Reverse Gyrase of *Sulfolobus*: Purification to Homogeneity and Characterization†

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Received March 11, 1988; Revised Manuscript Received May 18, 1988

ABSTRACT: By using hydrophobic interaction as the first chromatographic stage, we purified to homogeneity reverse gyrase, an ATP-dependent DNA topoisomerase I, isolated from the thermoacidophilic archaebacterium *Sulfolobus acidocaldarius*. This procedure allowed quick and complete separation of reverse gyrase from nucleases and DNA binding proteins present in *Sulfolobus*. The final product was revealed, by SDS–PAGE, as a unique band with an apparent molecular mass of 128 kDa, and the amino acid composition was determined. Western blotting experiments with antibodies raised against reverse gyrase indicate that no proteolysis occurred during the purification course. Gel filtration and sedimentation data gave a Stokes radius of 42 Å and a sedimentation coefficient of 5.7 S, suggesting a monomeric structure for the native enzyme which was confirmed by electron microscopy. Finally, pure reverse gyrase in a monomeric state was still able to promote positive supercoiling of the DNA.

DNA topoisomerases are enzymes that modify the topological state of the DNA [for a review, see Wang (1985, 1987) Maxwell and Gellert (1987)]. They act by introducing transient breaks into the DNA, and the nature of the break (single or double strand) defines the type (I or II) of the topoisomerase involved. In eubacteria, changes in the superhelicity of the DNA may activate or inhibit gene expression (Brahms et al., 1985). This process is regulated through a balance between two antagonistic topoisomerase activities: topoisomerase II (gyrase) increases the negative superhelicity of the DNA while topoisomerase I (protein ω) relaxes this structure. In eucaryotes, the status of topoisomerases is still unclear: the purified topoisomerases I and II do not show such antagonism, since both enzymes relax DNA and may act as a swivel during DNA replication. In addition, topoisomerase II seems necessary for chromosome segregation by its decatenation activity (Uemura & Yanagida, 1984) and appears as a structural component of the mitotic chromosome scaffold (Earnshow & Heck, 1985) and of the interphase nuclear matrix (Berrios et al., 1985). On the other hand, topoisomerase I seems involved in the transcription processes (Fleischmann et al., 1984).

Since a number of biochemical properties of eucaryotes are expressed in archaebacteria, we have searched for topoisomerases in this group of organisms now considered as the third kingdom [Woese (1981) and for a recent review see also Woese (1987)]. We have focused our study on *Sulfolobus acidocaldarius*, an extreme thermoacidophilic organism. It seemed interesting to investigate the structure of its DNA at high temperature and the properties of the enzymes which modulate this structure, i.e., topoisomerases. In previous works, we have isolated an ATP-dependent topoisomerase activity (Mirambeau et al., 1984) which turned out to be similar to that of the enzyme discovered by Kikuchi and Asai (1984) and named reverse gyrase. It was further demonstrated that reverse gyration was performed by a type I topoisomerase in a ATP-dependent reaction (Forterre et al., 1985; Nakasu & Kikuchi, 1985). Moreover, a high density of positive supercoiling could be obtained in the presence of poly(ethylene glycol) (Forterre et al., 1985). The discovery that high positive supercoiling can also take place in vivo (Nadal et al., 1986) prompted us to investigate the mechanism of reverse gyration by the purified enzyme. In this paper, we describe the purification to homogeneity of reverse gyrase and the physical properties of this enzyme. Since none of the previous purification procedures (Forterre et al., 1985; Nakasu & Kikuchi, 1985) allowed us to totally eliminate the important nuclease activity present in crude extracts, we devised a new purification scheme which involves hydrophobic chromatography. This procedure yields a pure enzyme without proteolysis and completely free of nuclease.

Experimental Procedures

Materials

Plasmid pBR322 was prepared as described by Duguet et al. (1983). ATP, NAD, and ethidium bromide were purchased from Boehringer (Mannheim). The protein markers used in this work were from Sigma Chemical Co., as well as poly(ethylenimine) (Polymin P), DTT,[1] and PMSF. Phenyl-Sepharose, heparin–Sepharose, and Sephadex G-150 were purchased from Pharmacia. Phosphocellulose P11 was from Whatmann. SDS was from Serva, pure sucrose from BDH, and agarose (A37 NA) from IBF. The other chemicals were purchased from Merck.

Netropsin was a generous gift of Pr. C. Zimmer (Iena, DDR).

Methods

Cell Culture. Sulfolobus acidocaldarius DSM 639 was obtained from Dr. Zillig (München, FRG). The cells were grown at 75 °C in a 300-L fermenter (Laboratoire d'Ex-

† This work was supported by grants from CNRS and the Association pour la Recherche sur le Cancer (ARC).

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[1] Abbreviations: DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

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traction et de Fermentation, CNRS, Gif/Yvette, France) as described previously (Mirambeau et al., 1984).

Topoisomerase Assay. The standard reaction mixture (20 μL) contained 50 mM Tris-HCl, pH 8, 0.5 mM DTT, 0.5 mM EDTA, 10 mM $MgCl_2$, 1 mM ATP, 120 mM NaCl, 30 μg/mL bovine serum albumin, and 0.25 μg of pBR322. 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 1% SDS, 0.25 mg/mL bromophenol blue, and 15% sucrose.

Dilutions of the various enzymatic fractions were performed in each case in the same buffer as the fraction tested, with the addition of 200 μg/mL bovine serum albumin. One unit of enzyme was defined as the amount of protein required to relax 50% of the negatively supercoiled pBR322 input under the standard assay conditions. The reaction products were analyzed by 1% agarose gel electrophoresis, stained with ethidium bromide, photographed under UV illumination, and quantified as previously described by Duguet et al. (1983).

Protein Determination. Protein concentration was determined according to Bradford (1976), with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. SDS–polyacrylamide gradient (5–20%) gel electrophoresis was performed according to Hames (1981), and proteins were revealed by silver staining (Oakley et al., 1980). Molecular weight markers were myosine (205 kDa), β-galactosidase (116 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Purification of Reverse Gyrase. All the purification steps were carried out at 4 °C.

S. acidocaldarius frozen cells (40 g) were disrupted by thawing in 240 mL of buffer A (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, 1 mM DTT, 1 mM EDTA) containing 1.2 M NH_4Cl, 1 mM EGTA, 1 mM sodium bisulfite, 1 mM PMSF, 1 mM leupeptin, and 1 mM pepstatin A. Cell lysis was achieved by homogenization with an Ultraturax (TP 18/2) apparatus. The resulting solution was centrifuged at 2400*g* for 10 min. The pellet was resuspended in the same buffer (100 mL), homogenized, and centrifuged as indicated for the lysis. The two supernatants were pooled (fraction I, 340 mL, 4360 mg of proteins).

Polymin P was added to fraction I to a final concentration of 0.36%. After gentle mixing during 15 min, the solution was centrifuged at 2400*g* for 30 min. The supernatant was further clarified by ultracentrifugation at 90000*g* for 1 h (fraction II, 405 mL, 3850 mg of proteins).

Ammonium sulfate was added to fraction II to a final concentration of 70% saturation and centrifuged at 24000*g* for 20 min. The pellet was dissolved with ammonium sulfate at 35% saturation in buffer A and centrifuged in the same conditions. The supernatant was diluted with 2.1 M NaCl in buffer A to give a final concentration of 0.8 M ammonium sulfate and 1.2 M NaCl. This solution was again clarified by centrifugation at 24000*g* for 20 min and saved as fraction III (270 mL, 1040 mg of proteins).

Fraction III was loaded on a phenyl-Sepharose column (2.6 × 18.5 cm) equilibrated with 0.8 M ammonium sulfate and 1.2 M NaCl in buffer A. The column was first washed with 1 L of the same buffer at a flow rate of 40 mL/h and then with 600 mL of 0.25 M NaCl in buffer A. It was further washed with 1 L of 30% ethylene glycol–0.25 M NaCl in buffer A and developed with a linear gradient of 30–60% ethylene glycol (2 × 500 mL) in buffer A containing 0.25 M NaCl. Finally, the phenyl-Sepharose column was washed with 1% Triton X-100 in buffer A (450 mL). Active fractions were pooled, dialyzed against buffer A containing 10% ethylene glycol (buffer B), and referred to as fraction IV (480 mL, 86.5 mg of proteins).

Fraction IV was loaded onto a phosphocellulose column (2.6 × 15 cm) equilibrated with buffer B and washed with 1 L of the same buffer at a flow rate of 40 mL/h. The bound proteins were eluted with 0.8 M NaCl in buffer B. Active fractions were pooled and dialyzed against buffer B containing 0.2 M NaCl (fraction V, 85 mL, 9.1 mg of proteins).

Fraction V was applied onto a heparin–Sepharose column (1.6 × 5 cm) equilibrated with 0.2 M NaCl in buffer B at a flow rate of 10 mL/h. The column was washed with 50 mL of the same buffer and developed with a 0.2–1 M NaCl (2 × 60 mL) linear gradient. Active fractions were pooled and saved as fraction VI (31 mL, 0.8 mg of proteins).

Fraction VI was concentrated and equilibrated with buffer A containing 0.6 M NaCl on an Amicon microconcentrator (Centricon 30). This fraction was loaded on a 5–20% sucrose gradient in buffer A containing 0.6 M NaCl and centrifuged at 175000*g* for 40 h in a SW 41 Beckman rotor. Fractions of 350 μL were collected. Active fractions were pooled, concentrated, and finally equilibrated with 25 mM NaH_2PO_4/Na_2HPO_4, pH 7, 0.5 mM DTT, 0.5 mM EDTA, and 100 mM NaCl (fraction VII, 0.36 mL, 0.08 mg of proteins).

Determination of the Sedimentation Coefficient and the Stokes Radius. The sedimentation value was determined by the procedure used for the last step of purification according to Martin and Ames (1961). The Stokes radius was determined by gel filtration through Sephadex G-150 (0.5 × 35.5 cm column) equilibrated with buffer B containing 0.6 M NaCl. A mixture of 60 μL containing 5 μL of fraction VI, 6.4 units of alcohol dehydrogenase, and 0.5% Triton X-100 was loaded onto the column and eluted at a flow rate of 0.6 mL/h. Fractions of 50–60 μL were collected. The excluded volume was determined by using blue dextran (M, 2 × 10^6). For calculation of the Stokes radius, we used the equation of Laurent and Killander (1964).

The protein standards used in these two experiments were as follows: catalase, 11.3 S, 52 Å; alcohol dehydrogenase, 7.4 S, 46 Å; bovin serum albumin, 4.3 S, 35 Å; ovalbumin, 3.6 S, 27 Å; cytochrome *c*, 1.9 S, 17 Å. The concentration of cytochrome *c* was determined in each fraction by spectrophotometry at 408 nm. Alcohol dehydrogenase activity was measured according to the instructions of the manufacturer. The concentration of the other protein markers was determined by the method of Bradford (1976).

Determination of the Isoelectric Point. The isoelectric point of reverse gyrase was determined by using pH 3–9 and pH 5–8 IEF PhastGels (Pharmacia) according to the instructions of the manufacturer.

Results

Choice of the Enzymatic Assay. We have previously reported that reverse gyrase was able, at low ionic strength, to promote positive supercoiling of the DNA in a processive manner (Forterre et al., 1985). This assay is highly specific for reverse gyrase. However, it is less sensitive than the relaxation assay and more difficult to quantitate, since many interferences, such as aggregations or nuclease activity, may occur, particularly in crude fractions. For these reasons, the relaxation assay, which is a general assay for topoisomerase activity, was preferred. This assay, performed at 120 mM NaCl (i.e., distributive conditions) and at pH 8.0 (optimum pH, not shown), allowed a precise measurement of the activity

Table I: Purification of Reverse Gyrase

fraction	step	volume (mL)	total protein (mg)	total activity ($\times 10^{-3}$ units)	sp act. ($\times 10^{-3}$ units/mg)
I	crude extract	340	4360	ND[c]	ND
II	Polymin P	405	3850	ND	ND
III	ammonium sulfate	270	1040	13000–18000	12.5–17.3
IV	phenyl-Sepharose	480	86.5	12200	140
V	phosphocellulose	85	9.1	4250	460
VI	heparin–Sepharose	31	0.8[a]	4030	5000
VII	sucrose gradient	0.36	0.08[b]	700	8750

[a] Amount of proteins estimated after silver staining of polyacrylamide gel. [b] Amount of proteins determined by amino acid composition. [c] ND, not determined.

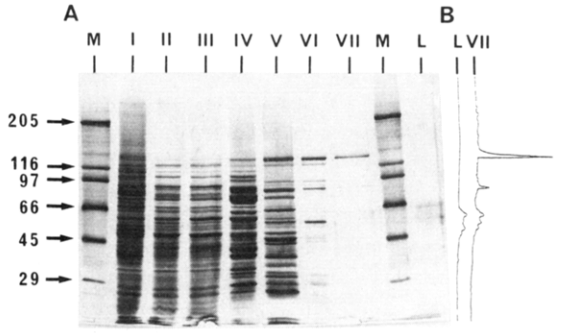

FIGURE 1: SDS–polyacrylamide gel electrophoresis of the fractions obtained at each stage of purification. (A) Silver staining of the gel: M, molecular mass markers; lane I, 3 μL of crude extract; lane II, 4 μL of Polymin P fraction; lane III, 4 μL of ammonium sulfate stage; lane IV, 150 μL of phenyl-Sepharose pool; lane V, 100 μL of phosphocellulose step; lane VI, 50 μL of heparin–Sepharose pool; lane VII, 1 μL of sucrose gradient pool; lane L, Laemmli buffer without protein. (B) Densitometric traces of lanes L and VII.

present in the various fractions obtained during the purification. Moreover, in these conditions, the large amount of nuclease activity present in the first three steps of the purification was partially inhibited.

Comments on the Purification. The reverse gyrase was purified as summarized in Table I and Figure 1. Cells of *S. acidocaldarius* were lysed at high ionic strength in a buffer containing protease inhibitors. These conditions prevented proteolytic degradation and decreased protein–nucleic acid interactions which allowed us to remove nucleic acids by precipitation with Polymin P (fraction II). This step appeared essential to prevent interferences in the subsequent steps of purification (Kikuchi & Asai, 1984; Forterre et al., 1985). In the course of ammonium sulfate precipitation, most of the topoisomerase activity was recovered in the fraction between 35 and 70% saturation in ammonium sulfate (fraction III). A large part of protein contaminants was eliminated during this fractionation (Table I), but the pattern of proteins remained complex (Figure 1A, lane III). At this point, a major nuclease activity was still present and prevented a precise measurement of topoisomerase activity.

Fraction III was directly loaded on a phenyl-Sepharose column. Washing of the phenyl-Sepharose column at low ionic strength (0.25 M NaCl) allowed the elimination of numerous proteins, including most of the nuclease activity. This is clearly visible by the production of DNA forms II and III after incubation of pBR322 with these fractions (Figure 2C, lanes b and c). A second wash of the column with 30% ethylene glycol again eliminated a large part of the proteins bound (Figure 2B, lanes c and d) including a DNA-binding protein (Figure 2C, lanes c and d). This protein was not removed by the standard buffer used to stop the reaction and remained tightly bound to DNA during the electrophoresis. An extensive extraction eliminated this protein (not shown). No significant topoisomerase activity eluted during these stages. The column

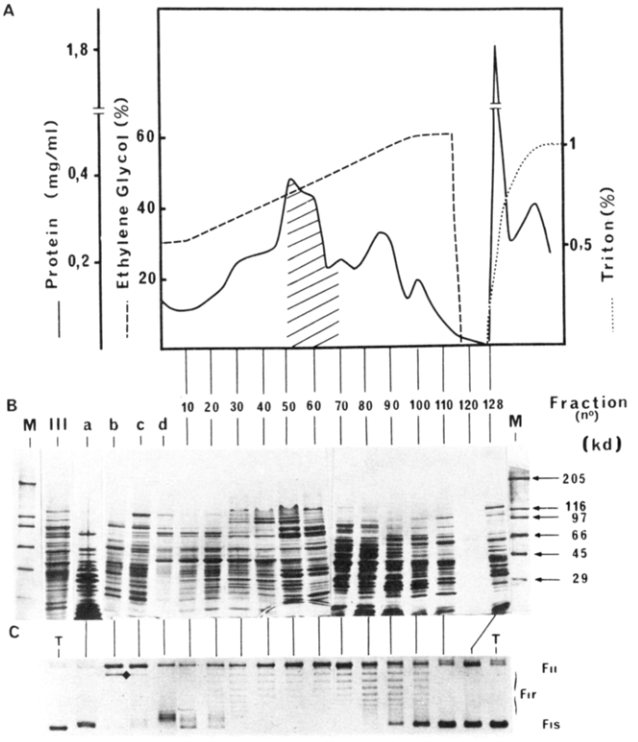

FIGURE 2: Phenyl-Sepharose column chromatography. (A) Elution profile of proteins. Fractions of about 12 mL were collected: (///) pooled topoisomerase fractions. (B) SDS–polyacrylamide gel electrophoresis of the fractions eluted: M, molecular mass markers; lane III, fraction III (4 μL); lane a, flow through (500 μL); lane b, fraction eluted at 0.25 M NaCl (100 μL); lane c, fraction intermediate between lanes b and d; lane d, fraction eluted at 30% ethylene glycol (100 μL); lanes 10–128, analysis of fractions 10–128. Fractions 10, 20, and 70–100, 100 μL each; fractions 30–60, 50 μL; fractions 110, 120, and 128, 200 μL. (C) Activity of the fractions visualized by agarose gel electrophoresis of the DNA: T, pBR322 DNA incubated without fraction; F_{IR}, partially relaxed topoisomers; F_{IS}, supercoiled DNA; F_{II}, DNA form II; (◆) DNA form III.

was finally developed with a 30–60% ethylene glycol linear gradient, and the topoisomerase activity eluted at 50% ethylene glycol. The most active fractions were pooled (fraction IV) and represented 9% of the proteins initially bound to phenyl-Sepharose. In this fraction, it was possible to quantitate precisely the topoisomerase (Table I). Moreover, the pattern of proteins present at this step (fraction IV) was largely different from those of the first steps (Figure 1A, compare lanes I, II, and III with lane IV). At the end of the ethylene glycol gradient, some proteins remained tightly bound to phenyl-Sepharose and were in part removed by Triton X-100 (Figure 2A,B, lane 128). No activity was observed in these fractions (Figure 2C, lane 128).

Fraction IV was loaded at low ionic strength on a phosphocellulose column. The use of a phosphate buffer seemed to increase the selectivity of protein binding. This resulted in the elimination of 90% of the proteins loaded, while reverse

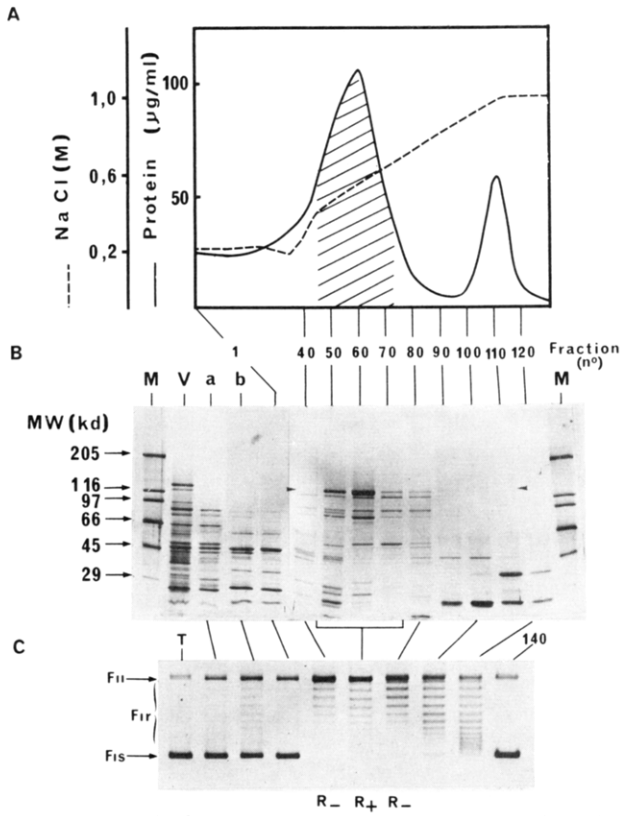

FIGURE 3: Heparin–Sepharose column chromatography. (A) Elution profile of proteins. Fractions of about 1 mL were collected. (///) Pooled topoisomerase fractions. (B) SDS–polyacrylamide gel electrophoresis of the fractions eluted: M, molecular mass markers; lane V, fraction V (100 µL); lane a, flow through (200 µL); lane b, fraction eluted at 0.2 M NaCl (100 µL); lanes 10–120, analysis of fractions 10–120. Fraction 1, 400 µL; fractions 40–70, 100 µL; fractions 80–100 and 120, 500 µL; fraction 110, 200 µL. The arrows indicated the band attributed to reverse gyrase. (C) Activity of the fractions visualized by agarose gel electrophoresis of the DNA: T, pBR322 DNA incubated without fraction; F_{IR}, partially relaxed topoisomers; F_{IS}, supercoiled DNA; F_{II}, DNA form II. R+ indicates topoisomers with moderate positive supercoiling: these topoisomers present an electrophoretic mobility which is slightly different from the ladder of negatively supercoiled topoisomers (R−).

gyrase remained bound and was further eluted with 0.8 M NaCl (fraction V). At this point, reverse gyrase represented a significant part of the protein content (Table I and Figure 1A, compare lanes IV and V). However, we noted a loss of activity which may be due to the removal of an activator during this step (Table I).

The subsequent chromatography was achieved on heparin–Sepharose (Figure 3). At 0.2 M NaCl, 75% of the protein content of fraction V did not bind to heparin–Sepharose, while reverse gyrase remained bound and was further eluted at 0.5 M NaCl (fraction VI). At this point, with use of the standard relaxation mixture, it was possible to reveal the positive supercoiling activity (Figure 3C, fractions 50–70), which is highly specific for reverse gyrase. Indeed, these fractions produced positively supercoiled DNA that corresponds to the peak of activity. In contrast, fractions 100–120 (Figure 3C) presented a weak relaxation activity. In fraction VI, reverse gyrase represented the major protein (Figure 1A, lane VI).

The purification was achieved by sedimentation on a sucrose gradient. During this step, two contaminants (120 and 95 kDa in SDS–polyacrylamide gel) present in fraction VI (Figure 1, lane VI) cosedimented at the bottom of the gradient and were eliminated. In addition, low molecular weight contaminants sedimented at the top of the gradient and were removed. Finally, reverse gyrase, sedimenting in the middle of the gradient, was purified (fraction VII).

Table II: Physical Properties of Reverse Gyrase

molecular mass, M (kDa)	128
structure	monomer
sedimentation coefficient, $s_{20,w}$ (S)	5.7
Stokes radius, a (Å)	42
frictional ratio, f/f_0[a]	1.27
isoelectric point, p_i	5.6–5.9

[a] The frictional ratio f/f_0 was calculated from the equation $f/f_0 = a/(3\nu M/4\pi N)^{1/3}$, where N = Avogadro's number.

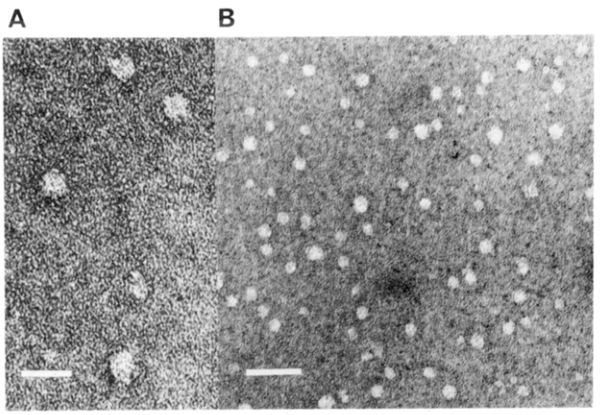

FIGURE 4: Electron microscopy of purified reverse gyrase. The reverse gyrase was visualized on a Philipps EM 400 electron microscope after negative staining with uranyl acetate. The magnification was 640000× in (A) and 300000× in (B). The bar represented 20 nm in (A) and 50 nm in (B).

Purity and Stability of the Protein. Analysis of fraction VII by SDS–polyacrylamide gel electrophoresis under denaturing and reducing conditions (Figure 1A, lane VII) indicated that reverse gyrase represented more than 95% of the proteins stained (see densitometric traces in Figure 1B). A minor polypeptide (about 90 kDa) was also present in fraction VII (Figure 1B). This polypeptide is likely a degradation product of reverse gyrase since it was also revealed by antibodies directed against the major 128-kDa polypeptide (see Figure 5, lane 7).

Partially purified fractions of reverse gyrase (fractions IV–VI) were stable at 4 °C for several months. The purified fractions of the sucrose gradients were also stable in the presence of sucrose. In contrast, fraction VII conserved without sucrose was stable only at protein concentrations higher than 200 µg/mL and in siliconized tubes or in the presence of 0.1% Triton X-100.

Molecular Weight and Subunit Structure. As determined by SDS–PAGE, reverse gyrase migrated, under denaturing and reducing conditions, as a unique polypeptide of 128 kDa (Figure 1A, lane VII).

The gel filtration and sedimentation data gave a Stokes radius of 42 Å and a sedimentation coefficient of 5.7 S (Table II). By using these values, we calculated, according to the method of Siegel and Monty (1966) and assuming a partial specific volume (ν) of 0.725 mL/g, an apparent native molecular mass of about 100 kDa. We concluded that native reverse gyrase is a monomer of 128 000 Da with a frictional coefficient of 1.27, suggesting a rather symetric protein (see Table II).

These results were in a good agreement with the observation of the enzyme by electron microscopy. At high magnification (640000×) reverse gyrase appeared as a globular protein with a diameter of 7–9 nm (Figure 4A). Finally, the observation at low magnification (300000×) confirmed the homogeneity of fraction VII (Figure 4B). We noted that some particles

Table III: Amino Acid Composition (%) of Reverse Gyrase[a]

	first determination	second determination		first determination	second determination
Asx	11.3	11.9	Met	0.7	0.5
Thr	5.3	5.1	Ile	6.6	7.7
Ser	10.4	8.8	Leu	10.2	11.2
Glx	11.0	11.4	Tyr	3.9	1.7
Pro	4.0	3.9	Phe	3.3	3.8
Gly	7.7	7.6	His	1.7	1.6
Ala	5.1	5.3	Lys	8.3	8.2
Cys	0.0	0.0	Arg	5.5	4.6
Val	5	6.7			

[a] An aliquot of fraction VII was hydrolyzed with 6 M HCl, 0.25% phenol, and 0.02% mercaptoethanol at 110 °C during 24 h. The amino acid mixture was analyzed by a Beckman 7003 system according to the recommendations of the manufacturer.

of larger size were occasionally observed especially at high protein concentration (see Figure 4B). When the fraction was diluted just before being loaded on the grids, these structures decreased, suggesting that they resulted from a nonspecific multimeric association of reverse gyrase molecules.

Amino Acid Composition. Two independent amino acid compositions of purified reverse gyrase were given in Table III. These two determinations were similar for most of the amino acids. We noted the absence of cysteine and the presence of numerous hydrophobic residues such as leucine or isoleucine. As suggested by the slightly acidic isoelectric point of reverse gyrase (p_i = 5.6–5.9, Table II), the large amount of basic residues was probably neutralized by acidic residues. This suggests that the aspartic and glutamic residues represented a large part of the Asx + Glx content (see Table III).

Antibodies Raised against Reverse Gyrase. A pure reverse gyrase protein band, revealed by KCl precipitation according to the method of Hager and Burgess (1980), was removed from preparative SDS–PAGE. The piece of acrylamide was directly injected to the rabbit. The specificity of the immune serum obtained was determined by slot–blot and Western blotting procedures. The serum can be directly used by dilution in a range of 1/400 to 1/1000. The different fractions obtained during the purification were submitted to SDS–PAGE, transferred to nitrocellulose, and probed with the immune serum. The results (Figure 5) show the high specificity of these antibodies, especially in the crude fractions (Figure 5, lane 1–3) where reverse gyrase was in very low amounts with regard to the other proteins (see also Figure 3A, lanes I–III). In addition, we noted that the molecular weight of the protein detected by antibodies in the bacteria lysed just prior electrophoresis (Figure 5, lane 1) was identical with that of the purified protein, suggesting that no proteolysis occurred during the purification.

Activity of the Purified Reverse Gyrase. In order to confirm that the purified enzyme was still able to sustain a reaction of gyration, we have incubated closed pBR322 circles at 75 °C with fraction VII. The reaction was carried out essentially as described under Materials and Methods, except that the NaCl concentration was reduced to 10 mM.

To clearly visualize positively supercoiled DNA, we have developed a new bidimensional agarose gel electrophoresis technique, where the second dimension was performed in the presence of netropsin. This drug binds to the minor groove of the DNA and increased the twist of the double helix of DNA (Snounou & Malcolm, 1983). As a consequence, the mobility of the negative topoisomers tends to increase; these topoisomers describe the right part of the arch in the experiment of Figure 6C. Conversely, the mobility of the positive topoisomers is reduced; those topoisomers described the left part of the arch in the experiment of Figure 6A.

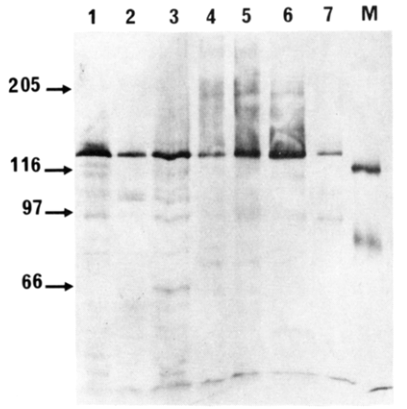

FIGURE 5: Western blot analysis of the fractions obtained at each stage of purification. Proteins were submitted to electrophoresis on a 8.5% SDS–polyacrylamide gel and electrotransferred on a nitrocellulose membrane according to the method of Svoboda et al. (1985). The membrane was incubated during 1 h with immune serum at a dilution of 1/800 and finally stained with an immunodetection kit (Bethesda Research Laboratories) using alkaline phosphatase. Lane 1, frozen *S. acidocaldarius* cells lysed in Laemmli buffer just prior to loading onto the gel; lane 2, 20 μL of Polymin P fraction; lane 3, 20 μL of ammonium sulfate stage; lane 4, 100 μL of phenyl-Sepharose pool; lane 5, 30 μL of phosphocellulose step; lane 6, 15 μL of heparin–Sepharose pool; lane 7, 0.6 μL of sucrose gradient pool; lane M, biotinylated β-galactosidase (116 kDa) and biotinylated lactoperoxidase (85 kDa).

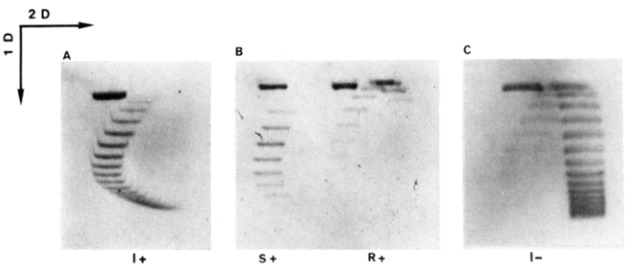

FIGURE 6: Two-dimensional minigel analysis of the topological conversions catalyzed by the purified reverse gyrase. Electrophoreses were carried out as described by Forterre et al. (1985) with the following modifications: 1.3% agarose gels were used and 13 μM netropsin was added in place of chloroquine in the second dimension. The temperature of the gel shown in (B) was maintained at 4 °C. (A) Positively supercoiled DNA pBR322 standard. (B) The reactions were carried out in the standard reaction mixture except that the substrate was relaxed pBR322 DNA and that the NaCl concentration was 10 mM. We used a molecular ratio of 2 reverse gyrase/DNA circle for the R+ reaction and 8 for the S+ reaction. (C) Negatively supercoiled pBR322 DNA standard.

The result of incubation of pBR322 relaxed at 37 °C by rat liver topoisomerase I (Mirambeau, 1987) with purified reverse gyrase is shown in Figure 6B. At a molecular ratio of two reverse gyrase monomers per DNA circle, about 10 positive superhelical turns (R+) were produced while up to 20 supercoils were obtained at a molecular ratio of 8 (S+). This result demonstrates that the reaction of reverse gyration is an intrinsic property of the purified enzyme.[2]

[2] For this calculation, we took into account the difference of temperature between the incubation (75 °C) and the electrophoresis (4 °C). Since the law of variation of the twist as a function of temperature (Depew & Wang, 1975; Pulleyblank et al., 1975) was verified in our conditions of incubation and in a range of temperature between 4 and 75 °C (Duguet, unpublished results), we could predict that the topoisomer which appeared relaxed during the electrophoresis (top of the arch) contained 10 positive superhelical turns at 75 °C.

Discussion

This paper described a purification procedure which allows highly purified reverse gyrase to be obtained. For this purpose, hydrophobic interaction chromatography was used as the first chromatographic stage. This choice presented several advantages: in particular, the dialysis ordinarily required after an ammonium sulfate precipitation was avoided. Moreover, the first purification steps were achieved at high ionic strength and in a very short time. These were important points to limit proteolysis. Thus, from the comparison of the size of the pure reverse gyrase in SDS–polyacrylamide gel (128 kDa) with the size of the enzyme in a crude extract by Western blotting procedure, it appeared that no proteolysis occurred during the purification. On the contrary, if the first stages of purification were performed at lower ionic strength and in the absence of protease inhibitors, other polypeptides, with a lower molecular weight, were detected in Western blots, which may represent proteolysis products (not shown).

Another advantage of phenyl-Sepharose was to efficiently separate reverse gyrase from nucleases. Indeed, neither anionic (DEAE-Sephacel) nor cathionic (Bio-Rex 70 or phosphocellulose) resins used in the previous purification procedures (Forterre et al., 1985; Nakasu & Kikuchi, 1985) allowed, at least in our hands, complete elimination of the nuclease(s). Nakasu and Kikuchi (1985) did not mention such nuclease activity; however, they used a strain of *Sulfolobus* which was different from ours (Kikuchi & Asai, 1984). Another explanation may be that they used spermidine (which partially inhibits nuclease) in all buffers, including the assay buffer.

Finally, the use of phenyl-Sepharose allowed complete separation of the DNA-binding protein previously reported (Forterre et al., 1985) from reverse gyrase: it was then possible to show that reverse gyration was not the result of the combined effect of this DNA-binding protein in the presence of a classical topoisomerase. The preparation of the enzyme as a single polypeptide thus permitted study of reverse gyration per se.

For this purpose, we have developed a new bidimensional agarose gel electrophoresis of the DNA by using netropsin (or related molecules) instead of chloroquine or ethidium bromide in the second dimension (Wang et al., 1983). This method allowed clear visualization of positively supercoiled topoisomers and more precise quantification of the superhelical density of such DNA. Indeed, pure reverse gyrase was able to produce positively supercoiled DNA from a relax DNA, confirming that gyration in reverse was an intrinsic property of this enzyme.

An important point was to decide if monomeric reverse gyrase can sustain the reaction of positive supercoiling or if it is performed by a multimeric structure. Although the Stokes radius (42 Å) and the sedimentation coefficient (5.7 S) did suggest a monomeric structure, aggregation products were often observed especially at low ionic strength and high protein concentration. This aggregation may be due to the hydrophobic property of reverse gyrase, as revealed by phenyl-Sepharose chromatography and amino acid composition. However, the monomeric structure is likely the active state of reverse gyrase. This is supported by the following remarks: (i) aggregation products were reduced by use of diluted reverse gyrase, as revealed by electron microscopy experiments; (ii) the use of Triton X-100 during gel filtration allows reverse gyrase to be obtained as a monomeric protein; (iii) reverse gyrase was still able to produce positive supercoiling of the DNA at a Triton concentration as high as 1% (not shown). It is remarkable to note that DNA gyrase, which catalyzes the same kind of reaction, is a large tetramer of 400 kDa. By using the pure enzyme, it is now possible to precisely determine the mechanism of reverse gyration and the role of ATP in this process.

Finally, it is of particular interest to investigate the role of reverse gyrase in vivo, since we have demonstrated the presence of positively supercoiled DNA in a virus-like particle (SSV1) of *Sulfolobus* (Nadal et al., 1986). The availability of antibodies against the pure enzyme will enable measurement of the amount of reverse gyrase during *Sulfolobus* growth and during the development of the virus SSV1 in this bacterium. This will provide new insight for the putative role of positive supercoiling in an adaptation for life at high temperature.

Acknowledgments

We thank A. Escaut (Laboratoire d'Extraction et de Fermentation, Gif/Yvette, France) and A. Meyer (IJM, Paris, France) for growth of *S. acidocaldarius*, F. Harper (IRSC, Villejuif, France) for electron microscopy, L. Camoin, J.-L. Guillaume, and M. Hattab (Laboratory of Pr. Strosberg, Institut Pasteur, Paris, France) for amino acid composition, N. Barre, M. Camier, and P. Kuks (Laboratory of Pr. P. Cohen, Université Pierre et Marie Curie, Paris, France) for serum prelevement, IEF analysis, and the Western blotting procedure, M. Jendubi (Institut Pasteur, Paris, France) for the immunization procedure, Pr. C. Zimmer (lena, DDR) for the generous gift of netropsin, C. Elie (IRSC, Villejuif, France) for valuable discussions, and A.-M. Lotti for preparation of pBR322 DNA.

Registry No. DNA topoisomerase, 80049-01-0.

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Specificity in Formation of Triple-Stranded Nucleic Acid Helical Complexes: Studies with Agarose-Linked Polyribonucleotide Affinity Columns†

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Received July 7, 1988; Revised Manuscript Received September 6, 1988

ABSTRACT: The binding of a variety of deoxyribo and ribo homo- and copolynucleotide complementary duplexes to agarose-linked homopolynucleotide affinity columns has been studied. The results provide information concerning the specificity of recognition of complementary base pairs of nucleic acids through a mechanism that involves triple-helix formation under physiological conditions of ionic strength, pH, and temperature. The method employed made it possible, for the first time, to survey the full range of base triplets conceivable from the canonical nucleic acid bases and, in addition, hypoxanthine and thereby to differentiate between those triplets which can and cannot form. Certain previously observed features of the stereochemistry of double-helical targets for third-strand binding are confirmed, and some unrecognized features are elaborated. These include a general requirement for clusters of purine residues in one strand, protonation of third-strand C residues, the ability of natural third-strand residues to distinguish between A·T/U and G·C base pairs, and a capacity of third-strand (unnatural) I residues to recognize all base pairs within such clusters. Thus, the basis for a third-strand binding code is demonstrated.

The possibility that RNA–DNA interaction might occur through a general mechanism involving triple-stranded helix formation (Broitman et al., 1987; Fresco et al., 1989) has led us to examine the specificity and extent of base triplet formation in nucleic acid structures by several different methods. Here we report a study of the binding of nucleic acid duplexes by agarose-linked third-strand homopolyribonucleotide affinity columns under conditions that closely approximate the biological milieu. While some triple-stranded polynucleotide structures have been recognized for a long time (Felsenfeld et al., 1957; Fresco, 1963; Lipsett, 1964), generally they have been viewed as peculiarities of homopolynucleotide interaction. Moreover, they have not been widely studied under comparable conditions, and the full range of potential base triplets that can be formed starting with the Watson–Crick pairs has not been generally recognized [e.g., Broitman et al. (1987)]. Earlier, however, columns of poly(U)[1] and of poly(C) linked to Sephadex had been employed to screen for A·T and G·C clusters, respectively, in some eukaryotic and viral DNAs (Flavell & Van den Berg, 1975; Zuidema et al., 1978).

In the present work, a high level of specificity of recognition of A·U/T and G·C base pairs in homo- or co-purine·homo- or co-pyrimidine residue clusters by specific third-strand residues is demonstrated. This specificity suggests the plausibility of such a mechanism of recognition of complex target sequences by third-strand intermolecular RNA binding or intramolecular DNA binding that could conceivably serve some important biological functions.

Experimental Procedures

Materials

Affinity Columns. Short columns (1.4–2.5 × 0.5 cm) of agarose (Sepharose 4B) covalently linked polyribonucleotides, several hundred residues long, [Pharmacia Ag–poly(A), Ag–poly(G), Ag–poly(I), Ag–poly(C), and Ag–poly(U)] were prepared containing 0.3–0.7 mL of matrix (0.4–1.4 mg of polynucleotide).

Potential Polynucleotide Ligands. All single-stranded polyribonucleotides and polydeoxyribonucleotide duplexes were obtained from Pharmacia. Poly(A·U) and poly(I·C) were prepared by mixing equimolar stock solutions ($\sim 10^{-4}$ M) of their homopolymer constituents in the standard buffer (see

†This work was supported by grants from the National Science Foundation (DMB8419060) and the American Cancer Society (NP-580). A.G.L. and E.F. were recipients of fellowships from the Rita Allen Foundation and the Dana Foundation, respectively. This is paper 17 in the series "Polynucleotides", of which paper 16 is Broitman et al. (1987).
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[1] Abbreviations: Polynucleotide single strands, duplexes, and triplexes are designated by the standard symbols for the residues of homopolymer strands in parentheses, preceded by the word poly; a dot is inserted between the symbols for each strand of a duplex or triplex and a dash between the symbols for the residues of an alternating repeating sequence. Whereas a d preceding the letter symbol of a residue indicates that the backbone is deoxyribo, the absence of any symbol indicates that it is ribo. Base pairs and triplets are indicated by the letter symbols for their residues, with a dot between them. Column matrices are indicated by the abbrevation for agarose, Ag, followed by a dash and the symbols for the covalently linked homopolynucleotide. When a residue is protonated, its letter symbol carrier a (+) as a superscript.

0006-2960/88/0427-9108$01.50/0