Quinolone-Resistant Mutations of DNA Gyrase Increase Sensitivity to Acriflavin

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DNA gyrase were constructed to possess the quinolone-resistant (D87N in GyrA or K447E in GyrB) and acrB (S759R-R760C in GyrB) mutations and their sensitivities to acriflavin and oxolinic acid were examined. Both quinolone-resistant mutations in GyrA and GyrB increased acriflavin sensitivities in the supercoiling assay irrespective of the co-presence of the acrB mutation. In the DNA binding assay, however, the hypersensitivity caused by the GyrB (K447E) mutation was observed only in the co-presence of the acrB mutation; the presence of the acrB mutation, which not affecting acriflavin sensitivity, reduces the extent of DNA binding, as reported previously. Thus, the quinolone-resistant mutation site in GyrB is likely to be involved in DNA binding which is not detectable in acrB' gyrase. Furthermore, oxolinic acid was found to enhance DNA binding of the gyrase having GyrB (acrB-K447E), supporting a recent proposal that quinolone binding to the DNA-gyrase complex does not require DNA breakage.

Key words: topoisomerase II; DNA binding; E. coli; supercoiling; acrB

Bacterial DNA gyrase is a type II topoisomerase that introduces negative supercoils into closed-circular DNA. The enzyme consists of two subunits, A (97 kDa) and B (90 kDa), and forms a tertiary complex A2B2. The gyrase A protein (GyrA) consists of NH2-terminal (59–64 kDa) and COOH-terminal (33 kDa) domains. The NH2-terminal domain contains the active site tyrosine residue (Tyr122) which covalently binds to the 5' end of cleaved DNA and is responsible for DNA break-reunion. The COOH-terminal domain is necessary for DNA wrapping which determines the polarity of supercoiling. The gyrase B protein (GyrB) has been deduced to contain an NH2-terminal ATPase domain and a COOH-terminal domain which interacts with GyrA and DNA.1–3) The amino acid sequence homology and domain structure suggests that bacterial DNA gyrase and eukaryotic type II topoisomerase possesses similar structure and enzyme mechanism (Fig. 1).

Several acriflavin-hypersensitive (acr) mutants from Escherichia coli4–7) have been isolated, and one of them, the acrB mutation, falls within the gyrb locus that encodes GyrB.8) In a previous study, we reported that the acrB mutation alters two amino acids (Ser759–Arg760 to Arg-Cys) in the C-TERM region, which consists of 76 amino acids and is located at the COOH-terminal end of GyrB (Fig. 1). This C-TERM region corresponds to the hinge region of yeast topoisomerase II and is probably essential for conformational change in enzymatic reaction.9) The mutation did not change the sensitivity to acriflavin in the supercoiling assay, but reduced the enzyme activity. The gel shift assay showed that acriflavin inhibits DNA binding of gyrase. Furthermore, the acrB mutation caused a significant reduction in the ability of the enzyme to bind the DNA fragment containing a gyrase cleavage site without altering acriflavin sensitivity, indicating that the acrB site in the GyrB protein is essential for DNA binding in the catalytic reaction.9)

DNA gyrase is also an intracellular target of quinolone drugs (see reference 10 for a recent review). A number of studies have shown that the quinolone drugs interact with the DNA-gyrase complex. Quinolone-resistant mutations were first found in gyrA, and later in gyrB of E. coli.10,12) The gyrA mutations occur within the region of amino acid residues 67 to 106, including Asn87 instead of Asp (D87N). Two quinolone-resistant mutations have been found in gyrB, and they alter the amino acid residues 426 and 447. The mutant gyrase enzymes show resistance to quinolone drugs in vitro as well as in vivo.13)

The mutation sites of quinolone resistance are located at

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the NH₂-terminal side of the Tyr122 active site in GyrA and also at the gap between the ATPase domain and the C-TERM region of GyrB. In the primary structure of type II topoisomerase, these mutation sites surround the corresponding C-TERM region (Fig. 1). Berger et al. 15 have suggested that the mutational hot spot for quinolone-resistance in GyrA corresponds to the conserved amino acid residues in the catalytic site activator protein (CAP)-like DNA-binding domain of yeast topoisomerase II (Fig. 1). Recently, Morais Cabral et al. 15 reported the crystal structure of the breakage reunion domain of the GyrA dimer. They also found DNA binding domain structure at the active tyrosine region as in yeast topoisomerase II. They proposed that the major DNA groove substantially widens to bring the phospholyl groups closer to the active tyrosines. The quinolone-resistant mutations are located at the point where the distortion of the DNA is expected.

The reported quinolone-resistant mutations of GyrB (D426N and K447E) fall in the sequence which is also conserved in the type II topoisomerases, indicating that these mutation sites also play an important role in the enzyme reaction, although evidence suggesting their role has not been reported. Quinolone traps the transient state of the DNA-gyrase complex in which the 5’ end of cleaved DNA and the Tyr122 residue of the GyrA subunit are covalently bound each other. These features have suggested that quinolone recognizes and inhibits the DNA break-reunion step of the enzyme reaction. Therefore, these conserved sequences containing quinolone-resistant mutations in GyrA and GyrB are expected to contribute to the DNA break-reunion step.

To investigate the relationship between quinolone-resistant mutation and DNA binding, we examined the effect of acriflavine, which interacts with DNA binding, on thegyrases containing GyrA (D87N) and GyrB (K447E). Double-mutant gyrase which contains the GyrB (acrB-K447E) was also constructed, and the relationship between these two mutational domains was investigated. The results suggest that the quinolone-resistant mutation region participates in DNA binding.

MATERIALS AND METHODS

Cloning of the gyrA (D87N) Gene Genomic DNA of the JM109 strain was prepared as described by Cosloy and Oishi, 6 and the DNA fragment containing the gyrA gene was amplified by the PCR method. The amplified fragment was subcloned into pBluescript II (KS+) and sequenced. The glutathione S-transferase (GST) fusion expression plasmid was constructed as described previously.

Introduction of the Quinolone-Resistant Mutation into the gyrB Genes The quinolone-resistant mutation (A1339 to G at Lys447 to Glu) was introduced into the gyrB genes of plasmid pJB11 (wild-type) and pSB1 (acrB), which were described previously. 19 The NH₂-terminal side of the gene containing the mutation site was amplified from pJB11 and pSB1 by PCR using, respectively, oligonucleotides 5’-TTATGGATCCATGTCCGAATCTTTATGACTCC-3’ ( gyrB ‘NH₂-terminal’ primer including the BamH I site) and 5’-CTCGACGTTGAGATTCTCCCTT-3’ (quinolone-resistant mutation site reverse primer). COOH-terminal side with mutation site was also amplified using the oligonucleotides 5’-AATACTCGAATATCGATATTCGC-3’ ( gyrB ‘COOH-terminal’ primer containing a Xho I site) and 5’-AAAGGTTAATATCTCAACGTCGAG-3’ (quinolone-resistant mutation site forward primer). These amplified DNA fragments were mixed for annealing, and a second PCR was performed using the gyrB ‘NH₂-terminal’ and ‘COOH-terminal’ primers to generate the whole gene fragment. The amplified DNA fragments were digested with the restriction endonucleases BamHI and XhoI, and the resultant 2415-bp fragments were cloned into the expression vector pGEX4T-3 (Pharmacia Biotech, Tokyo, Japan) at the sites of the same restriction endonucleases and named pKF1 (K447E) and pKF2 (acrB-K447E). Both insertion sequences were verified using a Taq DyeDeoxy Terminator Sequencing Kit (Perkin-Elmer, Fostercity, CA, U.S.A.) in an automated DNA sequencer (Model 373S, Perkin-Elmer).

Purification of Gyrase Subunits The GyrA and GyrB proteins were prepared as described in Funatsuki et al. 24 Briefly, each subunit was over-expressed in E. coli as GST fusion proteins, and was purified by glutathione affinity chromatography and thrombin cleavage. To eliminate the copurified partner GyrA or GyrB subunits, chromatographies with DEAE-Sepharose and Novobiocin affinity columns were carried out. GyrB (K447E) and GyrB (acrB-K447E) were eluted from the Novobiocin column with HEPES buffer containing 5 M and 7 M urea, respectively. A flow-through fraction was collected to obtain GyrA.

Enzyme Assays The supercoiling and gel shift assays with recombinant gyrases from GyrB and GyrA variants were performed as described by Funatsuki et al. 24 Briefly, for the supercoiling assay, 1 μg of relaxed pBluescript II (KS+) plasmid DNA was incubated with various amounts of recombinant gyrase in 60 μl of reaction mixture at 30°C for 30 min. One unit enzyme activity of gyrase was defined as the amount that brought 50% of relaxed DNA to the supercoiled position in 0.8% agarose gel electrophoresis. We used 100 unit enzyme activity of each recombinant gyrase for the supercoiling assay in the presence of oxolinic acid and acriflavine. For the gel shift assay, 204-bp double-stranded DNA containing the gyrase cleavage site of pBR322 (base position 886—1089) was labeled with [γ-32P]ATP by T4 polynucleotide kinase. The labeled fragment (300 pg) was incubated with 5 μg of recombinant gyrase (2.5 μg for each subunit) in the presence of 25 ng of double-stranded competitor DNA (poly[dIdC]-poly[dIdC], Sigma, St. Louis, U.S.A.) at 25°C for 60 min and subjected to polyacrylamide gel electrophoresis (PAGE) in 25 mm Tris-HCl buffer (pH 8.4) containing 192 mm glycine and 8 mm MgCl₂. To quantitate the DNA-gyrase complex, a gel containing the complex was excised and the radioactivity was counted by the Cerenkov method.

RESULTS

Enzyme Preparation To obtain GyrA protein with quinolone-resistant mutation, we cloned the gyrA (nalA96) gene from the JM109 strain. 18 The nucleotide sequence revealed that the 259th G from the starting point of the coding region was substituted by A. Consequently, the nalA96 mutation alters the Asp amino acid at 87 to Asn. This mutation corresponds to the N-113 mutation which has been reported by Yoshida et al. 19 To obtain the GyrB protein with the
quinoxolone-resistant mutation, the GyrB (K447E) mutation was introduced into gyrB (wild) and gyrB (acrB) by oligonucleotide-directed site-specific mutagenesis. This mutation corresponds to the type II mutation of gyrB reported by Yamagishi et al. Each subunit was over-expressed in E. coli, and purified to homogeneity. Absence of the partner subunit was shown by SDS-PAGE and the supercoiling assay (data not shown).

The specific activity of DNA supercoiling was determined for each reconstituted gyrase. The enzyme with the GyrB (K447E) mutation showed a 400-fold reduced specific activity (8.0×10^2 unit/mg) from the wild-type gyrase (3.2×10^6 unit/mg), and the GyrB (acrB-K447E) enzyme showed an additional 6-fold reduction in activity (1.4×10^5 unit/mg) of the GyrB (K447E) enzyme. Gyrase carrying the GyrA (D87N) mutation showed a 320-fold reduction in specific activity (1.0×10^4 unit/mg). Gyrase with the GyrA (D87N) and GyrB (acrB) mutations showed an additional 3-fold reduced activity (3.0×10^3 unit/mg). The acrB mutation alone also significantly reduced the specific activity (4.0×10^4 unit/mg) as reported previously. The GyrB (acrB) effectively associates with GyrA to form gyrase tertiary complex, whereas the effect of other mutations on association was not determined. Therefore, there is a possibility that the mutant subunits of our preparation reduced the efficiency of reconstitution.

The 50% inhibition doses (ID_{50}) of quinoxolone for these gyrase in the supercoiling assay are shown in Table 1. The GyrA (D87N) and GyrB (K447E) subunits significantly reduced the sensitivity to oxolinic acid of the corresponding reconstituted gyrase as expected. The gyrase with the acrB-K447E mutation also showed lower sensitivity to oxolinic acid than those of wild-type and acrB gyrase. On the other hand, the acrB single mutation did not significantly change the sensitivity to oxolinic acid.

### Table 1. Inhibitory Concentrations of Acriflavine and Oxolinic Acid on Supercoiling Activity of Reconstituted Gyrase

<table>
<thead>
<tr>
<th>Reconstituted gyrase</th>
<th>Acriflavine (μg/ml)</th>
<th>Oxolinic acid (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GyrA (Asn87) + GyrB (wild)</td>
<td>15</td>
<td>&gt;100</td>
</tr>
<tr>
<td>GyrA (Asn87) + GyrB (acrB)</td>
<td>7.5</td>
<td>&gt;100</td>
</tr>
<tr>
<td>GyrA (wild) + GyrB (wild)</td>
<td>&gt;40</td>
<td>9</td>
</tr>
<tr>
<td>GyrA (wild) + GyrB (acrB)</td>
<td>&gt;40</td>
<td>9</td>
</tr>
<tr>
<td>GyrA (wild) + GyrB (Glu447)</td>
<td>15</td>
<td>&gt;100</td>
</tr>
<tr>
<td>GyrA (wild) + GyrB (acrB-Glu447)</td>
<td>15</td>
<td>75</td>
</tr>
</tbody>
</table>

a) Asn87, D87N, b) Glu447, K447E.

### DNA Binding of Quinoxolone-Resistant Gyrase

DNA-binding activity was examined using the gel shift assay with 32P-labeled, 204-bp, double-stranded DNA containing the gyrase cleavage region of pBR322 (Fig. 3). Gyrase with GyrB (K447E) showed a similar extent of DNA binding as the wild-type gyrase. Gyrase with GyrB (acrB-K447E) showed a reduced DNA-binding level comparable to that of the Gyrase (acrB) enzyme. These results indicate that Gyrase (K447E) mutation does not alter the extent of DNA binding in the ab-

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Fig. 2. Effects of Acriflavine on the Supercoiling Activity of Mutant Gyrase

Relaxed pBluescript II DNA (1 μg) was incubated with the reconstituted gyrase (100 units) containing GyrA and GyrB variants (Glu447, K447E) and various concentrations of acriflavine. The samples were extracted with phenol-chloroform, precipitated with ethanol, and subjected to 0.8% agarose gel electrophoresis. The upper and lower bars indicate positions of relaxed and supercoiled DNA, respectively.
Fig. 3. Comparison of DNA-Binding Activities of Reconstituted Gyrase Containing GyrB Variants

The gel shift assay was performed with the reconstituted gyrase (5 μg) containing GyrB variants and the [3H]-labeled, 204-bp DNA fragment containing the gyrase cleavage region of pHBr122. Upper: Autoradiograph of gel shift assay on DNA binding. The arrow indicates the position of the DNA-gyrase complex. Lower: Radioactivities in the gel area corresponding to the DNA-gyrase complex were measured.

Fig. 4. Effects of Acriflavine on the DNA Binding of Gyrases with GyrB Variants

Exposure times for autoradiography varied with the experiment. Some details are the same as in Fig. 3.

Fig. 5. Effects of Oxolinic Acid on the DNA Binding of Gyrases with GyrB Variants

Some details are the same as in Fig. 3.

Hence of drugs.

The inhibitory effects of acriflavine on these gyrase are shown in Fig. 4. Acriflavine suppressed the DNA binding of the GyrB (acrB-K447E) enzyme at a lower concentration than that of the GyrB (acrB) enzyme. The ID_{50} for the DNA binding of the acrB-K447E enzyme was estimated to be 2 μg/ml which is significantly lower than that for the acrB enzyme (20 μg/ml). The increased sensitivity in DNA binding caused by the acrB-K447E mutation correlates with the sensitivity for the supercoiling activity. However, although the GyrB (K447E) single-mutant gyrase also increased the sensitivity to acriflavine in the supercoiling assay (Fig. 2), it retained normal DNA binding activity in the presence of acriflavine (Fig. 4).

The effect of oxolinic acid on DNA binding was also examined by the gel shift assay (Fig. 5). Oxolinic acid did not produce a significant effect on DNA binding of the wild-type enzyme, although a slight reduction in binding of the DNA-gyrase complex was observed as the concentration of oxolinic acid increased. Mutant gyrase with GyrB (K447E) showed a similar response to oxolinic acid as wild-type gyrase.

In the case of the GyrB (acrB) enzyme, oxolinic acid significantly increased the extent of the DNA-gyrase complex, especially at low concentration. At a concentration of 12.5 μg/ml, the extent of binding increased to almost the same level as that of the wild-type enzyme (Fig. 5). The gyrase with GyrB (acrB-K447E) also enhanced DNA binding in the presence of a low concentration of oxolinic acid, although the
increase was smaller than that of the GyrB (acrB) enzyme. These results indicate that quinolone binds to the DNA-gyrase complex containing the acrB and makes the complex more stable.

DISCUSSION

Acriflavine is a well-known DNA intercalating agent, and it changes the conformation of DNA. Though its precise inhibitory mechanism is not clear yet, acriflavine inhibits the DNA binding of gyrase in the gel shift assay. The acrB, mutation which causes hypersensitivity to acriflavine in vivo, does not alter the sensitivity to acriflavine in the supercoiling assay, but it reduces the extent of DNA binding in the gel shift assay (reference 6 and also Fig. 4). On the contrary, we found that both quinolone-resistant mutations introduced into GyrA and GyrB subunits increased the sensitivity to acriflavine in the supercoiling assay irrespective of the co-presence of the acrB mutation (Fig. 2). In the supercoiling assay all the gyrase that showed quinolone-resistance also displayed acriflavine hypersensitivity (Table 1). This finding implies that the mechanisms of quinolone-resistance cause the acriflavine hypersensitivity.

Gyrase with the GyrB (acrB-K447E) double-mutation increased the sensitivity of DNA binding to acriflavine as well as that of the supercoiling activity (Fig. 4). This finding indicates that the K447E mutation site is related to the DNA binding as is the acrB mutation site. Because the DNA binding is an essential step in the enzyme reaction, increase of sensitivity in DNA binding should directly affect the sensitivity of the supercoiling activity. Therefore, the acriflavine-hypersensitivity of this gyrase is most likely due to the alteration of DNA-binding property. However, K447E single-mutation gyrase retained normal DNA-binding activity in the presence of acriflavine (Fig. 4) although the mutation increased the sensitivity of the supercoiling activity (Fig. 2). The results indicate that the hypersensitivity of DNA binding caused by the K447E mutation appears only in the co-presence of the acrB mutation. We deduced that the K447E mutation in GyrB contributes to a distinct DNA binding that does not affect the DNA binding related to the acrB mutation site.

This hypothesis is consistent with the idea that quinolone interacts with the protein and the DNA bases adjacent to the cleavage site. Yoshida et al. have proposed that a ‘quinolone pocket’ appears in the DNA-gyrase complex during DNA break-reunion reactions, and that quinolone binding affinity with this pocket is determined by both of the GyrA and GyrB subunits in concert. The models imply that the mutation sites of quinolone-resistance in GyrA and GyrB come closer in the DNA break-reunion step to make a small pocket. Our results suggest that the conserved sequences containing quinolone-resistant mutation sites in GyrA and GyrB participate in DNA binding and that these mutations alter the mode of DNA binding to become more sensitive to acriflavine. Such DNA binding may be specific at the DNA break-reunion step distinct from the initial DNA binding which is normally observed in gel shift assay. In the crystal structure of topoisomerase II, however, the conserved sequences containing the quinolone-resistant mutation site of GyrB locate farther apart from the CAP-like DNA-binding domain, which corresponds to the hot spot region for the quinolone-resistant mutation in GyrA. Considering the crystal structure of the yeast topoisomerase II, we have pointed out that C-TERM region of GyrB correspond to the hinge region which cause the conformational change of the enzyme in the process of DNA binding (Fig. 1). To generate a small pocket for quinolone to bind to DNA and the mutation sites of GyrA and GyrB, gyrase would require a large conformational change during the initial DNA-binding step, which is probably mediated by the hinge motion of C-TERM region containing the acrB site.

In the gel shift assay, oxolinic acid increased the DNA binding of gyrase carrying the acrB mutation. The increase was also observed in the gyrase with GyrB (acrB-K447E) which exhibited resistance to quinolone in the supercoiling assay at the concentration examined. These results indicate that quinolone stabilizes the DNA-gyrase complex without complete inhibition of the supercoiling activity. Recently, a mutant gyrase with GyrA (Y122S), which is deficient in the DNA cleavage reaction, was constructed by Critchlow and Maxwell. They constructed GyrA (Y122S) mutant subunit that also lacks the COOH-terminal DNA wrapping domain of GyrA: this mutant subunit is 64 kDa protein and named 64 (Y122S). The gyrase reconstituted with 64 (Y122S) showed reduced DNA binding under normal conditions. However, quinolone increased the DNA binding of this gyrase, in spite of the deficiency of DNA. Their results suggest that quinolone binding to the DNA-gyrase complex does not require DNA breakage, which is necessary to generate covalently bound DNA and gyrase. Our results also support their report. However, the mechanism of quinolone binding to the quinolone-resistant gyrase and its relationship to DNA binding of the acrB site or the GyrB (K447E) site still remain to be elucidated.

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