Characterization of the Interaction between DNA Gyrase Inhibitor and DNA Gyrase of Escherichia coli*

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Escherichia coli DNA gyrase is comprised of two subunits, GyrA and GyrB. Previous studies have shown that GyrI, a regulatory factor of DNA gyrase activity, inhibits the supercoiling activity of DNA gyrase and that both overexpression and antisense expression of the gyrI gene suppress cell proliferation. Here we have analyzed the interaction of GyrI with DNA gyrase using two approaches. First, immunoprecipitation experiments revealed that GyrI interacts preferentially with the holoenzyme in an ATP-independent manner, although a weak interaction was also detected between GyrI and the individual GyrA and GyrB subunits. Second, surface plasmon resonance experiments indicated that GyrI binds to the gyrase holoenzyme with higher affinity than to either the GyrA or GyrB subunit alone. Unlike quinolone antibiotics, GyrI was not effective in stabilizing the cleavable complex consisting of gyrase and DNA. Further, we identified an 8-residue synthetic peptide, corresponding to amino acids 89–96 of GyrI, which inhibits gyrase activity in an 

in vitro

supercoiling assay. Surface plasmon resonance analysis of the ITGGQYAV-containing peptide-gyrase interaction indicated a high association constant for this interaction. These results suggest that amino acids 89–96 of GyrI are essential for its interaction with, and inhibition of, DNA gyrase.

DNA gyrase is a type II topoisomerase found in all bacteria and is essential for viability. It is involved in the replication, repair, recombination, and transcription of DNA. DNA gyrase consists of two subunits, GyrA and GyrB, and the active holoenzyme is an A2B2 complex. Mechanistic studies have revealed the steps involved in the gyrase supercoiling reaction. This process involves the wrapping of DNA around the A2B2 complex, cleavage of this DNA on both strands, and the passage of a segment of DNA through the double strand break. Religation of the break results in the introduction of negative supercoils. These processes require the binding and hydrolysis of ATP.

DNA gyrase is the target of several antibacterial agents, including the coumarin and quinolone families of antibiotics. Coumarins are potent inhibitors of gyrase supercoiling and ATPase reactions. Through the use of x-ray crystallography the molecular details of the ATPase reaction and its inhibition by coumarin have been revealed. Coumarin, on the other hand, stabilizes a conformation of the enzyme-DNA complex referred to as the “cleavable complex.” This complex blocks the passage of RNA polymerases.

Although the majority of compounds known to target DNA gyrase are either quinolones or coumarins, natural toxins also exist such as microcin B17 and CcdB. Microcin B17 is a peptide antibiotic secreted in the stationary phase by several strains of Escherichia coli and induces DNA cleavage in a fashion similar to that of the quinolones. The antibiotic efficacy of this polypeptide depends on the post-translational modification of eight of its cysteine and serine residues to thiazoles and oxazoles, respectively. CcdB is a bacterial toxin encoded by the E. coli F plasmid. These natural toxins target domains of the GyrA and GyrB proteins distinct from those targeted by the quinolones and coumarins. Microcin B17 targets GyrB, whereas CcdB targets GyrA. No cross-resistance to quinolones has been observed.

DNA gyrase inhibitor (GyrI) is the first chromosomally encoded DNA gyrase regulatory factor identified in E. coli. In previous work, we found that this purified protein inhibited DNA supercoiling in vitro and that abnormally high or low GyrI levels resulted in the suppression of cell proliferation in vivo. The promoter activity of gyrI increases in the late exponential phase and peaks in the stationary phase. Independently, Baquero et al. (26) reported that the gyrI gene (identical to the sbmC gene) belongs to the SOS regulon. The inactivation of GyrI causes cells to be more sensitive to microcin B17, whereas its overexpression yields microcin B17-resistant cells.

In this work, we examined the interaction of the GyrI protein with the DNA gyrase A2B2 tetramer. We show that the interaction of GyrI with DNA gyrase requires both the GyrA and GyrB subunits and that a specific region of GyrI mediates its interaction with, and inhibition of, DNA gyrase.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—E. coli strains used in this study were DH5α and KL16 (25). E. coli BL21(DE3) Gold prophage was purchased from Stratagene. The pET3a, pET16b, and pET24b plasmids were purchased from Novagen.

Construction of Expression Vectors—Genomic DNA was purified from the E. coli strain KL16. The plasmid vectors used in the cloning of gyrA and gyrI were pET16b and pET3a, respectively. The plasmid

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‡‡ The abbreviations used are: GyrI, DNA gyrase inhibitor; DTT, di-thiothreitol; SPR, surface plasmon resonance; PBS, phosphate-buffered saline.
Interaction between GyrI and Gyrase

Vectors used in the cloning of the gyrB gene were pET16b and pET24b. The pET16b and pET24b vectors contain a His tag sequence, which is a consecutive stretch of histidine residues which can be expressed at either the amino or carboxyl terminus of the target protein. The pET3a vector contains a T7-tagged sequence that can be expressed at the amino terminus of the target protein. The E. coli plasmid GyrI-gyrase complexes were formed by mixing recombinant GyrA and GyrB; however, the respective purities of these proteins were previously published [25]. Originally, we used Talon to purify histidine-tagged GyrA and GyrB, and 72°C. 30 cycles of PCR were carried out at 94°C for 1 min, and 72°C for 3 min. The gyrA gene was inserted into the NdeI-BamHI site of pET16b, the gyrB gene was inserted into the BamHI site of pET24b, and the gyrI gene was inserted into the NdeI site of pET3a, yielding pCA30, pCA31, and pCA32, respectively. All constructs were verified by DNA sequencing using an ABI Prism 377 DNA sequencer (PerkinElmer Life Sciences, Applied Biosystems).

DNA Gyrase Supercropping and Cleavage Assay Using Purified GyrI and DNA Gyrase—We engineered recombinant fusion genes expressing GyrA, GyrB, and GyrI proteins containing histidine or T7 tags on their COOH- or NH2-termini. These recombinant proteins were purified by chromatography as described under “Experimental Procedures.” SDS-PAGE analysis revealed that the sizes of GyrA, GyrB, and GyrI correlated with their estimated molecular masses (Fig. 1A). The holoenzyme, obtained by mixing recombinant GyrA and GyrB, possessed DNA supercoiling activity as determined using relaxed pBR322 DNA as substrate. DNA supercoiling was inhibited by novobiocin and oxolinic acid in a dose-dependent manner (Fig. 1B) and inhibited by the GyrI protein (Fig. 1C). We next verified the capacity of purified GyrI and oxolinic acid to facilitate DNA gyrase-mediated cleavage of pBR322 plasmid DNA. As shown in Fig. 1D, oxolinic acid facilitated the cleavage of pBR322 DNA by gyrase (lane 6), whereas GyrI could not position DNA gyrase into a cleavable complex, such that upon the addition of SDS, a double strand cleavage of the DNA was not observed (Fig. 1D, lanes 2, 3, and 4). Similarly, when GyrI was assayed for in vitro DNA gyrase activity in the absence of ATP, no DNA cleavage was observed (Fig. 1D, lane 1). However, inhibition of DNA supercoiling was observed when GyrI was added to the supercoiling reaction (Fig. 1C) (25).

Interaction between GyrI and Gyrase—The next step was to clarify the interaction between GyrI and the DNA gyrase holoenzyme as well as with the individual subunits GyrA and GyrB. Purified T7-tagged GyrI was incubated with His-GyrA, His-GyrB, or His-holoenzyme, and the reaction mixtures were immunoprecipitated with a His-tag-specific antibody. The immunoprecipitants were resolved by 12% SDS-PAGE and sub-

RESULTS

DNA Gyrase Supercoiling and Cleavage Assay Using Purified GyrI and DNA Gyrase—We engineered recombinant fusion genes expressing GyrA, GyrB, and GyrI proteins containing histidine or T7 tags on their COOH- or NH2-termini. These recombinant proteins were purified by chromatography as described under “Experimental Procedures.” SDS-PAGE analysis revealed that the sizes of GyrA, GyrB, and GyrI correlated with their estimated molecular masses (Fig. 1A). The holoenzyme, obtained by mixing recombinant GyrA and GyrB, possessed DNA supercoiling activity as determined using relaxed pBR322 DNA as substrate. DNA supercoiling was inhibited by novobiocin and oxolinic acid in a dose-dependent manner (Fig. 1B) and inhibited by the GyrI protein (Fig. 1C). We next verified the capacity of purified GyrI and oxolinic acid to facilitate DNA gyrase-mediated cleavage of pBR322 plasmid DNA. As shown in Fig. 1D, oxolinic acid facilitated the cleavage of pBR322 DNA by gyrase (lane 6), whereas GyrI could not position DNA gyrase into a cleavable complex, such that upon the addition of SDS, a double strand cleavage of the DNA was not observed (Fig. 1D, lanes 2, 3, and 4). Similarly, when GyrI was assayed for in vitro DNA gyrase activity in the absence of ATP, no DNA cleavage was observed (Fig. 1D, lane 1). However, inhibition of DNA supercoiling was observed when GyrI was added to the supercoiling reaction (Fig. 1C) (25).

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GyrA or GyrB subunits. These results indicated that all of the association and dissociation phases indicated that all of the association and dissociation phases were charactered by two-step equilibrium processes. Therefore, the binding of GyrA, GyrB, or the holoenzyme was measured.

In these experiments, GyrI was immobilized in the cuvette, and the binding of GyrA, GyrB, or the holoenzyme was measured. Analysis of these interactions using the FAST FIT program indicated that all of the association and dissociation phases were consistent with a monophasic reaction model. The association rate for the binding between the analyte (GyrA, GyrB, or the holoenzyme) and the immobilized substrate (GyrI) is expressed in the Equation 1.

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d_{R}/dt = K_a [C] (R_{max} - R_t) - K_d R_t
\]  
(Eq. 1)

**Fig. 1.** Characterization of purified DNA gyrase and GyrI.

Panel A, size determination of purified GyrA, GyrB, and GyrI. Aliquots of the indicated purified proteins were separated by 12% SDS-PAGE and stained with Coomassie Brilliant Blue. From left, first lane, GyrI-His; second lane, GyrA-His; third lane, GyrB-His; far right lane, molecular mass markers (sizes in kDa). Panels B and C, novobiocin, oxolinic acid, or GyrI inhibition of *E. coli* gyrase DNA supercoiling. Relaxed pBR322 DNA (0.4 μg) was incubated with gyrase (179 ng) and ATP in the presence of inhibitors. Reactions were stopped, and the DNA was examined by electrophoresis in 1.0% agarose gels in TAE buffer. Panel B, control supercoiled (lane 1) and relaxed pBR322 DNA without inhibitor and DNA gyrase (lane 2) are shown. Lanes 3, 4, 5, and 6 include novobiocin at 0.39, 0.78, 1.56, and 3.13 μg/ml, respectively; lanes 7, 8, 9, and 10, with oxolinic acid at 12.5, 25, 50, and 100 μg/ml, respectively. Lane 11 (Control) represents the reaction products without inhibitor.

Panel C, control linear (lane 1), supercoiled (lane 2), and relaxed pBR322 DNA without oxolinic acid, GyrI, and DNA gyrase (lane 3) are shown. Lane 4 includes 200 μg/ml oxolinic acid; lane 5 includes 3.5 μg/ml GyrI. Lane 6 (Control) represents the reaction products without oxolinic acid and GyrI. Panel D, the DNA cleavage assay was carried out as described under "Experimental Procedures." PstI-restricted pBR322 (0.2 μg) was incubated with gyrase (179 ng; lanes 2–4, 6–8) in buffer containing ATP for 50 min at 30 °C. Oxolinic acid (200 μg/ml; lanes 5 and 6) or GyrI protein (26 ng for lane 4, 52 ng for lane 3, and 105 ng for lanes 1, 2, and 8) was added to the reaction mixtures, and the samples were incubated for 50 min at 30 °C. Control represents the reaction products in the absence of gyrase (lane 8) or ATP (lanes 1 and 5). The reactions were stopped by the addition of SDS and proteinase K and were incubated further at 37 °C for 30 min. After removal of proteins, the samples were analyzed on 1.0% agarose gels in TAE buffer.

**Fig. 2.** Binding of GyrI to the gyrase holoenzyme, the GyrA subunit, or the GyrB subunit. Reaction mixtures (25 mM Tris-HCl (pH 7.5), 67 mM KCl, 5 mM MgCl₂, 1.25 mM spermidine hydrochloride, 1.7 mM ATP, 5 mM DTT, 75 μg/ml bovine serum albumin, and 150 μg/ml *E. coli* tRNA) containing various combinations of GyrI, GyrA, and GyrB were immunoprecipitated after by anti-His antibody (IP). The immunoprecipitants were separated on a 12% SDS-PAGE and immunostained (Blot) with anti-T7 or anti-His antibody. Panel A, GyrA (lanes 1 and 2), GyrB (lanes 1 and 2), and GyrI (lanes 1–3) were incubated with the reaction mixtures with (lane 1) or without ATP (lane 2). Precipitates were subjected to Western blot analysis using anti-T7 antibody. Panel B, GyrA (lanes 1, 4, 6, and 8), GyrB (lanes 2, 5, 6, and 9), and GyrI (lanes 3–7) were incubated with the reaction mixtures. Precipitates were subjected to Western blot analysis using anti-T7 or anti-His antibody. Panel C, GyrA (lanes 4 and 6), GyrB (lanes 2, 3, 4, and 7), and GyrI (lanes 1, 3, 4, and 5) were incubated with the reaction mixtures. The histidine tag of GyrB was positioned on its COOH-terminal rather than on its NH₂-terminal side. Precipitates were subjected to Western blot analysis using anti-T7 or anti-His antibody.

**SPR Kinetic Analysis of GyrI Binding**—To characterize further the interaction of GyrI with the holoenzyme, we determined the kinetic parameters governing the interaction of GyrI with either GyrA, GyrB, or the holoenzyme using SPR. For these experiments, GyrI was immobilized in the cuvette, and the binding of GyrA, GyrB, or the holoenzyme was monitored. Analysis of these interactions using the FAST FIT program indicated that all of the association and dissociation phases were consistent with a monophasic reaction model. The association rate for the binding between the analyte (GyrA, GyrB, or the holoenzyme) and the immobilized substrate (GyrI) is expressed in the Equation 1.
where $K_a$ is the association rate constant, $K_d$ is the dissociation rate constant, $R_{\text{max}}$ is the maximum binding capacity of the immobilized substrate surface as determined by saturation with analyte, $R_r$ (arc seconds) is the amount of bound analyte measured as the SPR response at time $t$, and $[C]$ is the concentration of analyte added to the cuvette. The affinity constant, $K_D$, is then calculated from $K_a / K_d$. In Fig. A, binding curves are depicted for the interaction of GyrA, GyrB, or the GyrA/B holoenzyme (250 nM) with immobilized GyrI. GyrI bound poorly to the individual GyrA and GyrB subunits compared with its binding affinity for the GyrA/B holoenzyme (Fig. 3A). In Fig. 3B, an overlay plot is shown for the association profiles used by the FAST FIT program to determine the $K_m$ value for each concentration of the holoenzyme (31.2–600 nM). As for the determination of $K_m$, we obtained it directly by measuring the dissociation phase using the FAST FIT program in Fig. 3A. The dissociation rate constant of GyrA was similar to that of GyrB for the interaction with the immobilized GyrI. On the other hand, in the presence of GyrA/B holoenzyme, binding to the immobilized GyrI exhibited slow dissociation. A series of profiles for GyrA, GyrB, and the holoenzyme binding to GyrI was used to generate the kinetic constants shown in Table I. These results clearly support those of the immunoprecipitation experiments, indicating that the interaction of GyrI with DNA gyrase is dependent on the presence of both the Gyr A and GyrB subunits.

### Table I

| Complex         | $K_a$ | $K_d$ | $K_D$ |
|-----------------|-------|-------|-------|
| GyrA-GyrI       | 1.2 x 10^4 | 1.6 x 10^-2 | 1.3   |
| GyrB-GyrI       | 5.2 x 10^3 | 1.6 x 10^-1 | 30.8  |
| GyrA/B-GyrI     | 3.3 x 10^3 | 1.3 x 10^-1 | 39.4  |

**Inhibition of DNA Gyrase by GyrI-derived Synthetic Peptides**—To identify the region of GyrI involved in its inhibitory interaction with DNA gyrase, we used the DNA gyrase supercoiling reaction to examine the effect of overlapping synthetic peptides (Fig. 4A) corresponding to various sequences in GyrI that ultimately covered its whole sequence. The peptides underlined in Fig. 4A inhibited DNA supercoiling in a dose-dependent manner (12.5–100 μM), reaching complete inhibition at a concentration of 100 μM (final concentration) (Fig. 4B). Importantly, these peptides did not inhibit the activity of topoisomerase I (Fig. 4C). To define further the region mediating the inhibitory effect of GyrI on DNA gyrase, we synthesized peptides of various sizes within the region bordered by residues 81–112 of GyrI (Table II). It was found that peptides containing the motif ITGGQYAV inhibited DNA supercoiling with maximal efficacy. The concentrations of the ITGGQYAV-containing peptides required for 50% inhibition were remarkably similar (12.3–23.6 μM), although the peptide corresponding to amino acids 89–96 of GyrI was especially potent.

**SPR Kinetic Binding Analysis of Synthetic Peptides Corresponding to Various Sequences within GyrI**—We next quantified the interaction between the ITGGQYAV-containing peptide and DNA gyrase using SPR. The peptide ITGGQYAVAVRVDG (GyrI residues 89–104) or the peptide LMIVWVSDKNIVPKEVW (GyrI residues 33–48), used as a negative control, was immobilized in the cuvette, and the binding of gyrase to the immobilized peptides was measured. The time course of gyrase binding to both peptides is shown in Fig. 5A. The complex between the ITGGQYAV-containing peptide and gyrase was found to be relatively stable compared with that of the peptide corresponding to GyrI residues 33–48 (Table III), which had no inhibitory effect on DNA gyrase activity. In Fig. 5B, a linear plot of $dR_t/dt$ versus $R_r$ yields

$$\text{slope} = -(K_a[C] + K_d)$$  

$$\text{y intercept} = K_a[C]R_{\text{max}}$$

where $dR_t/dt$ was obtained by measuring the response at multiple time points on the real time association curve. By plotting the response of the $dR_t/dt$ versus $R_r$ lines as a function of the GyrI concentration [C], a new line is generated. The response gave an association rate $(K_a)\text{ of 1.7 } \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the association of gyrase with the immobilized ITGGQYAV-con-
The dissociation rate ($K_d$) can be directly obtained by analyzing the dissociation phase using the FAST FIT program, as shown in Table III. A $K_d$ value of 2.2 μM was determined from the ratio of $K_d$ to $K_a$ for the ITGGQYAV-containing peptide, whereas a weaker dissociation constant $K_d$ (26.0 μM) was observed for the GyrI residues 33–48. (Table III).

**DISCUSSION**

We previously discovered GyrI during the purification of DNA gyrase from *E. coli* (25). Here, we wished to characterize the molecular interaction between GyrI and DNA gyrase. To do so, DNA gyrase was partially purified by affinity chromatography on a novobiocine-Sepharose column (25, 37, 38). The GyrA subunit was eluted with 2 M KCl, and the GyrB subunit was eluted with a combination of 2 M KCl and 5 M urea. The DNA gyrase holoenzyme, consisting of two subunits (i.e., A (GyrA) and B (GyrB)), was eluted with 5 M urea. The GyrI protein coeluted with the fraction containing the two subunits. This result suggests that GyrI interacts with the holoenzyme consisting of GyrA and GyrB, but not with GyrA or GyrB alone.
To investigate the interaction of GyrI with gyrase, we used SPR and immunoprecipitation. The results of the immunoprecipitation experiments suggest that GyrI binds directly to the holoenzyme and that binding is also observed, albeit to a lesser degree, with the individual GyrA and GyrB subunits. We also showed that ATP is not required for the interaction between GyrI and the gyrase holoenzyme. Kinetic parameters from SPR experiments used to examine the interaction between GyrI and GyrA, GyrB, or the holoenzyme supported the immunoprecipitation results. The association rate constant for the formation of the GyrI-holoenzyme complex was determined to be $10^4$ M$^{-1}$ s$^{-1}$. This is similar to that for the association of CcdB and GyrA. Like GyrI, CcdB is a natural toxin that targets gyrase. Analysis of the CcdB-gyrase interaction reveals that this complex forms through the direct binding of GyrA to CcdB (39). The inhibitory mode of action of CddB appears to be similar to that of the quinolones on gyrase (40). It would be very interesting to determine the inhibition mechanism of the ITGGQYAV peptide (89–96 of GyrI) into the cleavage assay, no cleaved DNA-gyrase complexes were observed (data not shown). This result suggests that the inhibitory mechanism of the ITGGQYAV peptide might be different from that of the quinolones. It would be interesting to modify this peptide to develop a more effective inhibitor of gyrase activity. The discovery and analysis of natural molecules with anti-gyrase activity hold promise for further investigations into mechanism of DNA gyrase activity. Finally, the GyrI peptides we describe here may form the basis for a novel class of peptide-based antibacterial agents.

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