).

In the type of analysis shown in Figure 3, the protein-to-DNA ratio was usually ~0.05 (w/w), and the production of positive supercoils was efficient with a ratio as low as 0.006 (w/w), suggesting that the reaction was clearly catalytic rather than simply due to stoichiometric binding. For comparison, negative supercoiling by the concerted action of a DNA-binding protein and a topoisomerase occurs at protein-to-DNA ratio in the range of 0.2 – 1.0 (w/w) for protein HU (Rouvière-Yaniv *et al.*, 1979) and for protein HMG1 (Duguet *et al.*, 1981). Positive supercoiling was fully efficient with ATP concentrations as low as 5 – 10 μM (Figure 4, lanes 4,5). In the same range of concentrations, the non-hydrolysable analogue adenosine-5′-O-(3 thiotriphosphate) (ATP-γS) also sustained the production of positive supercoils, but to a lesser extent (Figure 4, lanes 9,10), suggesting that ATP hydrolysis is necessary to perform efficient supercoiling.

Polyethylene glycol strongly stimulates positive supercoiling

To check the possibility of obtention of a higher degree of positive superhelicity, we have tested several agents known to condensate DNA and to stimulate topoisomerase activity: one of these, the hydrophilic polymer polyethylene glycol (PEG 6000) remarkably improved positive supercoiling activity. When adjusted to 10%, it allowed the reaction to proceed towards high positive superhelical density. In agarose gels, the reaction product appeared as a unique band with a mobility equivalent to that of negative form I DNA (Figure 3B, lane 12). When run in a second dimension in the presence of chloroquine, its mobility remained maximal (Figure 2, panel D), while that of negative form I was reduced (Figure 2, panel A), indicating that it was made of positive supercoils. Again, incubation of this species with type I topoisomerase yielded relaxed DNA, suggesting that it was not made of knots. In these new conditions, not only the superhelical density (compare Figure 2, panel D with E), but also the rate of the reaction (compare Figure 3B, lanes 8 – 12 with lanes 2 – 6) were considerably increased. The reaction was highly processive, with no intermediate topoisomers (Figure 3B, lane 12), negatively supercoiled pBR322 being directly converted to positively supercoiled DNA. In this reaction, the linking number of pBR322 was increased by nearly 50.

Positive supercoiling of relaxed DNA

In the experiments described so far, negatively supercoiled pBR322 was used as a substrate. Is the enzyme able to perform a true 'reverse gyrase' activity, i.e., to produce positive supercoils, starting from relaxed DNA? Figure 5 shows that indeed it can. The reaction was completely ATP-dependent (Figure 5, lanes 1,2) and considerably stimulated by PEG (Figure 5, lanes 3,4). In the presence of PEG, positive supercoiling was as efficient as negative supercoiling by eubacterial gyrase. Again, both the rate of the reaction and the superhelical density of the products were increased, and the reaction was highly processive, with no intermediate product (Figure 5, lane 4).

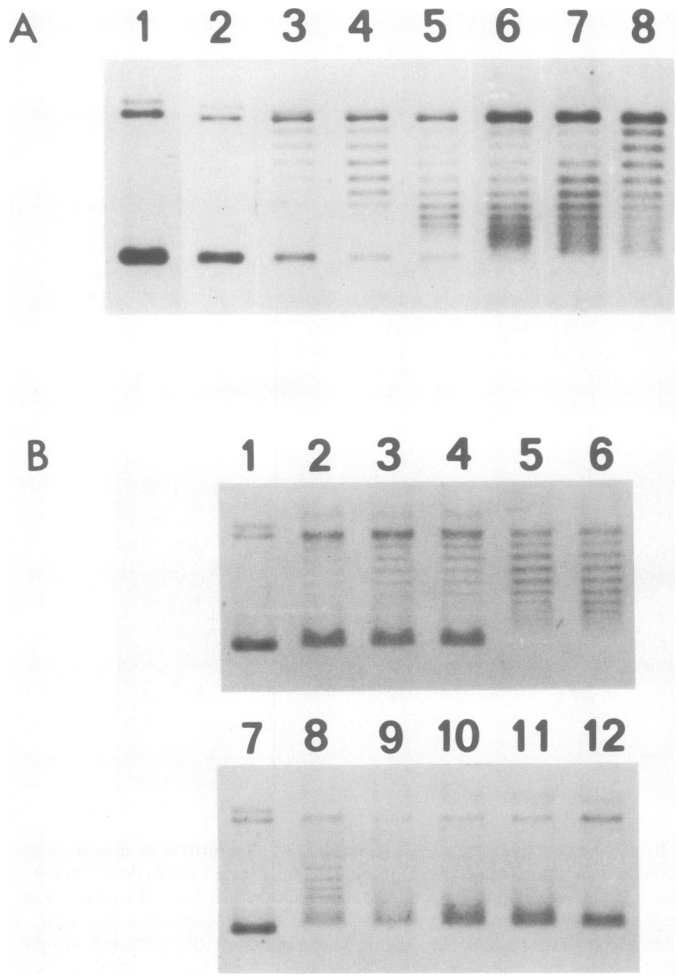

Fig. 3. Optimal conditions for positive supercoiling. 0.4 μg pBR322 form I was used in all the incubations. **(A)** Requirement for magnesium. DNA was incubated with 2 μl (0.02 μg) FPLC fraction in the standard assay for positive supercoiling (PEG omitted), with increasing concentrations of magnesium and analyzed in agarose gel. **Lane 1,** pBR322 control; **lanes 2–8** contained, respectively, 0.0; 0.05; 0.2; 1.0; 5.0; 15.0; 40.0 mM $MgCl_2$. The lower band in **lanes 1–5** is negatively supercoiled DNA. **(B)** Kinetics of positive supercoiling. DNA was incubated as above in the standard assay (5 mM $MgCl_2$) in the absence **(lanes 2–6)**, or in the presence **(lanes 8–12)** of 10% PEG and analyzed in agarose gels. **Lanes 1** and **7**, DNA control; incubation times were 1 min **(lanes 2,8)**; 2 min **(lanes 3,9)**; 4 min **(lanes 4,10)**; 10 min **(lanes 5,11)**; 30 min **(lanes 6,12)**. The lower band in **lanes 1–4** and **lane 7** is negatively supercoiled pBR322. All the other topoisomers in **B** are positively supercoiled, including the lower band visible in **lanes 8–12**.

Activities in the absence of ATP

An ATP-independent DNA relaxation activity co-eluted with positive supercoiling during our purification scheme. This activity was very low after phosphocellulose chromatography. In contrast, after an FPLC additional step, fractions active in positive supercoiling were able to relax efficiently the DNA substrate in the absence of ATP. Surprisingly, the products of this ATP-independent relaxation, analyzed on a two-dimensional gel (Figure 2, panel F) appeared to contain a few (3–5) positive superhelical turns.

ATP-driven topoisomerisations change the linking number of the DNA by step of 1

The above experiments could suggest that our FPLC fraction contained both an ATP-independent type I relaxation enzyme and an ATP-dependent type II 'reverse gyrase'. To detect the putative type II activity, we have analyzed the variations of the linking number of the DNA after 4 min reaction in 20 mM potassium, i.e., in conditions where topoisomerisation (relaxation and posi-

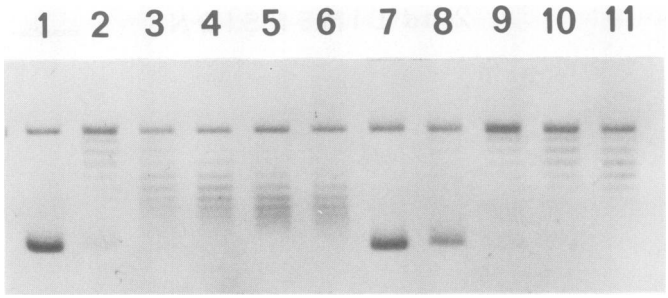

Fig. 4. ATP and ATP-γS requirements for positive supercoiling. 0.4 μg pBR322 DNA form I was incubated with 2 μl (0.16 μg) topoisomerase (phosphocellulose pool) in the standard assay for positive supercoiling (PEG omitted) with increasing concentrations of ATP **(lanes 2–6)** or ATP-γS **(lanes 7–11)**. **Lane 1,** ATP omitted; **lanes 2–11,** ATP and ATP-γS concentrations were 0.2 **(lanes 2,7)**; 0.8 **(lanes 3,8)**; 3.2 **(lanes 4,9)**; 12.8 **(lanes 5,10)**; and 50 μM **(lanes 6,11)**.

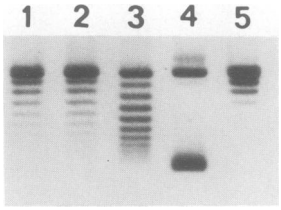

Fig. 5. Positive supercoiling from relaxed DNA. 0.4 μg covalently closed relaxed pBR322 was incubated with 2 μl (0.16 μg) topoisomerase (phosphocellulose pool) in the standard assay for supercoiling, and analyzed in agarose gel. **Lane 4,** complete reaction in the presence of 1 mM ATP and 10% PEG; **lanes 1** and **3,** PEG omitted; **lanes 1** and **2,** ATP omitted; **lane 5** is relaxed pBR322 control. The upper band visible in **lane 4** is positively supercoiled dimer.

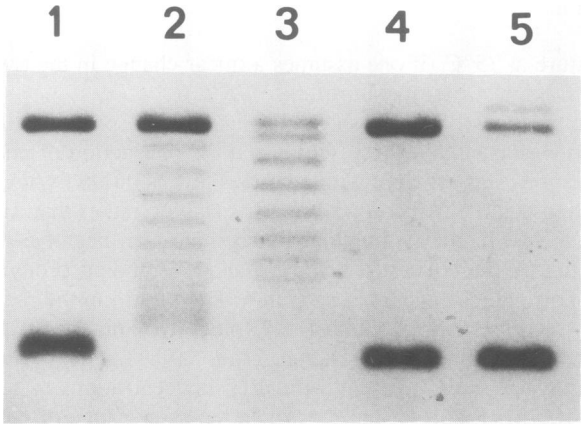

Fig. 6. Variations in the linking number of the DNA. 0.4 μg pBR322 DNA with a unique linking number, prepared as described in Materials and methods, was incubated with 2 μl (0.16 μg) topoisomerase (phosphocellulose pool) for 4 min at 75°C in the standard mixture, and topoisomers were analyzed in agarose gel. **Lane 1,** unique topoisomer incubated in the absence of ATP; **lane 2,** same incubation as in **1**, but in the presence of ATP; **lane 3,** pBR322 control incubated in the presence of ATP; **lanes 4** and **5** are unique topoisomer and pBR322 controls, repsectively. Note that topoisomers in **lane 2** are negatively supercoiled, while those in **lane 3** are positively supercoiled.

tive supercoiling) was completely dependent on ATP. Figure 6 shows the ladder of topoisomers (lane 2) obtained after 4 min incubation of a population of negatively supercoiled pBR322 topoisomers which had the same linking number (see Materials and methods). Comparison with pBR322 control (lane 3) indicates that, unexpectedly, the linking number of these topoisomers differs by increments of 1. In the absence of ATP, no reaction apparently occurred (lane 1). Analysis of the product of incubation shown in lane 1 in a chloroquine gel indicates that no redistribution of the topoisomer with a unique linking number occurred (not shown). This suggest that the above result was not due to the presence of a trace amount of ATP-independent relaxing activity. The enzyme was less active on the topoisomer with a unique linking number (presumably due to the method of preparation) than on native pBR322. Consequently, the DNA shown in Figure 6, lane 2 was only partially relaxed and not positively supercoiled, as was the DNA in lane 3. Nevertheless, Prunell *et al.* (in preparation), using small DNA circles (635 bp), showed that our fractions introduce positive supercoiling by step of 1. These results suggest that ATP-dependent relaxation and positive supercoiling are performed by the same enzyme, which is a type I DNA topoisomerase.

Discussion

We have described here the isolation of a 'positive supercoiling' activity in the archaebacterium *S. acidocaldarius*. A similar activity has been previously described by Kikuchi and Asai (1984) in another strain of the same organism. In addition, we have shown that: (i) positive supercoiling can be performed efficiently from relaxed DNA, as well as from negative supercoils; (ii) the reaction is strongly stimulated by polyethylene glycol and is clearly catalytic and processive; (iii) several lines of evidence suggest the action of a type I rather than a type II topoisomerase.

Stimulation by PEG extends the list of DNA-metabolizing enzymes activated by this compound, which includes DNA and RNA ligases, eukaryotic type II DNA topoisomerase and *E. coli* ω protein (type I topoisomerase) (Miller *et al.*, 1981; Zimmerman and Pfeiffer, 1983; Harrison and Zimmermann, 1984). PEG increased the reaction rate of positive supercoiling and allowed higher superhelical density to be obtained. This argues against the idea that the intrinsic structure of this DNA would prevent full supercoiling in some experiments (Snounou and Malcolm, 1984). This reaction allows for the first time the manufacture of positively supercoiled form I DNA in the absence of any intercalating drug. It is now possible to study the biological activity as well as the physicochemical properties of this fully supercoiled DNA without interfering material.

The experiments reported here, using pBR322 with a unique linking number, together with the results of Prunell *et al.*, using small relaxed DNA circles (635 bp) indicate that ATP-driven topoisomerisations (relaxation and positive supercoiling) increase the linking number of the DNA by step of 1. These results are consistent with the hypothesis that a single ATP-stimulated type I topoisomerase could account for the observed properties, i.e., ATP-dependent relaxation and positive supercoiling. Several other lines of evidence support this hypothesis: (i) ATP-independent relaxation co-purifies with ATP-dependent relaxation and positive supercoiling through phosphocellulose and FPLC column fractionation and by glycerol gradient sedimentation in 1 M salt. The products of this ATP-independent relaxation contain a few positive supercoils, suggesting a common basic mechanism with the ATP-dependent positive supercoiling. Clearly, a source of energy is necessary for ATP-independent positive supercoiling and may be supplied by the energy contained in the negatively supercoiled substrate. This idea is supported by the observation that positive supercoiling did not occur from relaxed DNA in the absence of ATP. (ii) All the observed topoisomerisations (relaxation and positive supercoiling with or without ATP) correspond to the same reaction, i.e., an increase in the linking number of DNA. ATP appears to stimulate both the rate and the extent of this increase. The reverse reaction, relaxation of positively supercoiled DNA (a decrease in the linking number), was never observed in our hands, neither in the presence, nor in the absence of ATP. (iii) Usually, ATP-dependence is considered to be characteristic of type II topoisomerase. However, the mode of ATP utilization by the *Sulfolobus* enzyme seems different from that used by type II topoisomerases where stimulation occurs at much higher concentrations (Sugino *et al.*, 1978; Duguet *et al.*, 1983), and ATP cannot be substituted by ATP-γS. An ATP-stimulated type I topoisomerase has been described in vaccinia virus (Foglesong and Bauer, 1984). (iv) Pefloxacin, a strong inhibitor of bacterial type II topoisomerase (Badet *et al.*, 1982) as well as the non-intercalating anti-cancer drug Epipodophyllotoxin, a strong inhibitor of eukaryotic type II topoisomerase (Chen *et al.*, 1984), did not inhibit positive supercoiling. Similarly, novobiocin, an inhibitor of both eukaryotic and prokaryotic type II topoisomerases did not inhibit positive supercoiling *in vitro*, although it inhibited *Sulfolobus* growth (Brock *et al.*, 1972). (v) In our hands, fractions active in positive supercoiling failed to perform catenation or decatenation of covalently closed circles, reactions which are characteristic of type II topoisomerases. (vi) The most active fractions on FPLC column contained only one polypeptide of high mol. wt. (> 100 000). The size of this polypeptide (132 000) is in the range of those of eukaryotic and eubacterial type I topoisomerase: it is interesting to note that the *Drosophila* type I topoisomerase is a monomer of 135 000 (Javaherian *et al.*, 1982). (vii) The sedimentation coefficient of ATP-dependent positive supercoiling activity is identical to that of ATP-independent activity (7.5S) and might correspond to a monomer of 132 000. At this stage of purification, we cannot yet conclude if positive supercoiling is an intrinsic property of the topoisomerase or if it is due to the catalytic stimulation of the enzyme by one or several factors present in our preparation. Kikuchi and Asai reported a variation by steps of 2 in the linking number of the DNA for the reverse gyrase. One hypothesis to explain the discrepancy with our results is to consider that *Sulfolobus* contains both a type I and a type II reverse gyrase.

In our search for DNA topoisomerases in *Sulfolobus*, we were puzzled by the report of Kikuchi and Asai describing four distinct enzymatic fractions (three type II ATP-dependent and one type I ATP-independent). Especially intriguing was the fact that two of these fractions (3 and 5) did not bind phosphocellulose: we repeatedly failed to detect topoisomerase activities in the non-adsorbed fraction of our phosphocellulose columns. Two non-exclusive explanations can account for the observation of multiple topoisomerase activities with different catalytic properties. (i) Because the purification scheme of Kikushi and Asai did not include a step which removed nucleic acids from the crude lysate, fractions 3 and 5 might correspond to DNA-bound topoisomerase: these fractions would no longer bind phosphocellulose. (ii) Other protein factors might interact with topoisomerase(s) and modify its chromatographic behavior, as well as its catalytic properties. Thus, the four activities described by Kikuchi and Asai could be explained by two or even only one ATP-stimulated 'reverse gyrase'. If the enzyme is a type I topoisomerase, then, *Sulfolobus*

will resemble eukaryotes and eubacteria with a type I prominent enzyme. However, a significant difference is the stimulation of this enzyme by ATP in *Sulfolobus*.

Possible physiological roles for positive supercoiling have been previously discussed by Kikuchi and Asai (1984). An attractive hypothesis is that 'normal' gyrase exists in *Sulfolobus* and that the level of superhelicity of the DNA in this organism is controlled by a balance between gyrase and 'reverse gyrase'.

Materials and methods

Purification of topoisomerase(s)

Preparation of the crude lysate and polyethyleneimine (polymin P) precipitation were performed as described (Mirambeau *et al.*, 1984) except that 15 g of bacteria were used and the lysis buffer contained 200 mM NH_4Cl instead of 22 mM. After centrifugation of the polymin P precipitate for 15 min at 20 000 *g*, the supernatant (40 ml) was kept and the pellet washed twice with 30 ml of lysis buffer containing 1 M NH_4Cl. The supernatants were clarified by centrifugation for 1 h at 50 000 r.p.m. in a 60 Ti Beckman rotor, pooled, and proteins were precipitated by addition of solid ammonium sulfate (0.564 g/ml). The precipitate was centrifuged for 15 min at 20 000 *g* and redissolved in 20 ml of 0.2 M potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 1 mM DTT, and 20% glycerol (buffer A). The fraction obtained (30 ml, 250 mg of proteins) was dialyzed against 1 liter of buffer A for 4 h, and applied to a 30 ml phosphocellulose column equilibrated in the same buffer at a rate of 15 ml/h. The column was washed with 60 ml of buffer A, and developed with a 0.2 – 0.8 M linear phosphate gradient (2 x 100 ml). Fractions active in positive supercoiling (Figure 1) were pooled (Fraction III, 80 μg/ml), dialyzed against 50 mM Tris-HCl, pH 7.0, 1 mM EDTA, 1 mM DTT, 5% glycerol (buffer B) and applied to a 1.5 ml FPLC column (sulfopropyl type, Pharmacia). The column was washed with 4 volumes of buffer B and developed by a linear KCl gradient (0.0 – 0.6 M). Fractions containing positive supercoiling activity were pooled (Fraction IV, 10 μg/ml). This fraction was stable for several months at 4°C.

Assay for positive supercoiling

The standard reaction mixture (20 μl) contained 50 mM Tris-HCl, pH 7.9, 0.5 mM DTT, 5 mM $MgCl_2$, 40 mM KCl, 1 mM ATP, 30 μg/ml bovine serum albumin and 0.4 μg pBR322 DNA (negatively supercoiled or relaxed). Eventually, 10% PEG 6000 was added. After addition of 2 μl of the fraction to be assayed, the mixture was incubated for 30 min at 75°C. The reaction was stopped by addition of 40 mM EDTA, 1% SDS, 0.25 mg/ml bromophenol blue, and 15% (w/v) sucrose. The products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide and photographed under u.v. illumination, as previously described (Duguet *et al.*, 1983). In some experiments, electrophoresis was performed in the presence of 3 μg/ml chloroquine.

Two-dimensional agarose minigels

Analysis of topoisomerisation products was occasionally carried out by two-dimensional agarose gel electrophoresis in a Biorad mini-sub cell. In the first dimension the DNA migrated at 60 V for 3 h in 36 mM Tris, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8 (TEP buffer). The gel was then soaked in TEP buffer containing 3 μg/ml chloroquine for at least 3 h. The second dimension was run in the same chloroquine containing buffer for 1 h 45 min at 60 V. The gel was stained with ethidium bromide and photographed as described (Duguet *et al.*, 1983).

Preparation of pBR322 with a unique linking number

Supercoiled pBR322 (90 μg) prepared as previously described (Duguet *et al.*, 1983) was submitted to electrophoresis in 1% agarose gel containing 9 μg/ml chloroquine for 15 h at 1.5 V/cm. After staining with ethidium bromide, topoisomers, visualized under u.v. illumination with low intensity, were cut out of the gel and electroeluted in dialysis bags for 16 h at 1 V/cm. Eluted DNA was extracted with phenol/chloroform, adjusted to 0.15 M NaCl, and ethanol precipitated. 3 – 4 μg each of four different topoisomers were recovered.

Chemicals and drugs

PEG 6000 was purchased from Merck, SDS was from Serva. Ethidium bromide, ATP, and ATP-γS were from Boehringer. Polyethyleneimine (polymin P), chloroquine, and Novobiocin were from Sigma. Pefloxacin and Epipodophyllotoxin were generous gifts of Professor F. Le Goffic, CNRS, Thiais and Professor J. C. David, Université de Rennes. Agarose ultra pure was from IBF France.

Other procedures

For glycerol gradient sedimentation of fraction III, 100 μl (40 μg) were loaded on the top of a 5 ml (15 – 40%) glycerol gradient in 0.2 M potassium phosphate, pH 7.4, 1.0 M KCl, 1.0 mM EDTA. After 20 h sedimentation in a SW 50.1 Beckman rotor at 50 000 r.p.m., 180 μl fractions were collected and tested for their activity of positive supercoiling.

SDS-polyacrylamide gel electrophoresis was run according to the method of Laemmli (1970) and proteins revealed by silver staining (Switzer *et al.*, 1979).

Protein concentration was determined according to Bradford (1976).

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References

Badet,B., Hughes,P., Kohiyama,M. and Forterre,P. (1982) *FEBS Lett.*, **145**, 355-359.

Bradford,M.M. (1976) *Anal. Biochem.*, **72**, 248-254.

Brock,T.D., Brock,K.M., Belly,R.T. and Weiss,R.L. (1972) *Arch. Microbiol.*, **84**, 54-68.

Chen,G.L., Yang,L., Rowe,T.C., Halligan,B.D., Tewey,K.M. and Liu,L.F. (1984) *J. Biol. Chem.*, **259**, 13560-13566.

Cozzarelli,N.R. (1980) *Science (Wash.)*, **207**, 953-960.

Di Nardo,S., Voelkel,K.A., Sternglanz,R., Reynolds,A.E. and Wright,A. (1982) *Cell*, **31**, 35-42.

Drlica,K. (1984) *Microbiol. Rev.*, **48**, 273-289.

Duguet,M., Bonne,C. and De Recondo,A.M. (1981) *Biochemistry (Wash.)*, **20**, 3598-3603.

Duguet,M., Lavenot,C., Harper,F., Mirambeau,G. and De Recondo,A.M. (1983) *Nucleic Acids Res.*, **11**, 1059-1075.

Fischer,L.M. (1984) *Nature*, **307**, 686-687.

Foglesong,P.D. and Bauer,W.R. (1984) *J. Virol.*, **49**, 1-8.

Gellert,M. (1981) *Annu. Rev. Biochem.*, **50**, 879-910.

Glikin,G.C., Ruberti,I. and Worcel,A. (1984) *Cell*, **37**, 33-41.

Goto,T., Laipis,P. and Wang,J.C. (1984) *J. Biol. Chem.*, **259**, 10422-10429.

Harland,R.M., Weintraub,H. and McKnight,S.L. (1983) *Nature*, **302**, 38-43.

Harrison,B. and Zimmerman,S.B. (1984) *Nucleic Acids Res.*, **12**, 8235-8281.

Javaherian,K., Tse,Y.-K. and Vega,J. (1982) *Nucleic Acids Res.*, **10**, 6945-6955.

Kikuchi,A. and Asai,K. (1984) *Nature*, **309**, 677-681.

Laemmli,U.K. (1970) *Nature*, **227**, 680-685.

Miller,K.G., Liu,L.F. and Englund,P.T. (1981) *J. Biol. Chem.*, **256**, 9334-9339.

Mirambeau,G., Duguet,M. and Forterre,P. (1984) *J. Mol. Biol.*, **179**, 559-563.

Pruss,G.J., Manes,S.H. and Drlica,K. (1982) *Cell*, **31**, 35-42.

Rouvière-Yaniv,J., Yaniv,M. and Germond,J.E. (1979) *Cell*, **17**, 265-274.

Ryoji,M. and Worcel,A. (1984) *Cell*, **37**, 21-32.

Shelton,E.R., Osheroff,N. and Brutlag,D.L. (1983) *J. Biol. Chem.*, **258**, 9530-9535.

Snounou,S.B. and Malcolm,A. (1984) *Eur. J. Biochem.*, **138**, 275-280.

Sugino,A., Higgins,N.P., Brown,P.O., Peebles,C.L. and Cozzarelli,N.R. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4838-4842.

Switzer,R.C., Merril,C.R. and Shifrin,S. (1979) *Anal. Biochem.*, **98**, 231-236.

Villeponteau,B., Lundell,M. and Martinson,H. (1984) *Cell*, **39**, 469-478.

Wang,J.C., Peck,L.J. and Becherer,K. (1983) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 85-91.

Woese,K. (1981) *Sci. Am.*, **244**, 98-122.

Zillig,W., Schnabel,R. and Stetter,K.O. (1984) *Curr. Top. Microbiol. Immunol.*, in press.

Zimmerman,S.B. and Pfeiffer,B.H. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 5852-5856.

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