Reverse Gyrase Functions as a DNA Renaturase

ANNEALING OF COMPLEMENTARY SINGLE-STRANDED CIRCLES AND POSITIVE SUPERCOILING OF A BUBBLE SUBSTRATE

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Reverse gyrase is a hyperthermophile-specific enzyme that can positively supercoil DNA concomitant with ATP hydrolysis. However, the DNA supercoiling activity is inefficient and requires an excess amount of enzyme relative to DNA. We report here several activities that reverse gyrase can efficiently mediate with a substoichiometric amount of enzyme. In the presence of a nucleotide cofactor, reverse gyrase can readily relax negative supercoils, but not the positive ones, from a plasmid DNA substrate. Reverse gyrase can completely relax positively supercoiled DNA, provided that the DNA substrate contains a single-stranded bubble. Reverse gyrase efficiently anneals complementary single-stranded circles. A substoichiometric amount of reverse gyrase can insert positive supercoils into DNA with a single-stranded bubble, in contrast to plasmid DNA substrate. We have designed a novel method based on phage-mid DNA vectors to prepare a circular DNA substrate containing a single-stranded bubble with defined length and sequence. With these bubble DNA substrates, we demonstrated that efficient positive supercoiling by reverse gyrase requires a bubble size larger than 20 nucleotides. The activities of annealing single-stranded DNA circles and positive supercoiling of bubble substrate demonstrate that reverse gyrase can function as a DNA renaturase. These biochemical activities also suggest that reverse gyrase can have an important biological function in sensing and eliminating unpaired regions in the genome of a hyperthermophilic organism.

Transactions of genetic information in DNA invariably require unwinding and rewinding of DNA double helix, processes usually resulting in creating topological problems. Nature’s solution to such problems is DNA topoisomerases that can reversibly cleave DNA strands and carry out strand passage through an enzyme-mediated DNA break (reviewed in Refs. 1–3). Based on the structure and mechanism of topoisomerases, they are grouped into two types, both of which use an active-site tyrosine to form a transient tyrosyl phosphate bond during the step of reversible DNA cleavage. Type I enzymes generate single strand cleavage, and type II enzymes make coordinated breaks in both DNA strands. Type I topoisomerase is divided into two structurally and mechanistically distinct subfamilies. Type IB enzymes, present in all eukaryotes, certain eukaryotic viruses, and some bacterial species (4), form a 3’-phosphotyrosyl covalent adduct at the DNA cleavage site. They can readily remove either positive or negative supercoils, capable of serving as an efficient swivel during DNA transactions (5). Type IA enzymes are ubiquitous, present in eukaryotic, eubacterial, and archaeabacterial species. Their enzymatic activity involves strand passage through a protein-mediated DNA gate formed with a tyrosyl linkage to a 5’ phosphoryl group at the cleavage site (6). These enzymes prefer DNA substrates with a single-stranded region and usually can only relax negative supercoils in a plasmid DNA. Type IA enzymes may have more divergent roles in DNA metabolism besides that as a swivel.

Reverse gyrase is the only topoisomerase that can insert positive supercoils into DNA (7). It has two closely linked domains: a helicase domain and a type IA topoisomerase domain (8–10). The crystal structure of reverse gyrase from Archaeoglobus fulgidus revealed that the structure of the topoisomerase domain is very similar to those from bacterial type IA topoisomerases, and the helicase domain shares the structure with several DNA helicases except for some residues involved in helicase translocation (11). Despite the availability of three-dimensional structures, the biochemical mechanism of reverse gyrase remains to be elucidated. Three mechanisms have been proposed. The first is the selective relaxation of the negative supercoils generated behind the translocating helicase moiety (8). However, there is no evidence that the helicase domain can translocate along DNA because no strand displacement activity was detected with either intact reverse gyrase or with the recombinant helicase domain (10). The second is that upon the unwinding of the DNA by the helicase domain, the reverse gyrase can separate the circular DNA into two topologically independent domains, one with the unwound base pairs, and the other with positive supercoils induced by the unwinding action in the other region (10). The reverse gyrase can reenate the unwound region without altering the positive supercoiling in the other domain, thereby leaving behind positive supercoils. How reverse gyrase can segregate the DNA molecule into two topological domains, and how the enzyme can switch between unwinding and rewinding action are not addressed here. The third is that reverse gyrase is capable of direction-specific strand passage, thus generating positive supercoils (11). The biochemical basis for this vectorial strand passage is unknown.

Our recent experiments with reverse gyrase have revealed several interesting new findings (12). Reverse gyrase is able to carry out different supercoiling reactions depending on the nucleotide cofactor. Besides the hallmark positive supercoiling activity by reverse gyrase with ATP, the enzyme is capable of generating hypernegatively supercoiled DNA in the presence of AMPPNP, and a mostly relaxed, but slightly positively supercoiled DNA in the presence of ADP. These supercoiling activities are inefficient and require an excess amount of enzyme to DNA. The final level of supercoiling depends on the enzyme to DNA ratio. This dependence is not because of a kinetic effect, since the reaction rate is the same with different amounts of enzyme in our reaction conditions. Furthermore, the negative supercoiling by reverse gyrase with AMPPNP is

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2 The abbreviation used is: AMPPNP, 5’-adenylyl-β,γ-imidodiphosphate.
resistant to relaxation by a potent topoisomerase I added to the reaction, suggesting that negative supercoils are constrained, possibly because of the binding of reverse gyrase/AMPPNP to the unwound region in DNA. Based on these results, we propose that reverse gyrase has affinity for either single- or double-stranded DNA, depending on whether the bound cofactor is ATP or ADP, respectively. Reverse gyrase with ATP can bind to the single-stranded region in the DNA, and upon the hydrolysis of ATP to generate ADP, the enzyme will then have a higher affinity for duplex DNA. This switch in binding specificity, coupled with the strand passage activity, facilitates rewinding of the unwound region. Because the linking number changes depend on the enzyme binding to DNA, the magnitude of final supercoiling is determined by the enzyme to DNA ratio.

In this article, we have analyzed the reactions that reverse gyrase can efficiently mediate with a substoichiometric amount of enzyme. They include supercoil relaxation, annealing of complementary single-stranded DNA circles, and positive supercoiling of DNA with a single-stranded bubble. These reverse gyrase activities provide new insight into their biochemical mechanism, and possibly their intracellular functions.

**EXPERIMENTAL PROCEDURES**

**Enzymes**—Reverse gyrase was purified from an *Escherichia coli* strain expressing cloned *A. fulgidus* reverse gyrase following the published procedure from Rodriguez and Stock (11). His-tagged Cre recombinase was purified from the bacterial expression strain provided by Shaikh and Sadowski (13). Isolation of a recombinant, His-tagged topoisomerase I from *Thermotoga maritima* was described earlier (12). Recombinant *Drosophila* topoisomerase I was described previously (14). All the restriction enzymes and T7 endonuclease I were from New England Biolabs (Beverly, MA).

**DNA Substrates**—Plasmid DNA including pUC19, pUC(AT)19, and pBS/SK were prepared through double CsCl/ethidium bromide density gradient ultracentrifugation. Their relaxed forms were prepared from reactions with *Drosophila* topoisomerase 1 (14). Positively supercoiled DNA substrates were generated by treating DNA with reverse gyrase at an enzyme/DNA ratio of 41 at 85 °C using the reverse gyrase reaction conditions described earlier (12). The bubble substrate, a covalently closed circular DNA molecule containing a single-stranded bubble, was made by annealing two single-stranded circles prepared from cells with phagemids pBS/SK and pBS/SK using the published protocol (15).
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Circular DNA substrates containing a bubble with a variable but defined size were synthesized with a modification of the technique described for making bubble substrate. Briefly, DNA sequences were introduced into phagemid vectors such that the single-stranded circles produced from them, after the annealing reaction, will give rise to DNA with two bubbles: one from the f1 replication origin (f1 ori), and the other from the cloned oligonucleotide sequences to form a bubble with the desired length (Fig. 1A). To remove the single-stranded bubble with the f1 ori sequence, we used the Cre/loxP site-specific recombination reaction to create the intramolecular deletion (16). The product will be a relaxed circular DNA with a unique single-stranded bubble of defined size and sequence. To facilitate the DNA manipulations described here, we created two phagemid DNA vectors, for plus and minus single-stranded circles, respectively, of a size of 6.1 kb and with two loxP sites bisecting the DNA circle and oriented in parallel direction.

For making DNA with a bubble of 50, 20, or 5 nucleotides in length, we synthesized 6 pairs of oligonucleotides (Fig. 1B). They can be annealed to form 6 oligonucleotide duplexes, each of which will have unique restriction sites of NheI and Xmal at the ends. The duplexes from annealing A and B series (P50, P20, and P5) were cloned into the NheI/Xmal sites in the phagemid vector for plus strand synthesis, and the duplexes from annealing C and D series (M50, M20, and M5) cloned into phagemid vector for minus strand synthesis. The DNA sequences of the single-stranded bubbles and their neighboring regions are shown in Fig. 1B. DNA constructs were sequenced to confirm the insertion of correct oligonucleotide sequences into the vector. Single-stranded DNA made from vectors with P50 and M50 can anneal and form a bubble of 50 nucleotides on one strand and 49 on the other (Bubble 50). Those from P20 and M20 will form a bubble of 20 nucleotides (Bubble 20), and P5 and M5 will result in a 5-nucleotide bubble (Bubble 5). The sequences flanking the bubble are GC-rich to limit the size of the bubble at elevated temperatures.

We used 250 μg each of the plus and minus strand pair and annealed them in 3 ml of reverse gyrase reaction buffer containing 1 mM ADP and 0.2 μM reverse gyrase at 80 °C for 1 h (reaction step A, Fig. 1A). To minimize the topological complexity in the products of the subsequent Cre recombinase reaction, restriction enzyme XhoI was added to cleave the annealed product at the single restriction site (reaction step B, Fig. 1A) to generate a full-length linear molecule. The Cre-mediated cyclization reaction was carried out in a buffer containing 50 mM Tris-HCl, pH 8.0, 33 mM NaCl, 10 mM MgCl₂, with 40 nM annealed DNA and 40 units/ml of Cre enzyme (step C, Fig. 1A). The reaction mixture was incubated at 37 °C for 1 h and DNA products were purified and concentrated through a MaxiPrep column from Qiagen (Valencia, CA). The intramolecular recombination reaction results in the cyclization of a 3.1-kb DNA sequence containing a single loxP site and the single-stranded bubble with defined sequence and length (bubble substrate, Fig. 1A). Because the cyclized DNA is the major covalently closed species in the reaction products, we used CsCl/ethidium bromide density gradient centrifugation to purify it away from the linear fragments of the recombination product containing the f1 ori bubble, and the unreacted linear, full-length DNA. The DNA sample after the banding run was extracted with n-butanol to remove ethidium bromide and dialyzed into TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA).

Reverse Gyrase Reaction—The reaction was usually carried out in a 30-μl buffer containing 10 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 50 mM KCl, 0.1 mM EDTA, 0.45 μg of DNA substrate, 1 mM adenine nucleotide cofactor, and a variable amount of reverse gyrase to give an enzyme/DNA ratio ranging from 0.1 to 100. The reaction was usually carried out at 80 °C unless otherwise indicated, and followed by the time course of 3.5, 7, 15, 30, and 60 min.

Gel Electrophoresis—The reverse gyrase reaction products were analyzed by agarose gel electrophoresis in 1.2% agarose in a buffer containing 36 mM Tris phosphate, pH 7.6, and 1 mM EDTA (TPE buffer). At the optimal temperature of 80 °C (Fig. 2, bottom panel), the reaction mixtures contained 1 mM AMP (lanes 2–6), ADP (lanes 7–11), ATP (lanes 12–16), and AMPPNP (lanes 17–21), and reverse gyrase at an enzyme/DNA ratio of 0.1. The reaction products were taken and stopped at 3.5 (lanes 2, 7, 12, and 17), 7 (lanes 3, 8, 13, and 18), 15 (lanes 4, 9, 14, and 19), 30 (lanes 5, 10, 15, and 20), and 60 min (lanes 6, 11, 16, and 21). The plasmid DNA substrate is in lane 1.

RESULTS

Plasmid DNA Relaxation—In our previous work, we noticed that when using the plasmid DNA as a substrate, there is an initial fast phase of supercoil relaxation, followed by insertion of DNA supercoils. Here we analyzed the supercoil relaxation reaction by reverse gyrase under stoichiometric conditions (enzyme/DNA ratio of 0.1), and in the presence of different adenine nucleotide cofactors (Fig. 2). At the optimal temperature of 80 °C (Fig. 2, top panel), reverse gyrase cannot relax DNA in the presence of AMP, but can relax DNA with ADP as a cofactor (lanes 2–6 and lanes 7–11, respectively). The relaxation reaction with ATP is extremely efficient; most of the negative supercoils were removed by the first time point at 3.5 min (lanes 12–16). Whereas the hallmark reaction for reverse gyrase is DNA positive supercoiling in the presence of ATP, we have shown that this reaction requires more than stoichiometric amounts of enzyme. In agreement with our earlier results, a substoichiometric amount of reverse gyrase cannot supercoil...
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DNA even after prolonged incubation. The relaxation reaction with AMPPNP as cofactor is only partial (Fig. 2, lanes 17–21, top panel), in part because of the ability of reverse gyrase to unwind DNA when AMPPNP is present, thereby stabilizing negative supercoils.

A unique feature of the reverse gyrase supercoiling reaction is temperature sensitivity. Raising the temperature from 80 to 85 °C greatly enhances both the negative and positive supercoiling reactions, and lowering to 75 °C drastically reduces them (12). In an interesting contrast, the supercoil relaxation with a substoichiometric amount of reverse gyrase is not exquisitely sensitive to the temperature. Raising the temperature from 80 to 85 °C does not increase the relaxation activity with either ADP or ATP as cofactors (Fig. 2, bottom panel, lanes 7–11 and lanes 12–16, respectively). The loss of partial relaxation with AMPPNP as a cofactor (Fig. 2, lanes 17–21, bottom panel) is presumably because of the fact that DNA unwinding is favored at the elevated temperature. At 85 °C, the plasmid DNA is in a pre-melting state with extensively unwound regions (12, 17). Similar relaxation reactions were carried out at 75 °C, and the activities were not significantly different from those observed at 80 °C (data not shown), again demonstrating that the relaxation reaction by reverse gyrase is not as sensitive to temperature as the supercoiling reaction.

Reverse gyrase is an atypical type IA topoisomerase, with both a topo I and DNA helicase domain present in the protein, and is capable of supercoiling and releasing DNA. Its negative supercoiling relaxation activity, similar to other type IA topoisomerases, likely requires an unwound region, in this case, promoted by negative supercoiling and elevated reaction temperature. However, it will be interesting to examine whether reverse gyrase has positive supercoil relaxation activity, and what the requirements are for such an activity. We have prepared 3 positively supercoiled DNA substrates, a plasmid DNA (pBS/SK+), DNA with single-stranded bubble (pBS/SK Bubble), and a plasmid with a 38-bp insert of alternating AT (pUC(AT)19). The bubble DNA was prepared by annealing two single-stranded circles produced from phagemids pBS/SK+ and pBS/SK− as described earlier (15). Because reverse gyrase will introduce positive supercoils into the bubble substrate in the presence of ATP (see later sections under “Results”), supercoil relaxation reactions were carried out with ADP as the cofactor. Neither positively supercoiled pBS/SK− nor pUC(AT)19 are relaxed by reverse gyrase (Fig. 3, lanes 1–5 and lanes 6–10, respectively). Whereas the AT-rich segment is expected to be unwound in a relaxed or negatively supercoiled DNA, the positive supercoiling in this DNA will promote base pairing in the AT-rich region (18, 19). The positive supercoils in the bubble substrate can be fully relaxed (Fig. 3, lanes 11–15). As a control, all three positively supercoiled DNA can be relaxed completely by a type IB topoisomerase, Drosophila topo I (lanes 19–21). These results demonstrate that in the mode of supercoil relaxation, reverse gyrase is similar to other type IA topoisomerases in that it can relax negatively supercoiled DNA, and only relax the positive supercoils provided the substrate contains a permanently denatured region.

Topological Linking of Complementary Single-stranded DNA Circles—One of the hallmark reactions for a type I topoisomerase is the linking of two complementary single-stranded circles through the transient single strand breaks generated by these enzymes. Both rat liver topo I, a type IB enzyme, and E. coli topo I, a type IA enzyme, can mediate this reaction (20, 21). We have prepared plus and minus strands of circular DNA from the corresponding phagemids, and incubated equal moles of two single-stranded circles with reverse gyrase in the elevated temperature or absence of cofactors. The DNA products were analyzed by agarose gel electrophoresis under either neutral (Fig. 4A) or alkaline (Fig. 4B) conditions. Without enzyme, incubation of the complementary circles resulted in the formation of DNA species migrating slower than the single-stranded DNA control (Fig. 4A, lanes 21 and 22), presumably because annealing complementary sequences to form circular DNA with duplex winding in both right-handed and left-handed senses, and with interruptions of single-stranded regions (Form V DNA). However, the two DNA strands are not linked topologically (linking number, Lk = 0). This is confirmed by the analysis of gel electrophoresis in alkaline buffer, in which the annealed circles were denatured and comigrated with single-stranded circles (Fig. 4B, lanes 21 and 22). With reverse gyrase, DNA species migrating slower than Form V appeared during the time course of the reactions, suggesting that topological linkage was introduced. However, this strand linking was most efficient with ATP as the cofactor (Fig. 4A, lanes 9–12). The topological linkage between single-stranded circles can be readily monitored with alkaline gel electrophoresis. The initial strand passage between two circles produces the largest mobility shift, and the interlinked dimer with a single crossover between two strands is the slowest migrating species on the gel (Fig. 4B). Subsequent topological linkage further compacts DNA under denaturing conditions, and the electrophoretic mobility correlates with the linking number between the intertwining strands. Using known supercoiled DNA markers, we established that the order of increasing mobility is hypernegatively supercoiled, negatively supercoiled, relaxed, and positively supercoiled DNA (data not shown, and Fig. 4B). Whereas reverse gyrase is inefficient in linking two single-

FIGURE 3. Reverse gyrase can relax positively supercoiled DNA with a single-stranded bubble. Time course for the reverse gyrase reactions (with 1 mM ADP and enzyme/DNA ratio of 0.5) with positively supercoiled substrates of pUC(AT)19 (lanes 1–5), pBS/SK− (lanes 6–10), and pBS/SK with a bubble (lanes 11–15). Time points of the reactions were identical to those shown in Fig. 2. The positively supercoiled substrates are in lanes 16–18, and Drosophila topo I (Dm topo I) could completely relax all three substrates (lanes 19–21).
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**FIGURE 4.** Topological linking of complementary single-stranded circles. Identical DNA samples were analyzed by agarose gel electrophoresis under either neutral (panel A) or alkaline (panel B) conditions. Time course of the reverse gyrase reactions without cofactor, or with 1 mM ADP, ATP, and AMPPNP are shown in lanes 1–4, 5–8, 9–12, and 13–16, respectively. Reverse gyrase was present in these reactions at a ratio of 0.8 enzyme/single-stranded circle. Equivalent amounts of topo I from *T. maritima* (Tm topo I), in terms of its relaxation activity, was used for the time course shown in lanes 17–20. Control reactions without enzyme and cofactors are shown in lanes 21 (with incubation) and 22 (without incubation). Single-stranded DNA (SS) can anneal and generate Form V (lane 21, panel A), but will migrate as a single-stranded circle under alkaline conditions because it is not topologically linked. The migration of annealing products in the alkaline gel is correlated with the linkage between the complementary circles (B), and they are in the order, from top to bottom, of hypernegative supercoiled (HNSC), negatively supercoiled (NSC), relaxed (RC), and positively supercoiled (PSC). The slowest migrating species is presumably the interlocked dimer (ID). In neutral gel (A), the relaxed DNA is the diffuse ladder migrating slower than the supercoiled species (PSC or HNSC).

The strand specificities of reverse gyrase reactions were analyzed by agarose gel electrophoresis under either neutral (panel A) or alkaline (panel B) conditions. Time course of the reverse gyrase reactions without cofactor, or with 1 mM ADP, ATP, and AMPPNP are shown in lanes 1–4, 5–8, 9–12, and 13–16, respectively. Reverse gyrase was present in these reactions at a ratio of 0.8 enzyme/single-stranded circle. Equivalent amounts of topo I from *T. maritima* (Tm topo I), in terms of its relaxation activity, was used for the time course shown in lanes 17–20. Control reactions without enzyme and cofactors are shown in lanes 21 (with incubation) and 22 (without incubation). Single-stranded DNA (SS) can anneal and generate Form V (lane 21, panel A), but will migrate as a single-stranded circle under alkaline conditions because it is not topologically linked. The migration of annealing products in the alkaline gel is correlated with the linkage between the complementary circles (B), and they are in the order, from top to bottom, of hypernegative supercoiled (HNSC), negatively supercoiled (NSC), relaxed (RC), and positively supercoiled (PSC). The slowest migrating species is presumably the interlocked dimer (ID). In neutral gel (A), the relaxed DNA is the diffuse ladder migrating slower than the supercoiled species (PSC or HNSC).

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**FIGURE 5.** Time course for positive supercoiling reactions with pBS/SK− plasmid or pBS bubble substrate DNA. Reverse gyrase was present at an enzyme/DNA ratio of 0.5 (lanes 1–10) or 0.1 (lanes 11–20). Relaxed DNA substrate is in lanes 21 (pBS/SK−) and 22 (pBS with single-stranded bubble). Under the conditions tested here, pBS/SK− plasmid DNA showed no change in supercoiling (lanes 1–5 and 11–15), whereas pBS bubble substrate can be positively supercoiled (lanes 6–10 and 16–20).

enzyme. For example, at an enzyme/DNA ratio of about 10, each DNA gyrase can introduce less than one positive supercoil, and the average number of supercoils introduced per reverse gyrase molecule decreases at higher enzyme to DNA ratios (12). It is therefore surprising to observe efficient positive supercoiling in the DNA products after strand annealing (Fig. 4, A and B, lanes 9–10), because the enzyme to DNA ratio was 0.8 in these experiments. However, the DNA products from single strand annealing experiments contain a single-stranded bubble because of the presence of identical, rather than complementary, sequence in the f1 replication origin (f1 ori) in the single-stranded circles. To examine if the presence of a single-stranded bubble may be responsible for such an efficient positive supercoiling reaction, we prepared relaxed DNA substrates from the bubble substrate generated from the strand annealing reaction, and its vector plasmid control, pBS/SK−. We used these relaxed DNA substrates in positive supercoiling reactions with a sub-stoichiometric amount of reverse gyrase (Fig. 5). Concordant with the previous results, at an enzyme/DNA ratio of either 0.5 or 0.1, the plasmid DNA substrate showed no change in DNA supercoiling over a time course up to 1 h (Fig. 5, lanes 1–5 and 11–15). With relaxed bubble substrate, the DNA was positively supercoiled by 15 min with an enzyme/DNA ratio of 0.5 (Fig. 5, lanes 6–10). Even with 5-fold less enzyme, the DNA was efficiently supercoiled after a 60-min incubation with reverse gyrase (Fig. 5, lanes 16–20). Positive supercoiling in the DNA products was confirmed by gel electrophoresis in the presence of chloroquine or netropsin. With chloroquine gel, positively supercoiled DNA still ran as a fast migrating band, whereas with netropsin gel, their electrophoretic mobility was retarded (data not shown). The efficient positive supercoiling of a bubble substrate suggests that reverse gyrase can increase the linking number by promoting duplex winding in a single-stranded region.

**Minimal Size of the Single-stranded Bubble for Efficient Positive Supercoiling**—The single-stranded region in the bubble substrate used in the above experiments is present because of the f1 ori sequence, and is 462 nucleotides in length. To determine the length requirement for a single strand bubble, we designed a novel method to synthesize a circular DNA substrate with a desired length and sequence context of the single-stranded region. The reaction steps and conditions for the synthesis were described under "Experimental Procedures." It is based on the same idea as the synthesis of the bubble substrate: annealing two complementary circles produced from phagemids. An insertion...
sequence was engineered into the DNA so that the annealed circle has two single-stranded bubbles, one from the inserted sequence to give a bubble with desired length, and the other from the f1 ori. We have also introduced a tandem set of loxP sites into the DNA vector sequence, thus allowing the Cre/loxP site-specific recombination to remove the f1 ori bubble and leave behind a covalently closed circle with only the engineered bubble from the inserted sequence (see Fig. 1A for a schematic diagram). We designed 6 sets of oligonucleotide sequences to insert into the phagemid vectors (Fig. 1B), which result in the synthesis of a 3.1-kb DNA substrate with a bubble size of 5, 20, or 50 nucleotides. The presence of a single-stranded region in these DNA substrates was also confirmed by their sensitivity toward a structure-specific endonuclease, T7 endo I, compared with either a control sequence without the bubble or a plasmid vector DNA (supplementary Fig. S1). These relaxed bubble DNA substrates were used in subsequent experiments with reverse gyrase.

We first examined the 5-nucleotide (Bub5) and 50-nucleotide (Bub50) bubble substrate in the positive supercoiling reactions (Fig. 6, A and B). With an enzyme/DNA ratio of 0.5, Bub5 showed no change in DNA supercoiling (Fig. 6A, lanes 1–5). In contrast, Bub50 was supercoiled with a similar rate and extent as the DNA with a much larger f1 ori bubble (Fig. 6A, lanes 6–10; compared with Fig. 5, lanes 6–10). The extent of DNA supercoiling was further analyzed by gel electrophoresis in the presence of netropsin in lanes 6–10 (Fig. 6, A and B). The gel electrophoresis demonstrated an insertion of about 9 nucleotides or more in the size of the single-stranded bubble for efficient positive supercoiling reactions, and the single-stranded region can be from either non-complementary heteroduplex sequence or from a stretch of AT sequence.

Whereas the heteroduplex (non-complementary) sequence in the DNA provides a single-stranded region for reverse gyrase to act upon, we tested if a segment of the AT-rich sequence can satisfy the requirement of the single-stranded bubble in the supercoiling reaction. A stretch of AT sequence in a relaxed DNA substrate is expected to be denatured under the reverse gyrase conditions at 80 °C. Using a DNA containing 38 nucleotides of AT sequence, pUC(AT)19, we showed that the rate and extent of DNA supercoiling are similar to the bubble substrate of Bub50 or f1 ori bubble (Fig. 8, lanes 6–10). Analysis by gel electrophoresis with netropsin demonstrated an insertion of about 9 superhelical turns into the DNA product (data not shown), a result similar to those obtained by using Bub50. Reverse gyrase showed no activity on the vector control, pUC19 (Fig. 8, lanes 1–5). Combining the results shown in Figs. 7 and 8 there is a requirement of at least 20 nucleotides or more in the size of the single-stranded bubble for efficient positive supercoiling reactions, and the single-stranded region can be from either non-complementary heteroduplex sequence or from a stretch of AT sequence.

DISCUSSION

Reverse gyrase is an unusual type IA topoisomerase both in terms of its structure and mechanism. It has two closely linked domains: a helicase domain at its N terminus and a type IA topoisomerase domain at its C terminus (8, 10, 11). Whereas the helicase domain displays DNA-dependent ATPase activity, neither reverse gyrase nor the isolated helicase

**FIGURE 6.** Relaxed DNA with a single-stranded bubble of 50 nucleotides can be positively supercoiled, but not the DNA with a 5-nucleotide bubble. A, time course of the positive supercoiling reactions for a 5-nucleotide bubble (lanes 1–5) and a 50-nucleotide bubble (lanes 6–10). Relaxed DNA substrates are in lanes 11 (5-nucleotide bubble) and 12 (50-nucleotide bubble). Reverse gyrase was present at an enzyme/DNA ratio of 0.5. B, reaction products from the 50-nucleotide bubble (lanes 6–10, panel A) were analyzed by gel electrophoresis in the presence of 5 μg/ml netropsin (lanes 1–5 in panel B) to resolve the positively supercoiled DNA. The starting material, relaxed DNA, is in lane 6.

**FIGURE 7.** Relaxed DNA with a 20-nucleotide bubble can be positively supercoiled, albeit to a lower level (lanes 6–10) than the 50-nucleotide bubble substrate, whereas control DNA without any bubble cannot be supercoiled (lanes 1–5). A time course of the reactions is shown here. Relaxed DNA substrate is in lanes 11 (M20 control DNA) and 12 (20-nucleotide bubble). Reverse gyrase was present at an enzyme/DNA ratio of 0.5.
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Reverse gyrase was present at an enzyme/DNA ratio of 0.5. Time course of the reactions for the control plasmid pUC19 is shown in lanes 1–5, and for a pUC19 vector containing 38 bp of AT sequence, pUC(AT)19, in lanes 6–10. The relaxed DNA substrates are shown in lanes 11 and 12 for pUC19 and pUC(AT)19, respectively.

We also demonstrated here that reverse gyrase can mediate two distinct reactions involving strand annealing: annealing of complementary single-stranded circles and positive supercoiling of DNA with a single-stranded bubble. Using gel electrophoresis under both neutral and alkaline conditions, we showed that single-stranded circles can be readily joined and wound into a double-stranded circle in the presence of reverse gyrase. Because the linking number of two separate circles is zero, during the course of the annealing reaction, the enzyme must mediate enough strand passage events to allow for the formation of double-stranded helical structure. This strand passage and rewinding activity are most efficient with reverse gyrase in the presence of a nucleotide cofactor, especially ATP (see Fig. 3). Therefore, conditions that favor positive supercoiling also promote strand annealing, both of which result in an increase in DNA linking numbers.

The positive supercoiling reaction with a bubble substrate is very efficient, capable of reaching a high level of supercoiling using a sub-stoichiometric amount of enzyme. This result is in marked contrast with earlier ones using plasmid DNA substrate, for which only a moderate level of positive supercoiling was reached with an excess amount of enzyme. Because the single-stranded region in the bubble substrate is unable to form stable base pairs, it will likely remain unwound and continue to serve as a site for the rewinding action by reverse gyrase. Besides a single-stranded bubble, the AT-rich segment can also serve as an efficient substrate for the positive supercoiling reaction. This is presumably because of the melting of the AT sequence at the incubation temperature of 80 °C, providing a site for reverse gyrase reaction. The DNA supercoiling can affect helical stability, and positive supercoiling facilitates the rewinding of unwound region (18, 19). Theoretical considerations have been developed to allow one to calculate the stability of the DNA segment with known base composition at a given superhelical density and temperature (19, 22). Precise calculations were not feasible for the system used in our experiments because some of the parameters used in this formulation are not available for the temperature range relevant in our experiments. However, it is possible that the positive supercoiling generated here can drive the rewinding of the AT-segment. A single-stranded bubble, unable to form base pairs, can also undergo pleconematic winding under positive supercoiling stress (19). This rewinding action by reverse gyrase can thus effectively diminish or eliminate the junctions of the single-/double-stranded region.

The strand annealing and positive supercoiling reactions described here support the mechanism we proposed for reverse gyrase: promotion of strand rewinding by a switch of binding affinity from single- to double-stranded DNA occurs after ATP hydrolysis, thus effectively promoting rewinding of single strands. This rewinding activity is further facilitated by its strand passage activity, allowing the enzyme to increase the linking number of DNA and to introduce positive supercoils. Because enzyme binding determines the magnitude of linking number changes, positive DNA supercoiling requires an excess amount of enzyme relative to DNA, and its extent depends on the stoichiometry of enzyme to DNA.

We presented here our analysis of efficient strand passage activities of reverse gyrase with a substoichiometric amount of enzyme. Similar to all type IA topoisomerases, reverse gyrase can readily relax negatively supercoiled DNA. In contrast to the supercoiling reactions we analyzed before, supercoil relaxation is not exquisitely sensitive to the reaction temperature. This supercoil relaxation activity is only limited to the negatively supercoiled DNA for the plasmid DNA substrate. Interestingly, reverse gyrase can relax positively supercoiled DNA, provided there is a single-stranded bubble present in the DNA substrate. Therefore, similar to other type IA topoisomerases, the supercoil relaxation activity of reverse gyrase requires the presence of an unwound or underwound region in the DNA substrate.

We also demonstrated here that reverse gyrase can mediate two distinct reactions involving strand annealing: annealing of complementary single-stranded circles and positive supercoiling of DNA with a single-stranded bubble. Using gel electrophoresis under both neutral and alkaline conditions, we showed that single-stranded circles can be readily joined and wound into a double-stranded circle in the presence of reverse gyrase. Because the linking number of two separate circles is zero, during the course of the annealing reaction, the enzyme must mediate enough strand passage events to allow for the formation of double-stranded helical structure. This strand passage and rewinding activity are most efficient with reverse gyrase in the presence of a nucleotide cofactor, especially ATP (see Fig. 3). Therefore, conditions that favor positive supercoiling also promote strand annealing, both of which result in an increase in DNA linking numbers.

The positive supercoiling reaction with a bubble substrate is very efficient, capable of reaching a high level of supercoiling using a sub-stoichiometric amount of enzyme. This result is in marked contrast with earlier ones using plasmid DNA substrate, for which only a moderate level of positive supercoiling was reached with an excess amount of enzyme. Because the single-stranded region in the bubble substrate is unable to form stable base pairs, it will likely remain unwound and continue to serve as a site for the rewinding action by reverse gyrase. Besides a single-stranded bubble, the AT-rich segment can also serve as an efficient substrate for the positive supercoiling reaction. This is presumably because of the melting of the AT sequence at the incubation temperature of 80 °C, providing a site for reverse gyrase reaction. The DNA supercoiling can affect helical stability, and positive supercoiling facilitates the rewinding of unwound region (18, 19). Theoretical considerations have been developed to allow one to calculate the stability of the DNA segment with known base composition at a given superhelical density and temperature (19, 22). Precise calculations were not feasible for the system used in our experiments because some of the parameters used in this formulation are not available for the temperature range relevant in our experiments. However, it is possible that the positive supercoiling generated here can drive the rewinding of the AT-segment. A single-stranded bubble, unable to form base pairs, can also undergo pleconematic winding under positive supercoiling stress (19). This rewinding action by reverse gyrase can thus effectively diminish or eliminate the junctions of the single-/double-stranded region.

The strand annealing and positive supercoiling reactions described here support the mechanism we proposed for reverse gyrase: promotion of strand rewinding by a switch of binding affinity from single- to double-stranded DNA, and by the coupled strand passage activity. These reverse gyrase reactions may have important intracellular functions as well. From the proteomic analysis, reverse gyrase is the only hyperthermophile-specific protein (23). Its biological functions are likely involved in maintaining the genome stability under the extreme growth temperature. The reverse gyrase mutant displays significant growth defects at high temperature (24). The winding of DNA strands by reverse gyrase, as demonstrated in the annealing of complementary single strand circles and positive supercoiling in bubble substrates, may be critical for its functions in the hyperthermophilic organisms. Reverse gyrase may thus serve as a “sentinel,” searching for unwound or bubble regions, and efficiently rewind the strands to reform DNA duplex. Future work on the biochemical basis of the differential affinity of reverse gyrase with DNA will be important for further understanding of its function and mechanism.

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