Acriflavine is an acridine dye that causes inhibition of cell division in microorganisms, plasmid loss of bacterial cells, and high mutation frequencies. The mutagenic activity of acridine dyes results mainly in frameshift mutation, which is derived from insertion or deletion of base(s) in the DNA (1). Although acriflavine is known as an antibiotic agent, almost all *Escherichia coli* K12 wild-type strains exhibit resistance to this reagent. For investigation of determinants in acriflavine resistance, five acridine-sensitive mutants have been isolated: *acrA* (2), *acrB* (3), *acrC* and *acrD* (4), and *acrE* (5).

The *acrA* mutant is highly cross-sensitive with membrane-attackable substances such as phenethyl alcohol, detergents, and some fatty acids (6, 7). The cloned *acrA* gene showed a gene dosage effect in *E. coli* (8). Sequence analysis revealed an operon encoding two membrane protein genes *acrA* and *acrE* (this is not the same gene described above) (9). This *acrAE* operon is homologous to the *envCD* operon mutant, which is also hypersusceptible to basic dyes, detergents, and antibiotics (10). These genes are deduced to be subunits of energy-dependent pump proteins.

Mutations that exhibit susceptibility to acriflavine have been isolated and classified as *acr* mutations in *Escherichia coli*. We cloned the *acrB* gene, which has been identified as a mutation of the *gyrB* gene, and found a double point mutation altering two consecutive amino acids (S759R/R760C) in the COOH-terminal region of the gyrase B subunit. The mutant B subunit was found to associate with the A subunit to make the quaternary structure, and the reconstituted gyrase showed an 80-fold reduction of specific activity in DNA supercoiling assay; the sensitivity to acriflavine was not different in the same unit of wild-type and mutant gyrase. The mutant enzyme retained intrinsic ATPase activity, but DNA-dependent stimulation was observed infrequently. A gel shift assay showed that acriflavine inhibited the DNA binding of gyrase. The *acrB* mutation also reduced significantly the DNA binding of gyrase but did not change the sensitivity to acriflavine. These results revealed that the *acrB* mutation is related to the inhibitory mechanism of acriflavine; and the acriflavine sensitivity of the mutant, at least in *vitro*, is caused mainly by reduction of the enzyme activity. Further, our findings suggest that the COOH-terminal region of the B subunit is essential for the initial binding of gyrase to the substrate DNA.

EXPERIMENTAL PROCEDURES

**E. coli Strains and Plasmids—**Wild-type strain W1895 (*metB, gyrA*, *gyrB*, *acrA*, *acrB*, *acrC*, *acrD*, *acrE*) and *acrB* strain NZ2879 (*acrA* mutant derived from W1895) were used in the acriflavine resistance test (3). The plasmid pJ1B11, which has 3.4-kilobase fragment containing the *gyrB* gene (22), was kindly provided by Dr. A. Hase (Osaka City Research Institute of Public Health and Environmental Science). Expression vector pGEX4T-3 was purchased from Pharmacia Biotech Inc. pBluescript II (KS+) was from Stratagene.

**Media, Chemicals, and Enzymes—**PGY medium—concentrated (1% Polypeptide (Difco Laboratories), 0.3% yeast extract, 0.3% NaCl, 0.1% glucose, adjusted pH 7.4) and PGY medium—dilute (0.5% Polypeptide, 0.1% yeast extract, 0.3% NaCl, 0.1% glucose, adjusted pH 8.0) were used for the acriflavine sensitivity test. Other media were constructed as described in Ref. 23. Restriction enzymes were from Takara Shuzo.

The *acrB* mutation located at the COOH-terminal region of the gyrase B subunit reduces DNA binding of DNA gyrase.*

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is defined by preferred trypsin cleavage site (Arg393) (41). The subunit and the region neighboring the inserted sequence designated as C-TERM (see “Discussion”). The mutation sites of acrB are in the C-TERM region: Ser779 and Arg800 (this paper). The expected A subunit association region is indicated by the cross-hatched box (see “Discussion”).

(Japan), the glutathione S-transferase gene fusion system was from Pharmacia, and oligonucleotides were from Biologica (Japan).

Cloning of the gyrB(acrB) Gene—Genomic DNA was purified from E. coli strain N2879 as described by Cosloy and Oishi (24). The DNA library was constructed with the pBlueScript II (KS+) plasmid, and the gyrB(acrB) gene was screened by colony hybridization using a wild-type gyrB gene as a probe. The DNA fragment was labeled with the ECL system (Amersham Corp.). The gyrB gene was cloned from strain W1895 by polymerase chain reaction using Vent DNA polymerase (New England Biolabs). DNA sequencing was performed using Sequenase version 2.0 (U. S. Biochemical Corp.).

Enzyme Purification—gyrA, gyrB, and gyrB(acrB) gene products were purified by the protocol of the glutathione S-transferase fusion protein system, DEAE-Sepharose column (25), and a Novobiocin-Sepharose column (26).

Each plasmid for overexpression of the gyr subunit was constructed using the expression vector pGEX4T-3 (Pharmacia). In the expression plasmid for the gyrA gene, an arginine codon AGA at the 6th codon, which is a rare codon in E. coli, was replaced by CGT. All of the gyrB(acrB) regions shown in Fig. 1 (Japan) are in the C-TERM region: Asp426, Lys447, denoted by a horizontal arrow and the region of conserved amino acid motifs which is denoted by the horizontally hatched box: EGDDSA (424–428), PLYR/KOKGL/L/MILN (445–452), IM(T/A)D(JQ/A)D (495–500). The COOH-terminal region neighboring the inserted sequence designated as C-TERM (see “Discussion”). The mutation sites of acrB are in the C-TERM region: Ser779 and Arg800 (this paper). The expected A subunit association region is indicated by the cross-hatched box (see “Discussion”).

TGED buffer without NaCl, concentrated by Centricon (Amicon), and stored at ~80 °C.

Supersothing Assay—Relaxed pBlueScript plasmid DNA was prepared by topoisomerase I enzyme from rat liver (27). Gyrase enzyme was reconstituted in 30 μl of reconstitution buffer (20 mM Tris-HCl, pH 7.6, 0.2 mM EDTA, 70 mM KCI, 10 mM MgCl2, 5 mM DTT, 500 μg/ml BSA, 20% glycerol) with purified A and B subunits (7.5 μg each). The supersothing assay was performed in 60 μl of the reaction mixture containing 50 mM Tris-HCl, pH 7.5, 20 mM KCI, 10 mM MgCl2, 10 mM DTT, 1.5 mM ATP, 5 mM spermidine, 50 μg/ml BSA, 500 μg/ml RNA, 10% glycerol, 1 μg of relaxed pBlueScript DNA, and various amounts of reconstituted gyrase. Samples were incubated at 30 °C for 30 min, then extracted with phenol solution and chloroform:isoamyl alcohol solution twice, respectively. DNA was recovered by ethanol precipitation, dissolved in TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and subjected to 0.8% agarose gel electrophoresis. One unit of enzyme activity of gyrase was defined as the amount that brought 50% of relaxed DNA to the supercoiled position in agarose gel electrophoresis.

ATPase Assay—The reaction mixture (100 μl) contained 50 mM Tris-HCl, pH 7.5, 20 mM KCI, 10 mM MgCl2, 50 μg/ml BSA, 1 μg of gyrase, and various concentrations of ATP with or without 2.5 μM of relaxed pBlueScript II (KS+) DNA. The mixtures were incubated at 30 °C for 10 min. The amount of generated ADP was measured by high performance liquid chromatography as described by Taylor et al. (28).

Gel Shift Assay—The gyrase cleavage site of pBR322 (29) (base position 886–1089) was amplified by polymerase chain reaction and subcloned into pBlueScript. The isolated DNA fragment was labeled with γ32P[ATP by T4 polynucleotide kinase. The binding reaction was performed with the method of Bachellier et al. (30) with modifications. Gyrase was reconstituted with 5 μg of A and B subunit, respectively, in 20 μl of the reconstitution buffer described above. Half of the reconstituted gyrase (10 μl) was added to 10 ml of 2× binding buffer (80 mM Tris-HCl, pH 7.6, 12 mM MgCl2, 40 mM KCI, 4 mM DTT, 20 mM ATP) containing 300 pg of labeled DNA and 25 ng of double stranded competitor DNA (poly[dI-dC]·poly[dI-dC], Sigma). Subsequently, samples were incubated at 25 °C for 60 min. Polyacrylamide gel (multigel 2–15% gradient, Daichi-kiagaku Co. Ltd.) was used, and running was done with 25 mM Tris-HCl, pH 8.4, 192 mM glycine, 8 mM MgCl2 at 90-V constant voltage for 3 h at 4 °C. Protein-bound and free DNA were separated electrophoretically with new buffer at 90-V constant voltage for 4 h at 4 °C. Gels were dried and contacted to the x-ray film. To quantitate the gyrase-DNA complex, a gel containing the complex was excised, and the radioactivity was counted by the Cerenkov method.
RESULTS

Identification of the acrB Mutation in the gyrB Gene— Using the gyrB(wild) gene as a probe, we isolated the gyrB(acrB) gene from N2879 strain. The nucleotide sequences of the gyrB(acrB) gene revealed two nucleotide substitutions. The 2275th A from the translation start was substituted for C by transversion, and 2278th C was substituted for T by transition. These point mutations alter amino acids in the B subunit: Ser\(^{759}\) \(\rightarrow\) Arg and Arg\(^{760}\) \(\rightarrow\) Cys. These mutations were located at the COOH-terminal region of the gyrB gene (Fig. 1). The nucleotide sequence of the other part was identical to the reported gyrB sequence (31).

Effect of Acriflavine on the Growth of Wild-type and Mutant E. coli— The bacterial growth curves in the presence of 20 \(\mu\)g/ml acriflavine are shown in Fig. 2. Wild-type strain (W1895) showed continuous growth without time lag. The wild-type cells carrying a high copy number of plasmids with wild-type gyrB (pJB11) or gyrB(acrB) (pSB1) had resistant features. However, the cells carrying pJB11 showed more intense growth than the cells without plasmid, whereas the cells carrying pSB1 showed a 2-h time lag for growth recovery. In the mutant strain (N2879), the cells without plasmid and with pSB1 did not show growth. On the contrary, mutant cells carrying pJB11 showed recovery of the survival after a 4-h decline. Such recovery after a limited time may result from the time lag for gyrB expression induced by decreased superhelicity of the genome DNA (32). The hypersensitivity to acriflavine in the N2879 strain has a complex feature that cannot be explained by the gene dosage effect alone. However, the semidominant character of the gyrB(acrB) gene, which would be caused by the stoichiometric effect of the gene product, is notable.

Purifications of Gyrase Subunits—To analyze the gene product of acrB, each subunit of gyrase was overexpressed and purified using the glutathione S-transferase-fusion protocol. After the glutathione S-transferase column procedure, however, a small amount of the other partner of the subunit was still found in purification of both A and B subunits, and each sample exhibited supercoiling activity. Therefore, these glutathione S-transferase-fusion proteins seem to retain the ability to associate with the partner subunit even though they contain a large polypeptide of glutathione S-transferase in the NH\(_2\)-terminal side. The copurified A and B subunits were absorbed and eliminated by a DEAE-Sepharose column and a Novobiocin affinity column, respectively. Absence of the partner subunit was confirmed by SDS-polyacrylamide gel electrophoresis and supercoiling assay. About 10 mg of protein was obtained from a 2.5-liter culture in each case.

Supercoiling Activity—The supercoiling activity in vitro was detected from gyrase proteins reconstituted with both wild-type and the mutant B subunit. However, their specific activities were quite different. The specific activity of wild-type gyrase was calculated to be \(3.2 \times 10^6\) units/mg of protein, and that of mutant gyrase was \(4.0 \times 10^4\) units/mg. Therefore, the activity of gyrase was reduced 80-fold by acrB mutation.
We also examined the effect of acriflavine in the supercoiling reaction (Fig. 3). Acriflavine caused partial inhibition against 1 unit of gyrase at a concentration of 1 μg/ml and complete inhibition at 6 μg/ml. Against 100 units of gyrase, little inhibition was observed at 10 μg/ml. These findings were common to the wild-type and mutant gyrase. Thus, the sensitivity of the supercoiling activity to acriflavine was not affected by the mutation.

Assembling of Gyrase Tetramers—We examined whether the mutant B subunit had lost the ability to associate with the A subunit. The molecular weight of the reconstituted mutant gyrase protein was estimated by a Superdex-200HR (Pharmacia) column, and the eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). Both wild-type gyrase and mutant gyrase were eluted at the same position with a molecular mass of 400 kDa, which corresponds to that of regular gyrase tetramer. SDS-polyacrylamide gel electrophoresis revealed an equal amount of gyrA and gyrB subunits. These results showed that the mutant B subunit was able to construct a gyrase tetramer, indicating that the lower supercoiling activity of the mutant gyrase was not due to the loss of the affinity with the A subunit.

ATPase Activity—The NH₂-terminal region of the B subunit contains an ATPase domain. We examined the ATPase activity of wild-type and mutant gyrase (Fig. 5). In the absence of DNA, these enzymes showed a similar level of intrinsic ATPase activity. The Lineweaver-Burk plot indicated that both Kₘ for ATP was 1.7 mM as reported (26). On the other hand, in the presence of DNA, the ATPase activity of the mutant gyrase was significantly lower than that of the wild-type gyrase. This result suggested that the lower supercoiling activity of the mutant gyrase was due to a decrease in its ATPase activity.

Acriflavine Inhibits ATPase Activity—Acriflavine inhibited the ATPase activity of both wild-type and mutant gyrase in a concentration-dependent manner (Fig. 6). The inhibitory effect was more pronounced in the mutant gyrase, indicating that the mutation in the B subunit affected the ATPase activity.

DNA Binding—Acriflavine also affected the DNA binding activity of the gyrase (Fig. 7). The gel mobility shift assay showed that acriflavine inhibited the formation of the DNA-gyrase complex. The quantitative analysis revealed that the inhibition increased with the concentration of acriflavine.
presence of DNA, the ATPase activity was quite different in the wild-type and mutant; the ATP hydrolysis of wild-type gyrase was significantly stimulated by DNA, whereas that of mutant gyrase was hardly stimulated.

Fig. 6 shows the inhibitory effect of acriflavine on ATPase activity. Although slight inhibition was observed under the conditions without DNA, the inhibitory effect was the most marked in the case of wild-type enzyme with DNA. In the case of both wild-type and mutant gyrase, the activity was reduced by the high concentration of acriflavine (~3 μg/ml) to the same level as the intrinsic ATPase activity. These results indicate that acriflavine specifically inhibited the DNA-dependent ATPase activity.

DNA Binding of Gyrase Proteins—We examined DNA binding of wild-type and mutant gyrase by gel shift assay. The wild-type gyrase showed notable binding to a 204-base pair DNA fragment containing the cleavage region of gyrase (29) (Fig. 7A). On the other hand, the gyrase with gyrB(acrB) subunit showed very little binding capacity (Fig. 7B). The extent of gyrase-DNA complex formation decreased as the concentration of acriflavine increased in the case of wild-type and mutant gyrase. Fifty percent inhibition of binding was observed at a similar concentration of the acriflavine (~20 μg/ml) in these experiments, indicating that the mutation did not change the sensitivity to acriflavine in DNA binding. Fig. 8 shows the DNA binding of gyrase under the competitive conditions of B subunits to associate with the A subunit. Various amounts of wild-type and mutant B subunits were mixed with constant amount of the A subunit, and the mixtures were subjected to the gel shift assay. With an increasing amount of the mutant B subunit, the amount of the gyrase-DNA complex decreased (Fig. 8A). On the other hand, an excess amount of wild-type B subunit did not inhibit DNA binding and rescued the defect of the mutant gyrase (Fig. 8B).

**DISCUSSION**

Gyrase is a target protein of several antibiotics, some of whose mechanisms have been analyzed in detail. Coumarin drugs bind the NH$_2$-terminal domain of the B subunit and inhibit ATP hydrolysis of the enzyme. Quinolone antibiotics are deduced to bind a transient complex consisting of cleaved DNA and gyrase, which are bound covalently to each other. Analysis of the resistant mutations of the gyrase gene revealed that mutations occurred in the corresponding domains where these antibiotics act. Isolated mutant gyrases showed resistant features in vitro as well as in vivo (33–35).

The present work showed that acriflavine suppressed DNA binding of gyrase (Fig. 7), and the acrB mutation caused the reduction of DNA binding (Figs. 7 and 8). These results suggest that the acrB mutation is also related to the antibiotic mechanism as the resistant mutations against coumarin and quinolone. Acriflavine is well known as a DNA intercalate substance, and it changes the superhelical density of plasmid as ethidium bromide does, implying that it changes the conformation of the DNA strand. We found that similar concentrations of acriflavine were effective in inhibiting the wild-type gyrase and an 80-fold higher amount of the mutant gyrase in the supercoiling assay (Fig. 3). Therefore, acriflavine is most likely to interact with DNA and not directly with the gyrase protein. As the DNA binding is the initial step of gyrase function, it is reasonable to consider that acriflavine suppresses the subsequent processes such as ATPase activation (Fig. 6) and DNA supercoiling (Fig. 3).

The acrB mutation, however, did not change the sensitivity of gyrase to acriflavine in vitro, unlike the resistant mutations against coumarin and quinolone, which change the sensitivity
of the gyrase to them, respectively. Supercoiling assay and gel shift assay (Figs. 3 and 7) indicated that the mutant enzyme reduced the activity without becoming more sensitive to acriflavine. These findings suggest that the acriflavine-sensitive feature of acrB is mainly the result of a reduction of specific activity. When we prepared the cell extract from strain N2879 with partial purification by polyamine P precipitation (27), no supercoiling activity was detected in that extract, whereas the expected activity was found in the extract from the wild-type strain (data not shown). Thus, gyrase activity in vivo also seemed to be decreased. The mechanism of the susceptibility to acriflavine in vivo, however, appears too complex to be elucidated. For example, the reduction of supercoiling activity may cause a decrease in the superhelical density of genome DNA which allows acriflavine to have greater access to DNA.

The acrB mutation alters two amino acids (S759R/R760C) near the COOH-terminal end of the gyrase B subunit (Fig. 1). The region designated as C-TERM in Fig. 1 is separated from the NH2-terminal part of the B subunit by an insertion of about 170-amino acid sequence (black boxed in Fig. 1), which is absent in gyrase of Gram-positive bacteria, bacterial topoisomerase IV (parE gene), and eukaryotic topoisomerase II. The C-TERM is expected to be a separable structural domain, but the relationship to DNA binding has not been reported in the region. Fig. 9A shows the homology of the C-TERM region to the corresponding regions of B subunit of Bacillus subtilis gyrase and parE protein of E. coli topoisomerase IV. As the deletion of 29 amino acids from the COOH-terminal end of parE protein (double underlined in Fig. 9A) results in a loss of ability to associate with parC protein (counterpart of the gyrase A subunit) (36), C-TERM potentially includes a structure necessary for association with the A subunit. Thus we examined whether the acrB mutation affects the association with the A subunit. The mutant B subunit, however, showed normal assembly with the A subunit in the gel filtration assay (Fig. 4) and the gel shift assay (Fig. 8). These results suggest that the reduction of DNA binding is caused by an alteration in the C-TERM region itself, not by loss of the quaternary structure.

Berger et al. (37) have recently reported the crystal structure of yeast topoisomerase II and pointed out that a region including a highly conserved sequence (Fig. 9A), which is located at about 20 residues NH2-terminal from the acrB site, is involved in the contact with the other B subunit in the gyrase tetramer. Considered together with the A subunit-associated region described above, the C-TERM region appeared to serve the two functional structures that are related to contact with the partner A and B subunits, respectively. The crystal structure of topoisomerase II indicates that the counterpart of the region between the conserved sequence and acrB is composed of an α-helix and forms an axis that extends to the linker between the A’ and B’ subfragments. Therefore, the axis-like structure in the NH2-terminal part of C-TERM may structurally support the B’ subfragment as a backbone. Further, during the enzyme reaction of topoisomerase II, a hinge motion of a polypeptide segment located between the A’ and B’ subfragments is suggested by an SV9 proteolysis experiment (38). The COOH-terminal part of C-TERM is expected to serve as the hinge region because it corresponds to the disordered linker region between the A’ and B’ subfragments in the crystal structure (37). These features of C-TERM led us to the hypothesis that one of the roles of this region is to provide a structure necessary for the conformational change of gyrase. During the enzyme reaction, the B subunits have to come apart for DNA entering. The contact to the other B subunit must be lost in this step, and the hinge motion of the COOH-terminal part of C-TERM is thought to separate each B subunit (37, 39), which is probably supported structurally by the NH2-terminal part of C-TERM. Therefore, a mutation in C-TERM is expected to cause a disorderly arrangement of the whole B subunit and/or disadvantage in conformational change of gyrase.

Several possibilities are expected to reduce the DNA binding affinity. One possibility is that the acrB mutation affects DNA recognition and/or the subsequent conformational change of gyrase which is induced during DNA binding. From the analogy of crystal structure of topoisomerase II, acrB seems to be located near the active site tyrosine and CAP-like DNA binding domain in the A subunit. The hydrophobicity plot shows that the mutation region is hydrophilic (Fig. 9B), implying that the amino acid residues are located at the surface of the protein. Further, the acrB mutation changes the location of a positive charge (Arg760) which might interact directly with DNA. Therefore, it is possible to speculate that this region is related directly to the recognition and/or transportation of DNA to the DNA binding domain of the A subunit. In fact, DNA binding is suggested to cause a structural change of the region corresponding to the COOH-terminal part of C-TERM (38). The amino acid sequence near the acrB mutation region is well conserved in the E. coli parE gene product as well as in B. subtilis gyrase (Fig. 9A). DNA binding characteristics of topoisomerase IV are thought to be more similar to those of type II eukaryotic enzymes than those of the gyrase from the observation that topoisomerase IV protects only short region (34 base pairs) of DNA from micrococcal nuclease digestion as eukaryotic topoisomerase II (40). Thus, the acrB mutation region seems to be related to general DNA binding and not to the gyrase-specific DNA wrapping, which is thought to be caused by the 33-kDa domain of the A subunit (17).

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