Heat Shock-induced DNA Relaxation in Vitro by DNA Gyrase of Escherichia coli in the Presence of ATP*

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Genetic studies revealed that DNA gyrase seems to catalyze immediate and transient DNA relaxation after Escherichia coli cells are exposed to heat shock (Ogata, Y., Mizushima, T., Kataoka, K., Miki, T., and Sekimizu, K. (1994) Mol. Gen. Genet. 244, 451–455). We have now obtained biochemical evidence to support this hypothesis. DNA gyrase catalyzed an increase in the linking number of DNA and relaxation of negatively supercoiled DNA, under physiological concentrations of ATP. Analyses by gel filtration chromatography of each subunit revealed that DNA relaxation activity co-migrated with each subunit. The linking number of DNA increased as the temperature increased. Further, the reaction was inhibited by nalidixic acid or by oxolinic acid. Based on these results, we propose that DNA gyrase participates in a concerted reaction with DNA topoisomerases in the immediate relaxation of DNA in cells exposed to heat shock.

We also reported that relaxation of DNA after heat shock in a topA deletion mutant was blocked by nalidixic acid or by oxolinic acid, while the reaction was not affected by these drugs in the wild-type strain (12). DNA relaxation after heat shock in cells carrying both the topA deletion and a nalidixic acid-resistant gyrA mutation (nalA26) was not inhibited by nalidixic acid (12). Thus, not only DNA topoisomerase I but also DNA gyrase seems to catalyze DNA relaxation after heat shock. The literature suggests that DNA topoisomers are under thermal equilibrium in the presence of ATP and DNA gyrase. There is, however, no report of direct evidence that DNA gyrase catalyzes DNA relaxation under physiological concentrations of ATP; the enzyme was found to catalyze the reaction of DNA relaxation only in the absence of ATP (15, 16).

We analyzed reaction products of purified DNA gyrase by agarose gel electrophoresis in the presence of chloroquine and found that DNA gyrase catalyzes an increase in the linking number of DNA in the presence of ATP, and that the linking number of the product DNA increases as the temperature increases. These findings provide biochemical evidence for the participation of DNA gyrase in DNA relaxation, after exposure of cells to heat shock.

EXPERIMENTAL PROCEDURES

Bacterial Strain—E. coli strain DM800 (ΔtopA-cysB204 gyrB225 acrA13) (17) was kindly provided by H. Ikeda (University of Tokyo).

Materials—ATP, chloroquine, and oxolinic acid were purchased from Sigma; nalidixic acid was from Dai-ichi Pharmaceutical Co., Ltd.; chloramphenicol was from Sankyo Co., Ltd.; bovine serum albumin (fraction V) was from Boehringer Mannheim; and molecular mass markers for SDS-polyacrylamide gel electrophoresis and calf thymus DNA topoisomerase I were from Life Technologies, Inc. Both the A and B subunits were purified from the overproducers, RW1053 harboring pMK90 (18) and N4830 harboring pMK481,† respectively, which were kindly provided by T. Ogawa (Nagoya University), using the following method.

Analysis of Linking Number of Plasmid DNA in Cells Treated with Chloramphenicol—DM800 (ΔtopA) cells harboring pUC118 were grown as described previously (12), but with some modifications. Exponentially growing cells (OD 650 of 0.5) were incubated with 100 μg/ml chloramphenicol at 30 °C for 16 h. Nalidixic acid (200 μg/ml) or oxolinic acid (100 μg/ml) was added, and incubation was continued at 30 °C for 20 min. Cells were heat-treated at 50 °C for 2 min. Plasmid DNA was extracted using the alkaline method (19) and analyzed by 1% agarose gel (15 × 15 × 0.60 cm) electrophoresis in Tris borate-EDETA buffer (19) at 60 V for 16 h in the presence of 15 μg/ml chloroquine. The gel was stained with 1 μg/ml ethidium bromide, and a photograph was taken on a UV illuminator with a Polaroid apparatus (Polapan 32, Polaroid), as described elsewhere (12, 20).

Purification of A and B Subunits of DNA Gyrase—A and B subunits of DNA gyrase were purified by measuring the supercoiling activities using a plasmid DNA, pKP1110 (21), that relaxed with calf thymus DNA topoisomerase I. The A subunit was purified by ammonium sulfate precipitation of a cell lysate, and chromatography on DEAE-cellulose and phenyl-Sepharose. The B subunit was purified by the method

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† T. Ogawa, personal communication.
DNA Relaxation by DNA Gyrase

Influence of DNA gyrase inhibitors on the heat-induced DNA relaxation in a topA deletion mutant treated with chloramphenicol. DNA gyrase catalyzes a reaction which increases the linking number of DNA in the presence of ATP, of negatively supercoiled DNA (24, 25). We therefore treated cells (12). We suggested that the DNA relaxation after heat shock is not the result of a heat shock response, since the reaction was observed in an rpmH amber mutant where the heat shock response does not occur (10). As protein synthesis was blocked by chloramphenicol, under the present conditions, the present result confirms our previous conclusion.

When the topA deletion mutant cells treated with chloramphenicol were further treated with nalidixic acid or oxolinic acid, inhibitors of DNA gyrase, no change in the pattern of the topoisomers by heat shock was observed (lanes 5 and 7). This implies that newly synthesized proteins are not involved in the DNA gyrase-dependent DNA relaxation and that the heat-induced DNA relaxation can probably be reconstituted with purified DNA gyrase and other proteins. DNA gyrase may have the potential to catalyze a reaction which changes the linking number of DNA, in both negative and positive directions. However, there are no data available to support this theory.

The results in Fig. 1 show that nalidixic acid, but not oxolinic acid, induces DNA relaxation in the topA mutant treated with chloramphenicol at 30°C. We assume that nonidentified DNA topoisomerases other than DNA topoisomerase I and DNA gyrase catalyze this reaction.

Increase in the Linking Number of DNA Catalyzed by E. coli DNA Gyrase in the Presence of ATP—To determine whether DNA gyrase catalyzes a reaction which increases the linking number of closed circular duplex DNA in the presence of ATP, we examined the reaction product isolated by agarose gel electrophoresis in the presence of chloroquine. As exposure to chloroquine leads to a positive supercoiling of double-stranded DNA, in a concentration-dependent manner (22, 23), one can distinguish small differences in the linking number of negatively supercoiled DNA by agarose gel electrophoresis in the presence of appropriate concentrations of this antimalarial drug. We incubated the negatively supercoiled form of pUC118 with A and B subunits of DNA gyrase in the presence of 2 mM ATP. Fig. 2A shows the results of agarose gel electrophoresis in the presence of various concentrations of chloroquine. Since the reaction products are negatively supercoiled, they migrated almost as a single band in the gel in the absence of chloroquine (lane 2). We also observed minor ladder bands in the gel in which migrations were slower than that of the major band (lane 2). Since the appearance of the slower migrating species depended on both A and B subunits of DNA gyrase (lanes 3 and 4), they were probably produced by the reaction with DNA gyrase.

In the gels with chloroquine, it was even more apparent that the pattern of topoisomers differed between the substrate and the product: the latter migrated slower than the former in the gel with 1 μg/ml chloroquine (lanes 5 and 6), and faster in the presence of 15 μg/ml chloroquine (lanes 9 and 10). A change of the electrophoretic pattern of the substrate DNA depended on both A and B subunits of DNA gyrase (lanes 7, 8, 11, and 12). These observations suggest that the linking number of the substrate DNA increases after the reaction with DNA gyrase in the presence of ATP; that is, purified DNA gyrase catalyzes the relaxation of negatively supercoiled duplex DNA in the presence of ATP. Fig. 2B shows titrations of A and B subunits of DNA gyrase in the reaction in the presence of a fixed amount of the B (120 ng) or A subunit (74 ng), respectively. The linking number of the substrate DNA increased, depending on each subunit. The amount of A and B subunits necessary for the relaxation of 1 μg of DNA was 37 and 120 ng, respectively, the same amount required for the supercoiling activity of this enzyme (data not shown).

A degradation product of the B subunit has relaxation activity that is dependent on the A subunit and independent on ATP, of negatively supercoiled DNA (24, 25). We therefore asked whether the reaction which increased the linking number of DNA with ATP was catalyzed by contaminated DNA.
topoisomerases, including the degradation product of the A and B subunits produced during the preparation of DNA gyrase (purity of our preparations exceeded 90%). Preparations of A or B subunits of DNA gyrase were separately loaded into a gel filtration column of Superose 12, and elution profiles of the activity of the increase caused in the linking number of DNA was compared to that of each subunit. The eluted fractions were incubated with the counterpart subunit, substrate DNA, and ATP. The reaction products were analyzed by agarose gel electrophoresis in the presence of 15 μg/ml chloroquine.

DNA relaxation induced by heat treatment in a topA mutant treated with chloramphenicol (Fig. 1). We examined the influence of these drugs on the increase in the linking number of DNA observed in the presence of ATP and DNA gyrase. As shown in Fig. 4, these drugs inhibited potently the reaction and concentrations required to inhibit completely the reaction were approximately 100 and 50 μg/ml, respectively. The concentrations of these drugs required for inhibition of the reaction were similar to levels required for DNA relaxation induced by heat shock in the topA mutant cells treated with chloramphenicol. These results are consistent with the hypothesis that DNA relaxation after heat shock in the topA mutant is catalyzed by DNA gyrase.

Influence of Nalidixic Acid and of Oxolinic Acid on the Reaction That Increased the Linking Number of DNA Caused by DNA Gyrase in the Presence of ATP—In vitro, nalidixic acid and oxolinic acid inhibit the supercoiling of closed circular duplex DNA induced by DNA gyrase. These drugs also inhibit the DNA relaxation induced by heat treatment in a topA mutant treated with chloramphenicol (Fig. 1). We examined the influence of these drugs on the increase in the linking number of DNA observed in the presence of ATP and DNA gyrase. As shown in Fig. 4, these drugs inhibited potently the reaction and concentrations required to inhibit completely the reaction were approximately 100 and 50 μg/ml, respectively. The concentrations of these drugs required for inhibition of the reaction were similar to levels required for DNA relaxation induced by heat shock in the topA mutant cells treated with chloramphenicol. These results are consistent with the hypothesis that DNA relaxation after heat shock in the topA mutant is catalyzed by DNA gyrase.

Influence of Temperature on Linking Number of DNA after the Reaction with DNA Gyrase in the Presence of ATP—The results in Figs. 1 and 4 suggest that DNA gyrase catalyzes the relaxation of plasmid DNA in cells after heat shock. Thus, we considered the possibility that the relaxation can be explained by an intrinsic characteristic of DNA gyrase. We next examined the influence of temperature on the linking number of DNA relaxation induced by heat treatment in a topA mutant treated with chloramphenicol (Fig. 1). We examined the influence of these drugs on the increase in the linking number of DNA observed in the presence of ATP and DNA gyrase. As shown in Fig. 4, these drugs inhibited potently the reaction and concentrations required to inhibit completely the reaction were approximately 100 and 50 μg/ml, respectively. The concentrations of these drugs required for inhibition of the reaction were similar to levels required for DNA relaxation induced by heat shock in the topA mutant cells treated with chloramphenicol. These results are consistent with the hypothesis that DNA relaxation after heat shock in the topA mutant is catalyzed by DNA gyrase.
after the reaction with purified DNA gyrase. Negatively supercoiled pUC118 DNA was incubated with DNA gyrase in the presence of ATP, at 30 or 45 °C, and was analyzed by agarose gel electrophoresis in the presence of chloroquine. As shown in Fig. 5, the reaction increasing the linking number of DNA reached saturation after 5 min, either at 30 or at 45 °C. The level of saturation of the linking number of DNA at 45 °C was larger than that at 30 °C. The extent of DNA relaxation observed in vitro by the reaction with purified DNA gyrase after a thermal pulse is much the same as that in the topA mutant cells treated with chloramphenicol (Fig. 1). Thus, temperature-induced DNA relaxation was simulated with plasmid DNA and with DNA gyrase. As DNA gyrase is not a heat-stable enzyme, one must consider the possibility that irreversible denaturation of the supercoiling activity of the enzyme after incubation at 45 °C could explain the increase in the observed linking number of the reaction product. To exclude this possibility, we changed the temperature of the reaction mixture at intervals of 10 min between 30 and 45 °C, and determined the linking number of DNA by agarose gel electrophoresis in the presence of chloroquine (Fig. 6). The linking number of DNA was increased after elevating the temperature from 30 to 45 °C (lanes 4 and 5). When the temperature was decreased again to 30 °C, the linking number of DNA concomitantly decreased to the initial level at 30 °C (lanes 6 and 7). Further, when the temperature was again elevated to 45 from 30 °C, the linking number increased again (lanes 8 and 9). These results indicate that the linking number of products produced with DNA gyrase reversibly increases and decreases with changes in temperature. Therefore, the increase in the linking number of products is not due to the irreversible denaturation of DNA gyrase, rather it is caused by an intrinsic characteristic of the enzyme.

**DISCUSSION**

We provide evidence that _E. coli_ DNA gyrase catalyzes not only a decrease but also an increase in the linking number of DNA in the presence of a physiological concentration of ATP (Fig. 2). We found that the average linking number of the reaction product with DNA gyrase was greater at higher temperatures (Fig. 5) and that the number reversed with temperature (Fig. 6). These results provide biochemical evidence suggesting that DNA gyrase participates in the reaction which causes the observed immediate increase in the linking number of DNA in cells after heat shock (10). In other words, DNA gyrase senses the temperature shift and changes the linking number of DNA. Our previous genetic studies also support this notion (12).

The linking number of reaction products with DNA topoisomerases is considered to be smaller at high temperatures (26–28). Thus, the larger linking numbers of the reaction products observed at high temperatures seem to be a special characteristic of DNA gyrase. The angle between two adjacent bases in double-stranded DNA increases with an increase in temperature (27). As a result, a temperature increase intro-
duces positive supercoiling in closed circular duplex DNA followed by a decrease in the linking number of DNA if DNA topoisomerases are present. We reported that the linking number of DNA increases with an increase in temperature in the presence of mouse DNA topoisomerase I and ethidium bromide (26). The DNA binding of ethidium bromide, which is necessary for the introduction of negative supercoiling after the reaction with mouse topoisomerase I, is sensitive to high temperatures (26). At high temperatures, the number of ethidium bromide molecules bound to DNA decreases, the result being less positive supercoiling of DNA. This factor may contribute more than the effect of temperature to the supercoiled structure of closed circular duplex DNA. As a result, the linking number of DNA is larger at higher temperatures. Thus, the DNA relaxation caused by a temperature increase is explained by the temperature-sensitive DNA binding of ethidium bromide. In the case of DNA gyrase, the enzyme has two distinct DNA binding domains (29, 30), a domain with a catalytic site for a double strand DNA break and rejoining and a domain responsible for creating positive supercoiling of DNA. The function of the former domain does not require ATP, since DNA gyrase catalyzes DNA relaxation in the absence of ATP (15, 16). The latter domain is necessary to introduce negative supercoiling of substrate DNA, and the interaction of this domain and DNA may be sensitive to high temperatures, as is the case for ethidium bromide.

In E. coli, there is DNA topoisomerase IV, another type II DNA topoisomerase, that relaxes DNA in the presence of ATP and is inhibited by quinolones (31, 32). Thus, our results do not rule out a role for this enzyme in relaxation of DNA in cells after heat shock. We earlier suggested that DNA topoisomerase I also contributes to the reaction (12). We propose here that DNA relaxation in cells induced by heat shock is a concerted reaction of these DNA topoisomerases together with DNA gyrase.

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