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Reverse gyrase; ATP-dependent type I topoisomerase from *Sulfolobus*

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Reverse gyrase, a topoisomerase which introduces positive superhelical turns into DNA, has been purified from *Sulfolobus* to near homogeneity. It is a single polypeptide with a mol. wt. of 120 000 as determined by denaturing gel electrophoresis. Contrary to a previous report, it is a type I topoisomerase as judged by the linking-number change of closed circular DNA topoisomer. Unlike other known type I topoisomerases, ATP or dATP is required for introducing positive superhelical turns. In order to relax negatively supercoiled DNA, other nucleotide triphosphates (XTP) are also effective with low efficiency. In the absence of either XTP or divalent cations, the enzyme introduces nicks into closed circular DNA when the reaction is stopped by SDS. This suggests that reverse gyrase cuts one of the two strands of DNA in the course of its enzymatic reaction.

Key words: ATP requirement/nicked intermediate/positive supercoiling/*Sulfolobus*/type I topoisomerase

Introduction

Superhelicity is one of the factors which affect biological functions of genes (Cozzarelli, 1980). Negative superhelicity is favored in many biological processes (reviewed by Drlica, 1984). The various topoisomerases affect the degree of superhelicity of each DNA molecule by changing the linking numbers (Gellert, 1981; Wang, 1982). Three kinds of reaction are known; one to remove negative or positive superhelicity and another to introduce negative superhelical turns into DNA. A third type of topoisomerase, reverse gyrase, found in the extract of *Sulfolobus*, a thermoacidophilic archaebacterium, introduces positive superhelical constraints (Kikuchi and Asai, 1984).

The topoisomerases are divided into two categories according to their reaction mechanisms. Type I enzymes cut and seal one of the two strands of the DNA substrate, and change the linking number of the topoisomer by an integral number, while type II topoisomerases cause breakage and reunion of both strands, and change the linking number of the topoisomer by integral multiples of two. It is by this mechanism of type II topoisomerase that knotted or catenated double-stranded DNA molecules can be disentangled (Goto and Wang, 1982). The enzymatic reaction of type II topoisomerases requires ATP hydrolysis regardless of the addition or removal of superhelicity, while all the type I enzymes do not require ATP as the energy source (reviewed by Gellert, 1981).

We purified reverse gyrase from *Sulfolobus* to near homogeneity. The enzyme consists of a single polypeptide moiety. Using this pure material we re-examined the properties of this unique enzyme and found that reverse gyrase is a type I topoisomerase: therefore the previous interpretation with partially purified materials (Kikuchi and Asai, 1984) was apparently not correct. Nevertheless, as reported previously, the enzyme requires ATP to introduced positive superhelical turns into DNA. In this respect it is unlike all the other type I topoisomerases. We report here the purification of this enzyme, the properties of its enzymatic reaction and the formation of a nicked intermediate.

Results

Purification

Each purification step is described in detail in Materials and methods and summarized in Table I. Negatively supercoiled pBR322 was used as a substrate and the reaction was terminated by addition of SDS and EDTA. It was previously reported that reverse gyrase was eluted between 0.15 and 0.3 M NaCl by DEAE Sephacel chromatography (Kikuchi and Asai, 1984). When we included a Polymin P precipitation step under high ionic strength (0.6 M NaCl) to remove DNA prior to ammonium-sulfate precipitation (fraction II), most activity then flowed through a DEAE Sephacel column (fraction III). Some activity was still bound to the column and could be eluted by 0.25 M NaCl buffer. This material cross-reacted with anti-reverse gyrase serum (data not shown). Fraction III was fractionated by phosphocellulose column chromatography. At this step, reverse gyrase was separated from the bulk of proteins (Figure 1, fractions 40–45; fraction IV). Another topoisomerase, type II, which could unknot knotted DNA extracted from defective particles of P4 phage was eluted with the major protein peak (Figure 1, fractions 19–22) and separated from reverse gyrase. Relaxing activity of negative supercoiled DNA by this enzyme was 100-fold less than that of reverse gyrase. Type II topoisomerase was further purified by hydroxylapatite chromatography (manuscript in preparation). This enzyme can relax negatively or positively supercoiled DNA (see Figure 5 for relaxation of negative supercoils).

Fraction IV was fractionated by heparin-Sepharose column chromatography. At this step, the activity coincided with the major protein peak (Figure 2, fraction V). It was further purified by gel filtration under high ionic strength (0.5 M NaCl) (fraction VI) because at low ionic strength (<0.2 M), reverse gyrase was eluted with a broad peak beginning at the void volume (data not shown). The mol. wt. of reverse gyrase estimated by gel filtration was 120 000 (Figure 3). The protein composition of each fraction was examined by SDS-polyacrylamide gel electrophoresis (Figure 4). A single polypeptide with a mol. wt. of 120 000 was observed as a major constituent in fraction IV and other bands were hardly detectable in fraction VI. Combined with the result of gel filtration, we concluded that the polypeptide of 120 000 was reverse gyrase and that it was monomeric under high ionic strength.

Linking-number change

We previously reported that reverse gyrase was a type II topoisomerase because a partially-purified fraction of reverse gyrase

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Table I. Summary of reverse gyrase purification

Fraction	Total protein (mg)	Total activity ($\times$ 10^6 units)	Specific activity ($\times$ 10^6 units/mg)	Yield[a] (%)
I. Crude lysate	2457	1935	0.78	100
II. Polymin P and ammonium sulfate precipitation	910	1950	2.14	100.8
III. DEAE Sephacel	711	1380	1.94	71.3
IV. Phosphocellulose	5.28	1080	204.5	55.8
V. Heparin Sepharose	0.828	720	869.5	37.2
VI. Sephacryl S-300	0.204	298	1460.8	15.4

[a]Total activity of crude lysate taken as 100%.

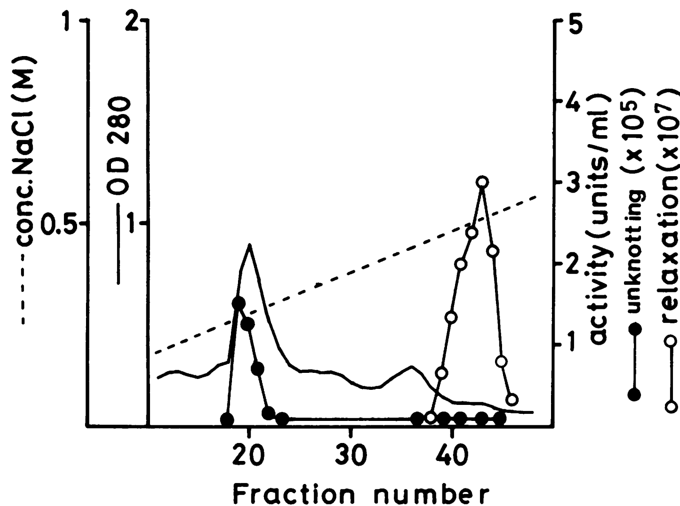


Fig. 1. Phosphocellulose column chromatography. Fractions of phosphocellulose column chromatography were assayed by unknotting of P4 DNA (●) and relaxation of negatively supercoiled pBR322 (○) under the standard assay conditions for type II topoisomerase and for reverse gyrase, respectively. The relaxation activity can be seen in the fractions where unknotting activity was detected. However, the activity was too small (of the order of 10^5) to be presented in this scale (10^7). The enzyme units were determined by serial dilutions of the fractions.

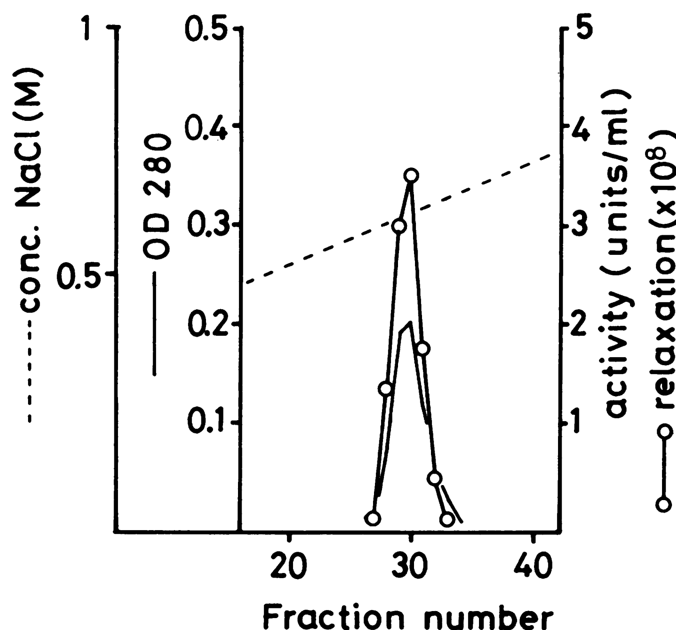


Fig. 2. Heparin-Sepharose column chromatography. Fractions of heparin-Sepharose column chromatography were assayed by relaxation of negatively supercoiled pBR322 (○) under the standard assay condition for reverse gyrase.

changed the linking number of a circular DNA, which had unique negative superhelicity, by integral multiples of two (Kikuchi and Asai, 1984). However, purified reverse gyrase had no unknotting activity. Unknotting P4 duplex DNA requires the transient breakage and rejoining of double-stranded DNA, the specific reaction catalyzed by type II topoisomerase. To solve this discrepancy we repeated the experiment using the purified reverse gyrase. Type II topoisomerase of *Sulfolobus* (see above) was used as a control. As shown in Figure 5, reverse gyrase changed the linking number of the topoisomer with a definite negative superhelicity by an integral number while type II enzyme changed it by integral multiples of two. Judging by two-dimensional gel electrophoresis (Figure 5B), the ladder formed by reverse gyrase was positively supercoiled. Also, the topoisomer with a unique positive superhelicity was shifted to more positive forms by the pure enzyme and the linking number increased one by one (data not shown). Therefore, we conclude that reverse gyrase is a type I topoisomerase. The experiment shown below indicating that nicked circles were recovered from reaction intermediates supports this conclusion.

Effect of salt

The optimal salt concentration was from 160 mM to 200 mM NaCl where the molar ratio of enzyme (if monomeric) to substrate DNA was ~0.01 (Figure 6A). KCl was also effective (data not shown). At high enzyme concentrations (molar ratio = 2.0–5.0), DNA attained higher positive superhelicity at lower salt concentrations from 5 mM to 50 mM (Figure 6B).

Effect of divalent cation

The topoisomerization by reverse gyrase required divalent cations and was inhibited by EDTA. The optimal Mg^{2+} concentration was from 3 mM to 10 mM. Ca^{2+} could be substituted but was slightly less effective (data not shown). Without Mg^{2+}, reverse gyrase introduced nicks into DNA when the reaction was stopped by SDS.

Effect of nucleotide triphosphate

Reverse gyrase relaxes negatively supercoiled DNA and can further introduce positive superhelical turns. It was essential to have ATP or dATP for positive supercoiling (Figure 7), while other XTPs, GTP, CTP, UTP, dGTP, dCTP and dTTP were effective to the extent that every negatively supercoiled molecule became relaxed (Figure 7). This relaxation activity with XTPs other than ATP or dATP was that of type I enzyme and coincided with reverse gyrase activity in the course of purification (data not shown). Only ATP was hydrolyzed to ADP in a DNA-

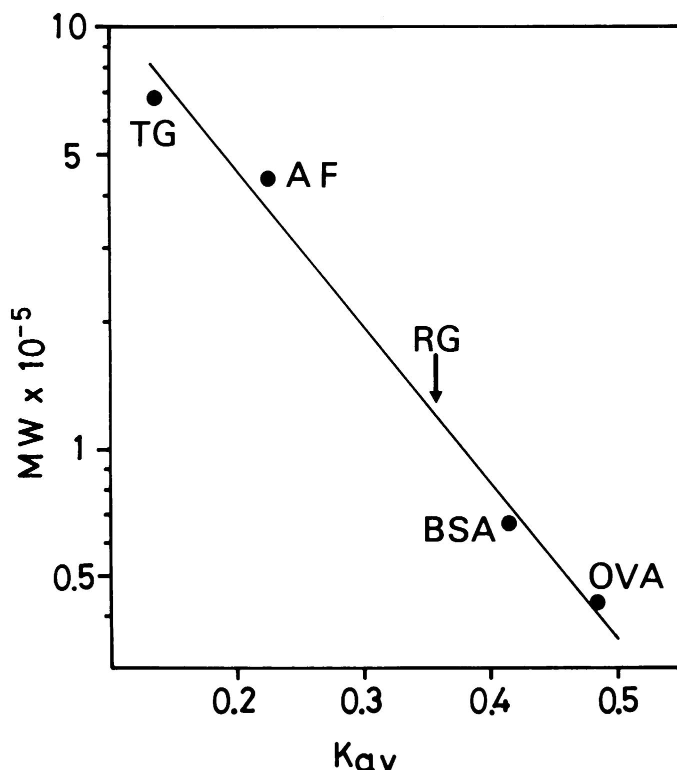


Fig. 3. Sephacryl S-300 gel filtration. Gel filtration was carried out under high ionic strength as described in Materials and methods. Thyroglobulin (TG, 668 000), apoferritin (AF, 440 000), bovine serum albumin (BSA, 68 000) and ovalbumin (OVA, 43 000) were added as internal markers. Each protein was identified by protein assay and SDS-polyacrylamide gel electrophoresis. The position of reverse gyrase was determined by relaxation of pBR322 with serial dilutions of each fraction. Kav = (Ve−Vo)/(Vt−Vo) Vt; column bed volume (Vo; void volume, Ve; elution volume).

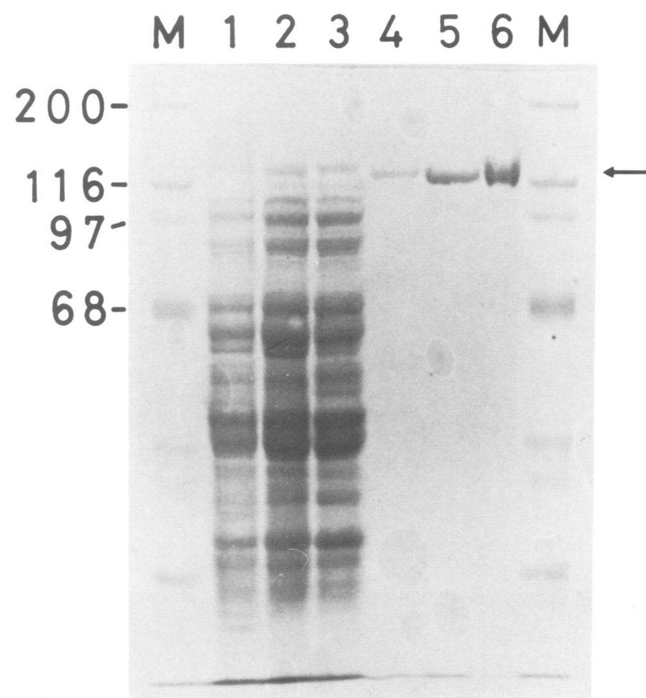


Fig. 4. Proteins in each purification step. Proteins of each purification step (see Table I) were examined by SDS-polyacrylamide (10%) gel electrophoresis. The amounts of protein applied to the gel were 20 μg for fractions I, II, III (**lanes 1, 2, 3**, respectively) and 2 μg for fractions IV, V, VI (**lanes 4, 5, 6**, respectively). Myosin (H chain), β-galactosidase, phosphorylase b, bovine serum albumin, α-chymotrypsinogen, and lysozyme were used as mol. wt. standards (lane M). Mol. wts. × 10^{-3} are indicated on the left of the figure. The arrow on the right of the figure indicates the position of reverse gyrase.

dependent manner, while UTP, CTP or GTP was not (T.Shibata, personal communication). A detailed study of the ATPase activity will be published elsewhere. The kinetics of the ATP-requiring reaction were compared with that of the XTP-requiring reactions shown in Figure 8. Relaxation of negatively supercoiled DNA was slow with GTP although DNA was rapidly relaxed and positively supercoiled with ATP. The minimum concentration of ATP for positive supercoiling was of the order of 10 μM which was far less than the amount of ATP required by type II topoisomerase of *Sulfolobus* (>300 μM).

Effect of pH, temperature, drugs

Reverse gyrase was active in the neutral pH range from pH 6.5 to pH 8.5 (data not shown). The effect of temperature was examined in the range from 55°C to 75°C. The activity was hardly detectable below 60°C as described previously (Kikuchi and Asai, 1984) and was maximum at 75°C (data not shown), which coincided with the growth condition of *Sulfolobus*.

Reverse gyrase was inhibited by several drugs, namely coumermycin, berenil, adriamycin and ethidium bromide (Figure 9). We could not observe any inhibitory effect with novobiocin and nalidixic acid (100 μg/ml) (data not shown). It is not known, however, whether these drugs are stable at 75°C.

Nicked intermediate

Reverse gyrase introduced nicks into negatively supercoiled DNA in the absence of Mg^{2+} or XTP, when the molar ratio of the enzyme to the substrate was 2−6 and the reaction was stopped with SDS (Figure 10). The amount of nicked circles increased with the amount of enzyme (Figure 10A, lanes 4−6, 8−10), and this nicking reaction was inhibited by EDTA (Figure 10A, lanes 2, 3). It was not caused by contamination with DNase because the amount of nicked circles did not increase significantly when the reactions were stopped with EDTA only (Figure 10A, lanes 12,13). When ATP or Mg^{2+} was added back to reaction mixtures for further incubation and the reactions were stopped afterwards with SDS, the amount of nicked circles decreased and the DNA was positively supercoiled (Figure 10A, lanes 7, 11, 10B, lanes 2, 4). These observations suggest that a nick is introduced when reverse gyrase is inactivated by SDS, or alternatively a nick has been introduced by reverse gyrase but the DNA strands are held together by the enzyme, unless SDS denatures the complex.

Discussion

We previously reported a novel topoisomerase, in an extract of *Sulfolobus*, which introduced positive superhelical turns into covalently-closed circular DNA molecules (Kikuchi and Asai, 1984). Although this was a catalytic reaction, the positive supercoiling might have been caused by a single unique enzyme or by the combined action of a DNA-binding protein and a regular topoisomerase (Brown *et al.*, 1979). It was therefore important to define the molecular constituents of reverse gyrase. As shown above, reverse gyrase is a single polypeptide of mol. wt. 120 000 (Figure 4), and it catalyzes positive supercoiling (Figure 8).

The extensively purified enzyme was unable to unknot P4 DNA. This was contradictory to the previous observation with a partially-purified enzyme preparation (Kikuchi and Asai, 1984).

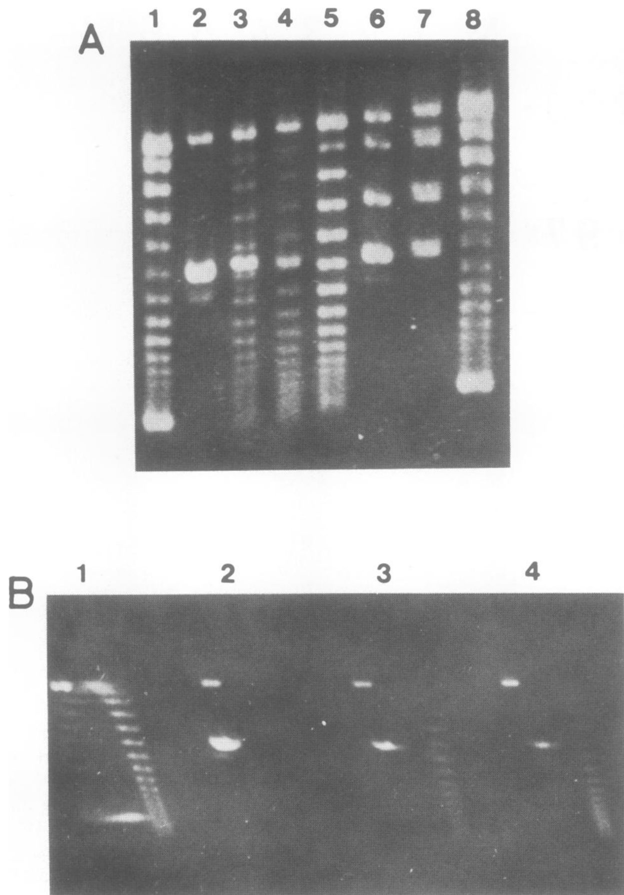


Fig. 5. Change in the linking number by reverse gyrase. A negatively supercoiled topoisomer with a unique linking number was incubated with reverse gyrase or type II topoisomerase of *Sulfolobus* at various times and electrophoresed on a 1% agarose gel. (**A**) **Lanes 1, 8**, negatively supercoiled pBR322 marker; **lane 2**, single topoisomer used as a substrate; **lane 3**, incubated with reverse gyrase for 1 min; **lane 4**, incubated with reverse gyrase for 3 min; **lane 5**, positively supercoiled pBR322 marker; **lane 6**, incubated with type II topoisomerase for 1 min; **lane 7**, incubated with type II topoisomerase for 3 min. (**B**) Topoisomers produced by reverse gyrase were examined by two-dimensional gel electrophoresis. Samples were electrophoresed first in the vertical direction (from up to down) in the standard condition and then re-electrophoresed in the horizontal direction (from left to right) in the same buffer containing 0.02 μg/ml ethidium bromide (Kikuchi and Asai, 1984). In arch-shape ladders, negatively supercoiled topoisomers were on the left side and positive ones were on the right side. The relaxed circles were at the top of the arch, while nicked circles were located as a separated spot on the left of the relaxed one. **1**, topoisomer markers of negatively and positively supercoiled pBR322. **2**−**4**, the same samples of **lanes 2**−**4** in **A**.

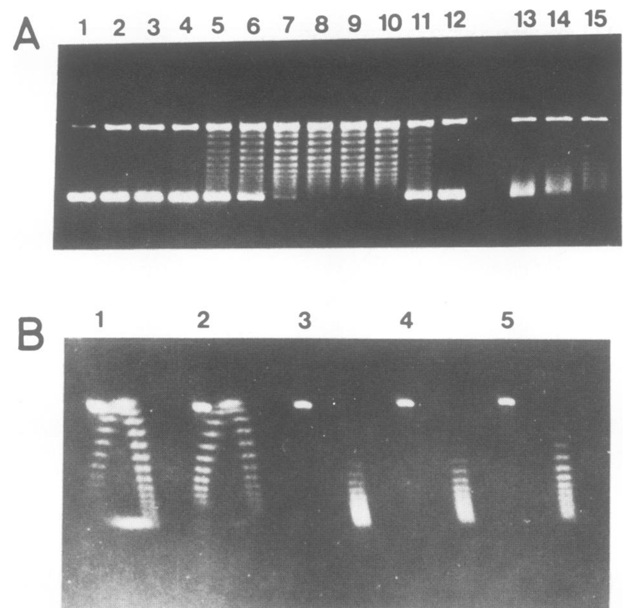
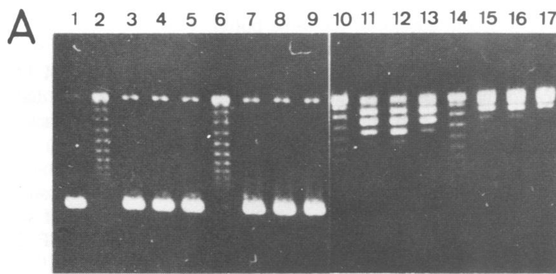


Fig. 6. Effect of NaCl concentration. (**A**) Reverse gyrase was incubated with negatively supercoiled pBR322 under the standard assay condition except that the concentration of NaCl was changed. **Lane 1**, pBR322 control (no enzyme). The concentration of NaCl was 5 mM (**lanes 2, 13**), 20 mM (**lanes 3, 14**), 50 mM (**lanes 4, 15**), 100 mM (**lane 5**), 120 mM (**lane 6**), 140 mM (**lane 7**), 160 mM (**lane 8**), 180 mm (**lane 9**), 200 mM (**lane 10**), 300 mM (**lane 11**), and 400 mM (**lane 12**). The amounts of enzyme added to the mixtures were 1.2 ng/ml (**lanes 2**−**12**) and 0.6 μg/ml (**lanes 13**−**15**). The molar ratios of the enzyme to DNA were 0.01 and 5, respectively, based on the mol. wt. of reverse gyrase as 120 000 and assuming a monomeric enzyme. (**B**) Samples were electrophoresed through two-dimensional gel as described in the legend of Figure 5B. (**1**) Topoisomer markers of positively and negatively supercoiled pBR322. (**2**) The same sample as **lane 10** in **A**. (**3**−**5**) The same sample as **lanes 13**−**15** in **A**.

Therefore we re-examined the linking-number change caused by the pure enzyme. A topoisomer with definite negative or positive superhelicity was converted by reverse gyrase to a series of topoisomers increasing their linking number one by one (Figure 5). On the other hand, the type II topoisomerase of *Sulfolobus*, which was characterized by the unknotting reaction of P4 knotted DNA, was clearly shown to change the linking number of a topoisomer in steps of two (Figure 5A, lanes 6, 7). We noticed that some preparations of a single topoisomer purified from agarose gel could not serve at all as the substrate for reverse gyrase, but were a good substrate for the type II topoisomerase (data not shown), indicating that the DNA preparation might occasionally contain a specific inhibitor of reverse gyrase, but not of the type II enzyme. This may explain the previous misinterpretation of the experiment; the partially-purified enzyme preparation might have been contaminated by a small amount of type II topoisomerase which was active, while the reverse gyrase reaction was blocked by the inhibitor in the topoisomer preparation. Therefore, reverse gyrase is a type I topoisomerase.

Eukaryotic type I topoisomerase acts in the absence of Mg^{2+} and moreover even in the presence of EDTA, while the prokaryotic enzymes, such as ω-protein and topo III of *Escherichia coli* (Dean *et al.*, 1983; Srivenugopal *et al.*, 1984), are both strictly dependent on the presence of Mg^{2+}. The reverse gyrase belongs to the latter class; it requires Mg^{2+} (or Ca^{2+}) for activity, and EDTA is a strong inhibitor of the enzyme reaction (Figure 10A). All the known type II topoisomerases require ATP hydrolysis for their reaction, while type I enzymes have no such requirement. Reverse gyrase seems to be the first exception to the rule, for its enzymatic reaction requires ATP (Figure 7). Vaccinia virus type I topoisomerase is stimulated by ATP but its activity does not depend on it (Foglesong and Bauer, 1984).

When either Mg^{2+} or XTP was removed during incubation and SDS was added afterwards to terminate the reaction, circular DNA was found to be nicked (Figure 10). This only ocurred with the addition of SDS (Figure 10A, lanes 12, 13), and the second incubation with deficient factors resulted in recovery of unbroken DNA (Figure 10B, lanes 2, 4), suggesting that this nicked circle was derived from an intermediate of the type I topoisomerase reaction (reviewed by Gellert, 1981; Wang, 1981). Whether the nicked circle formed by the reverse gyrase is



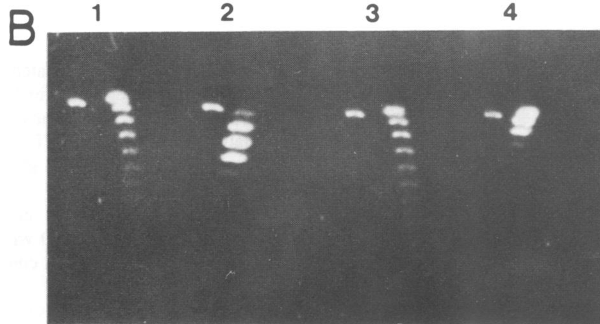


Fig. 7. Effects of XTP and dXTP. (**A**) Reverse gyrase was incubated with pBR322 under the standard assay condition except that ATP was substituted with the other XTP or dXTP. **Lane 1**, control; **lanes 2, 10**, 10 μM ATP; **lanes 3, 11**, 10 μM GTP; **lanes 4, 12**, 10 μM CTP; **lanes 5, 13**, 10 μM UTP; **lanes 6, 14**, 10 μM dATP; **lanes 7, 15**, 10 μM dGTP; **lanes 8, 16**, 10 μM dCTP; **lanes 9, 17**, 10 μM dTTP. Amounts of the enzyme added to mixtures were 0.67 ng/ml (**lanes 2–9**) and 0.2 μg/ml (**lanes 10–17**). (**B**) Some of the above samples were analysed by two-dimensional gel electrophoresis as in Figure 5B. **1** (lane 10), **2** (lane 12), **3** (lane 14), **4** (lane 16).

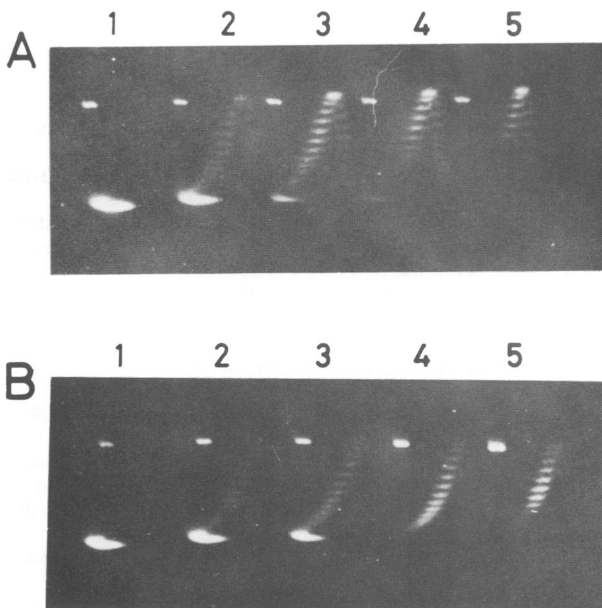


Fig. 8. Kinetics of reverse gyrase. Reverse gyrase (6 ng/ml) was incubated with pBR322 under the standard assay condition except that 1 mM ATP was substituted with 10 μM ATP (**A**) or GTP (**B**). Mixtures were incubated at 75°C for various times, as indicated below, and topoisomers were separated by two-dimensional gel electrophoresis as in Figure 5B. (**A**) **1**; control (no enzyme), **2**; 1 min, **3**; 2 min, **4**; 5 min, **5**; 10 min. (**B**) **1**; control, **2**, 5 min, **3**; 10 min, **4**; 20 min, **5**; 40 min.

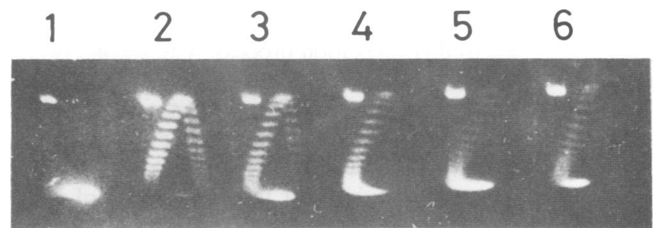


Fig. 9. Effect of drugs. Various drugs were added in the standard reaction mixture. DNA was extracted twice with phenol in order to remove drugs which interfered with gel electrophoresis, and was examined by two-dimensional gel electrophoresis as in Figure 5B. The enzyme concentration added to the mixture was 2.4 ng/ml. **11**, control (no enzyme); **2**, without drugs; **3**, 100 μg/ml coumermycin; **4**, 200 μg/ml berenil; **5**, 1 μg/ml adriamycin; **6**, 3 μg/ml ethidium bromide.

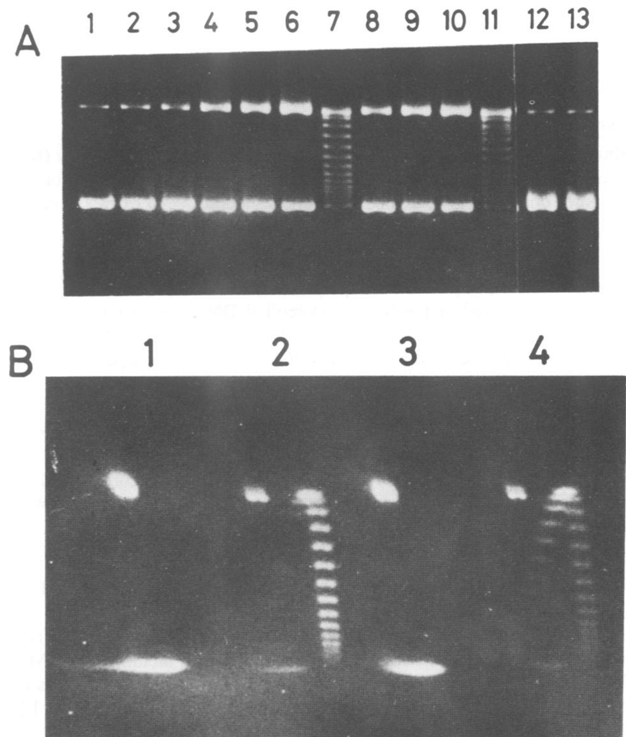


Fig. 10. Formation of nicked circle under deficient conditions. (**A**) Negatively supercoiled pBR322 was incubated with various amounts of reverse gyrase under the standard assay condition except that Mg^{2+} or ATP was excluded as indicated below. **Lane 1**, control; **lane 2**, 10 mM EDTA, no ATP; **lane 3**, 10 mM EDTA, 1 mM ATP; **lanes 4, 5, 6**, no $MgCl_2$, 1 mM ATP; **lanes 8, 9, 10**, 10 mM $MgCl_2$, no ATP. Amounts of the enzyme added to the mixtures were 0.24 μg/ml (**lanes 4, 8**), 0.48 μg/ml (**lanes 5, 9**) and 0.96 μg/ml (**lanes 2, 3, 6, 10**). The same sample as **lane 6** was made to 10 mM $MgCl_2$ and incubated at 75°C for an additional 2 min (**lane 11**). The reaction of the same samples as **lane 6** and **10** were stopped by addition of EDTA (final 20 mM) (**lanes 12, 13**, respectively). (**B**) Some of the samples were examined by two-dimensional gel electrophoresis as in Figure 5B. **1** (lane 6), **2** (lane 7), **3** (lane 10), **4** (lane 11).

covalently linked to the enzyme molecule or is completely dissociated from it by SDS remains to be shown.

In the reaction mechanism of reverse gyrase, we could distinguish two steps; the first step involves relaxation of the negative supercoil and the second step involves supercoiling of the relaxed circle to yield a positively supercoiled product. The first step requires Mg^{2+} and XTP. Although ATP is preferable, other XTPs can also serve as a cofactor and are not necessarily

hydrolyzed. We are not yet certain whether any hydrolysis of ATP takes place in this relaxation process. The second process requires Mg^{2+} and hydrolysis of ATP. Other XTPs cannot support this process (Figures 7,8) and are not hydrolyzed (T.Shibata, personal communication). ATP is the sole energy source for this process. The exact point where ATP is required has yet to be determined. It may be required for the dissociation of the enzyme from the complex and its recycling as presumed from the mechanism of gyrase (Liu and Wang, 1978; Brown and Cozzarelli, 1979).

Reverse gyrase is a monomer at 0.5 M NaCl as judged by the gel-filtration profile (Figure 3). But at 0.2 M NaCl, which is the optimal salt concentration for the reaction (Figure 6), it seems to form aggregates. As the enzyme concentration is far less in the reaction mixture than that in the chromatography, we cannot predict how many enzyme molecules are required for an active complex with a DNA substrate.

Materials and methods

Culture

Medium and growth conditions for *Sulfolobus* were described previously (Kikuchi and Asai, 1984). Cells were harvested by centrifugation at 8000 r.p.m. for 20 min and washed once with growth medium without glucose, yeast extract or casamino acid and stored at −20°C.

DNA

Negatively supercoiled pBR322 was prepared by a conventional method (Maniatis *et al.*, 1982). Relaxed or positively supercoiled DNA was prepared by type II topoisomerase or reverse gyrase of *Sulfolobus*, respectively. DNA topoisomer with a unique superhelicity was prepared as follows: negatively supercoiled pBR322 DNA was partially reacted with type II topoisomerase and topoisomers were separated by agarose gel electrophoresis with low-melting agarose (Sigma type VII), and a unique band was cut out. After melting at 65°C, DNA was extracted twice with phenol and once with chloroform then precipitated by ethanol. Knotted DNA was prepared from defective heads of P4 phage according to Liu and Davis (1981) and nicks at cohesive ends of the DNA were sealed by T4 ligase (Goto and Wang, 1982; Kikuchi and Asai, 1984).

Assay

The standard assay mixture (20 µl) for reverse gyrase contained 0.2 M NaCl, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM spermidine, 1 mM dithiothreitol, 1 mM ATP, 2.6 µg/ml negatively supercoiled pBR322. The enzyme solution was diluted with 50 µg/ml bovine serum albumin, 50 mM Tris-HCl (pH 7.5), 1 mM spermidine (dilution buffer) and 1/10 volume was added to the mixture. The reaction was carried out by incubation at 75°C for 10 min and stopped by cooling at room temperature followed by addition of 1/5 vol. of 5% SDS, 50 mM EDTA, 50% glycerol, 0.05% bromophenol blue. Samples were loaded on a 1% agarose gel and electrophoresed as described previously (Kikuchi and Asai, 1984). One unit of the enzyme was defined as the amount of protein required for 80% relaxation of negatively supercoiled DNA under the standard assay condition.

The standard assay mixture (20 µl) for type II topoisomerase contained low salt (24 mM KCl) as described previously (Kikuchi and Asai, 1984) and 3 µg/ml P4 knotted DNA was used instead of pBR322. The reaction was carried out and stopped as described above. One unit of the enzyme was defined as the amount of protein required for 50% unknotting of knotted DNA under the standard assay condition.

Purification procedure

All procedures were carried out below 4°C unless otherwise stated. 40 g wet-weight cells were suspended in 400 ml of 50 mM Tris-HCl (pH 7.5), 0.6 M NaCl, 1 mM spermidine, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM mercaptoethanol and lysed by three cycles of freezing and thawing. An appropriate amount of 2 M Tris base was added in order to adjust the pH between 7.3 and 7.8. The lysate was centrifuged at 40 000 r.p.m. for 2 h in a 60 Ti rotor (Beckman) and the supernatant was saved (fraction I).

Fraction I was made 0.3% in Polymin P by dropwise addition of 20% Polymin P solution (pH 7.5) with stirring in an ice bath. The precipitate was removed by centrifugation at 8000 r.p.m. for 30 min. Solid ammonium sulfate was added to the supernatant to 40% saturation and the suspension stirred for 30 min in an ice bath. The suspension was centrifuged as described above and the supernatant was saved. Solid ammonium sulfate was added to the supernatant to 60% saturation. The suspension was stirred and centrifuged as described above and the pellet suspended in 200 ml of the same buffer. Proteins were precipitated in 60% saturation of ammonium sulfate. The pellet was suspended in 50 ml of 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM spermidine, 1 mM PMSF, 2 mM mercaptoethanol (buffer A), and dialyzed against buffer A. The dialysate was centrifuged at 8000 r.p.m. for 10 min and the supernatant saved (fraction II).

Fraction II was applied to a 150-ml DEAE Sephacel (Pharmacia) column equilibrated with buffer A and washed with three column volumes of buffer A. Active fractions were collected and proteins were precipitated in 60% saturation of ammonium sulfate. The pellet was suspended in 40 ml of 20 mM sodium phosphate buffer (pH 7.4), 0.1 M NaCl, 1 mM spermidine, 1 mM PMSF, 2 mM mercaptoethanol (buffer B), and dialyzed against buffer B (fraction III).

Fraction III was applied to a 50-ml phosphocellulose (Whatman P11) column equilibrated with buffer B. The column was washed with 2 column volumes of buffer B and eluted by a 700 ml linear gradient from 0.1 M to 0.8 M NaCl in buffer B. Active fractions (Figure 1) were collected and dialyzed against 50 mM Tris (pH 7.5), 0.4 M NaCl, 1 mM spermidine, 0.1 mM dithiothreitol (buffer C) (fraction IV).

Fraction IV was applied to a 5-ml heparin-Sepharose column equilibrated with buffer C. The column was washed with three column volumes of buffer C and eluted by an 80 ml linear gradient from 0.4 M to 0.8 M NaCl in buffer C. Active fractions (Figure 2) were collected and dialyzed against 50 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 1 mM spermidine, 0.1 mM dithiothreitol, 10% glycerol (buffer D) (fraction V).

Fraction V was applied to a Sephacryl S-300 (Pharmacia) column (1.4 × 132 cm) at a flow rate of 5 ml/cm^2 h and eluted with buffer D without glycerol. Active fractions were collected and dialyzed against buffer A containing 50% glycerol and stored at −20°C (fraction VI).

When concentration of the samples was desired during the purification steps, fractions were dialyzed against 0.1 M potassium phosphate buffer (pH 7.4), 1 mM spermidine, applied to a 1−5 ml hydroxylapatite column and concentrated by elution with 0.5 M potassium phosphate buffer (pH 7.5), 1 mM spermidine.

Proteins of each fraction were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Protein concentration was determined by Protein assay (Bio Rad) according to Bradford (1976) with bovine serum albumin as a standard.

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