DNA gyrase is the only topoisomerase that can introduce negative supercoils into DNA. It is thought that the binding of conventional type II topoisomerases, including topoisomerase IV, to DNA takes place at the catalytic domain across the DNA gate, whereas DNA gyrase binds to DNA not only at the amino-terminal catalytic domain but also at the carboxyl-terminal domain (CTD) of the GyrA subunit. The binding of the GyrA CTD to DNA allows gyrase to wrap DNA around itself and catalyze the supercoiling reaction. Recent structural studies, however, have revealed striking similarities between the GyrA CTD and the ParC CTD, as well as the ability of the ParC CTD to bind and bend DNA. Thus, the molecular basis of gyrase-mediated wrapping of DNA needs to be reexamined. Here, we have conducted a mutational analysis to determine the role of the “GyrA-box,” a 7-amino acid-long motif unique to the GyrA CTD, in determining the DNA binding mode of gyrase. Either a deletion of the entire GyrA-box or substitution of the GyrA-box with 7 Ala residues abolishes the ability of gyrase to wrap DNA around itself and catalyze the supercoiling reaction. However, these mutations do not affect the relaxation and decatenation activities of gyrase. Thus, the presence of a GyrA-box allows gyrase to wrap DNA and catalyze the supercoiling reaction. The consequence of the loss of the GyrA-box during evolution of bacterial type II topoisomerases is discussed.

DNA topoisomerases are ubiquitous enzymes that alter the linking number of double-stranded DNA. There are two classes of topoisomerases, type I and type II (1, 2). Type I topoisomerases alter the linking number in steps of one by breaking one strand of duplex DNA, passing the other strand through the break, and then resealing the broken strand, whereas type II topoisomerases alter the linking number in steps of two by breaking both strands, passing another segment of the helix through the break, and then resealing the broken strands. The discovery of a novel type II enzyme from Sulfolobus shibatae (3, 4) prompted the division of the type II topoisomerases into the type IIA and type IIB subtypes. All type II topoisomerases, except the S. shibatae topoisomerase VI, belong to type IIA subtype.

DNA gyrase (5–7) and topoisomerase (Topo) IV (8) are type IIA topoisomerases. Gyrase and Topo IV consist of GyrA and GyrB subunits and ParC and ParE subunits, respectively. GyrA and ParC subunits catalyze strand-breakage and reunion reactions, whereas GyrB and ParE subunits hydrolyze ATP. The active forms of gyrase and Topo IV are an αβ tetramer, and these topoisomerases bind double-stranded DNA. Topo IV can relax both positive and negative supercoils and catenate and decatenate covalently closed, negatively supercoiled, double-stranded circular (form I) and nicked or gapped, double-stranded circular DNA molecules. Gyrase can supercoil covalently closed, relaxed, double-stranded circular (form I') DNA molecules, catenate and decatenate DNA rings, and, in the absence of ATP, relax negative supercoils (1, 2).

Among all topoisomerases, gyrase is the only enzyme that can introduce negative supercoils into DNA (1, 2). It is thought that the binding of Topo IV to DNA takes place only at the amino-terminal catalytic domain (NTD) of the ParC subunit across the DNA gate, whereas DNA gyrase binds to DNA at both the NTD and the carboxyl-terminal domain (CTD) of the GyrA subunit. It is the binding of the GyrA CTD to DNA that causes the wrapping of DNA and enables gyrase to catalyze supercoiling reaction (9–11). Recent structural studies, however, run counter to this view. Both GyrA (12, 13) and ParC (14, 15) CTDs adopt a “β-pinwheel” fold, which is reminiscent of the predicted “β-propeller” fold built of 6 blades (16), and not only the GyrA CTD but also the ParC CTD can bind and bend DNA (12). In addition, a single-molecule experiment has demonstrated that Escherichia coli Topo IV bends DNA upon its binding to DNA (17). Thus, the functional roles of GyrA and ParC CTDs need to be reexamined to determine the molecular basis of gyrase-mediated wrapping of DNA. It is noteworthy that gyrase-mediated wrapping of DNA, but not Topo IV-mediated bending of DNA, induces the constraint of supercoils in DNA and that the binding of the ParC CTD to DNA has not been detected by footprinting methods (18).

Is there any motif that distinguishes the GyrA CTDs from ParC CTDs? An amino acid sequence alignment supplemented with the secondary structure predictions has revealed that there are two structural differences between E. coli GyrA and E. coli ParC CTDs (12). One is the presence of a GyrA-box, QQRGGK (19), in the first (the amino-terminal-most) blade of the GyrA CTD and the other is the fact that the β-pinwheel fold of the GyrA CTD consists of six blades, whereas that of the ParC CTD contains only five blades. Although all GyrA CTDs possess full six blades, ParC CTDs exhibit significant structural diversity with various numbers of blades (12, 14, 15). Thus, the GyrA-box is the only motif unique to the GyrA CTD. Unfortunately, the GyrA-box locates in a disordered loop in the E. coli GyrA CTD (13).

We conducted a mutational analysis of the GyrA-box to determine its role in gyrase-mediated wrapping of DNA. We constructed two mutant E. coli GyrA proteins, GyrA A-del and GyrA-Ala, which contain the deletion of the entire GyrA-box (residues 560–566), and the substitution of the GyrA-box with 7 Ala residues, respectively. Either the deletion or Ala-substitution of the GyrA-box abolished the ability of E. coli gyrase to wrap DNA around itself and catalyze the supercoiling reaction. However, these mutations did not affect the relaxation and decatenation activities of gyrase. Thus, the GyrA-box is required for gyrase-mediated wrapping of DNA and gyrase-catalyzed supercoiling reaction.
Role of the GyrA-box

**MATERIALS AND METHODS**

**DNAs and Proteins**—pBR322 form I DNA was purchased from New England Biolabs (Beverly, MA). Kinetoplast DNA (kDNA) was a generous gift of Yuzhen Wang and Junghuei Chen (University of Delaware).

Two mutations were introduced into the *E. coli* gyrA gene using the overlap expansion PCR technique (20). One is the deletion of the GyrA-box, gyrA A-del, and the other is the substitution of the entire GyrA-box with Ala residues, gyrA A-Ala (Fig. 1). DNA sequences of the open reading frames were confirmed by dideoxy DNA sequencing (data not shown). The gyrA A-del and gyrA A-Ala genes were cloned into the pET-11c vector (21), and the mutant GyrA proteins were expressed in *E. coli* BL21(DE3) (21). Mutant GyrA proteins were purified by the protocol used to purify the wild type GyrA protein (22, 23). The final preparations of mutant GyrA proteins were greater than 97% homogeneous for a single band on an SDS-polyacrylamide gel (see Fig. 2A). Purified GyrA A-del and GyrA A-Ala were mixed with the wild type GyrB to reconstitute GyrA A-del gyrase and GyrA A-Ala gyrase, respectively.

GyrA59 gyrase, which was reconstituted with GyrA59, the 5-kDa GyrA NTD (residues 1–523) (11), and the wild type GyrB, was prepared as described in our previous studies (22, 23). GyrA59 gyrase no longer wraps the DNA strand and cannot catalyze the supercoiling reaction (11, 23). However, GyrA59 gyrase is still capable of catalyzing decatenation and relaxation reactions. *E. coli* Topo I was purified as described previously (24).

**Limited Trypsinolysis of GyrA**—Digestion of either the wild type or a mutant GyrA protein with trypsin was performed by a protocol similar to that developed by Reece and Maxwell (25). Briefly, reaction mixtures (50 μl) containing 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 5 mM dithiothreitol, 10% (v/v) glycerol, 15 μg of either the wild type or a mutant GyrA protein, and 1 μg of trypsin (Sigma, St. Louis, MO) were incubated at 37 °C. At the indicated times, a portion (10–μl each) of the reaction mixtures was taken, mixed with the same volume of 10% SDS solution, and then heated at 100 °C for 5 min. Reaction products were analyzed by electrophoresis through 10% polyacrylamide gels containing 0.1% SDS. Gels were stained with Coomassie Brilliant Blue and photographed using an Eagle Eye II system (Stratagene, La Jolla, CA).

**Gyrase-induced Constraint of Supercoils in DNA (Wrapping Assay)**—Form I DNA was prepared by incubating pBR322 form I DNA with *E. coli* Topo I. Form I DNA was used as a substrate in both the wrapping and supercoiling (described in the following section) assays.

Reaction mixtures (20 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 μg/ml bovine serum albumin, 10% (v/v) glycerol, 0.1 pmol (as molecule) pBR322 form I DNA, and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase were incubated at 37 °C for 10 min. Then, 50 fmol of *E. coli* Topo I was added to the reaction mixtures, and the incubation was continued at 37 °C for 30 min. SDS was added to 1% to terminate the reaction, and the reaction mixtures were further incubated at 37 °C for 5 min. EDTA and proteinase K were then added to 25 mM and 50 μg/ml, respectively, and the incubation was continued for an additional 15 min. The DNA products were purified by extraction of the reaction mixtures with phenol-chloroform (1:1, v/v) and analyzed by electrophoresis through vertical 12% agarose (14 × 10 × 0.3 cm) at 2 V/cm for 12 h in TAE buffer. Gels were stained with ethidium bromide and photographed using an Eagle Eye II system.

**Supercoiling Reaction**—Standard supercoiling reaction mixtures (12.5 μl) contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM potassium glutamate, 10 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1 mM ATP, 100 fmol (as molecule) of pBR322 form I DNA, and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase, and reaction mixtures were incubated for 15 min at 37 °C. Reactions were terminated by the addition of EDTA to 25 mM, and the DNA products were analyzed as described in the previous section.

**ATP-independent Relaxation of Negatively Supercoiled Plasmid DNA**—Reaction mixtures (12.5 μl) containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM potassium glutamate, 10 mM dithiothreitol, 50 μg/ml bovine serum albumin, 100 fmol (as molecule) of pBR322 form I DNA, and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase were incubated at 37 °C for 30 min. Reactions were terminated by adding EDTA to 25 mM, and the DNA products were analyzed as described in the previous section.

**Decatenation of kDNA**—Reaction mixtures (12.5 μl) contained 50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂, 100 mM potassium glutamate, 10 mM dithiothreitol, 50 μg/ml bovine serum albumin, 1 mM ATP, 0.3 μg of kDNA, and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase. Reaction mixtures were incubated at 37 °C for 30 min and terminated by adding EDTA to 25 mM. The DNA products were analyzed by electrophoresis through vertical 1.2% agarose gels (14 × 10 × 0.3 cm) at 2 V/cm for 12 h in TAE buffer. Gels were stained with ethidium bromide and photographed.

**RESULTS**

**Alterations of the GyrA-box**—Structural studies have revealed two differences between *E. coli* GyrA and *E. coli* ParC CTDs (12–16). One is the presence of a GyrA-box, QRRGGKG (19), and the other is the fact that both GyrA A-del and GyrA A-Ala were comprised of the same amino-terminal 64-kDa and the carboxyl-terminal 33-kDa fragments (24). Amino acid sequences of the β-CA loop (13) in the wild type (residues 555–576) and mutant *E. coli* GyrA CTDs are shown. Amino acid residues in the GyrA-boxes (residues 560–566) are typed in bold. A vertical arrow represents the trypsin digestion site between residues 571 and 572 to generate the amino-terminal 64-kDa and the carboxyl-terminal 33-kDa fragments (24). wt, the wild type GyrA; A-del, GyrA A-del; A-Ala, GyrA A-Ala.

![Figure 1. Deletion and Ala-substitution of the GyrA-box.](image)

We hypothesized that the presence of a GyrA-box alters the interac-

![Figure 2. Role of the GyrA-box.](image)

To directly examine this possibility, we conducted a mutational analysis of the GyrA-box using the *E. coli* gyrase as a model enzyme. We con-

![Figure 3. Chromatographic properties of these mutant GyrA proteins.](image)

thus, these alterations were compared to those of wild-type gyrase as a model enzyme.

We constructed two mutant GyrA proteins: GyrA A-del and GyrA A-Ala, which contain the deletion and Ala substitution of the entire GyrA-box, QRRGGKG (residues 560–566), respectively (Fig. 1). Chromatographic properties of these mutant GyrA proteins were identical to those of the wild type protein (data not shown) and limited trypsinolysis showed that both GyrA A-del and GyrA A-Ala were comprised of the same globular domains as the wild type GyrA (Fig. 2B). Thus, these alterations...
Role of the GyrA-box

The GyrA-box is required for the supercoiling activity of gyrase. The standard supercoiling reaction mixtures containing 100 fmol (as molecule) of pBR322 form I DNA and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase were incubated and the DNA products were analyzed as described under “Materials and Methods.” Form I and form I indicate the substrate and the final reaction product, respectively. wt, the wild type gyrase; As9, GyrA59 gyrase; A-del, GyrA A-del gyrase; A-Ala, GyrA A-Ala gyrase.

The GyrA-box is required for the supercoiling activity of gyrase. The standard supercoiling reaction mixtures containing 100 fmol (as molecule) of pBR322 form I DNA and the indicated amounts (as tetramer) of either the wild type or a mutant gyrase were incubated and the DNA products were analyzed as described under “Materials and Methods.” Form I and form I indicate the substrate and the final reaction product, respectively. wt, the wild type gyrase; As9, GyrA59 gyrase; A-del, GyrA A-del gyrase; A-Ala, GyrA A-Ala gyrase.

of the GyrA box did not appear to perturb the structural integrity of the GyrA protein.

The GyrA-box is required for gyrase-mediated wrapping of DNA. The GyrA-box is required for gyrase-mediated wrapping of DNA. As positive supercoils are generated as a result of gyrase-mediated wrapping of DNA, negative supercoils must be generated in other regions of the DNA molecule to maintain the overall linking number. Addition of E. coli Topo I, which is capable of relaxing, negative, but not positive, supercoils, would result in the relaxation of negative supercoils, and the subsequent removal of proteins from the DNA would leave positive supercoils in the DNA (9, 10). Thus, by measuring the constraint of supercoils introduced in the DNA, we can directly determine the extent of gyrase-mediated wrapping of DNA.

Form I DNA was either by the wild type or a mutant gyrase in the absence of ATP (Fig. 3). As expected, the wild type gyrase could wrap DNA around itself (Fig. 3, lanes 2 and 3). In contrast, no wrapping of DNA was detected when GyrA59 gyrase was bound to form I DNA (Fig. 3, lanes 4 and 5). We found that either GyrA A-del gyrase or GyrA A-Ala gyrase could not wrap DNA (Fig. 3, lanes 6–9). These results demonstrated that the GyrA-box was required for gyrase-mediated wrapping of DNA.

The Loss of the GyrA-box Abolishes the Supercoiling Activity of Gyrase—Next, we examined the effect of the loss of the GyrA-box on supercoiling activity of gyrase. The wild type gyrase was capable of catalyzing the supercoiling reaction efficiently, and 4 fmol (as tetramer) of the wild type enzyme was sufficient to completely supercoil 100 fmol (as molecule) of form I DNA (Fig. 4A, lane 4). Either the deletion or Ala substitution of the GyrA-box essentially abolished the supercoiling activity of gyrase (Fig. 4B). Based on the amounts of form I DNA produced by 1 fmol of the wild type gyrase (Fig. 4A, lane 2) and 64 fmol of either GyrA A-del gyrase (Fig. 4B, lane 6) or GyrA A-Ala gyrase (Fig. 4B, lane 12), the supercoiling activities of these mutant gyrase were at least 320-fold lower than that of the wild type gyrase. These results showed that the GyrA-box was essential for a gyrase-catalyzed supercoiling reaction. These results, together with those described in the previous section, showed that it was the presence of a GyrA-box that allowed gyrase to wrap DNA around itself and catalyze the supercoiling reaction.

Both GyrA A-del gyrase and GyrA A-Ala gyrase exhibited extremely low levels of the supercoiling activity (Fig. 4B). It is possible that the final preparations of GyrA A-del and GyrA A-Ala were contaminated with a trace amount of the endogenous wild type GyrA protein. However, no supercoiling activity of GyrA59 gyrase was detected (Fig. 4A, lanes 7–12), although GyrA59, GyrA A-del, and GyrA A-Ala were expressed and purified in the same manner. Another possibility is that low levels of
the supercoiling activity are intrinsic to these mutant gyrases. A mutant gyrase reconstituted with the amino-terminal 64-kDa trypsin fragment of the GyrA subunit, which lacks the majority of the CTD (Fig. 1), exhibits a low level of the supercoiling activity (25, 26). Thus, both GyrA A-del gyrase and GyrA A-Ala gyrase could possess low levels of the supercoiling activity.

The Absence of the GyrA-box Does Not Affect Either the Relaxation or Decatenation Activity of Gyrase—It seemed possible that, as is the case with GyrA59 gyrase (11), the inability of either GyrA A-del gyrase or GyrA A-Ala gyrase to wrap DNA around itself would affect its supercoiling activity but not relaxation and decatenation activities. It is also possible, although less likely, that the loss of the GyrA-box would affect not only the supercoiling but also the relaxation and decatenation activities of gyrase. To distinguish these possibilities, we examined the effect of the loss of the GyrA-box on the relaxation and decatenation activities of gyrase.

The ATP-independent relaxation (Fig. 5) and ATP-dependent decatenation (Fig. 6) activities of these mutant gyrases were measured using pBR322 form I DNA and kDNA as a substrate, respectively. Both GyrA A-del gyrase and GyrA A-Ala gyrase were capable of relaxing the negatively supercoiled plasmid DNA (Fig. 5). The specific activity in the relaxation assay of either GyrA A-del gyrase or GyrA A-Ala gyrase was only slightly lower (<2-fold) than that of the wild type gyrase. The decatenation activity of either GyrA A-del gyrase or GyrA A-Ala gyrase was virtually identical to that of the wild type gyrase. As expected, the wild type gyrase decatenated and supercoiled kDNA (Fig. 6, lanes 2–5). As a result, the majority of the DNA products were the supercoiled monomer DNA molecules (minicircles decatenated from kDNA). In contrast, the majority of minicircles decatenated by either GyrA A-del gyrase or GyrA A-Ala gyrase were relaxed, although small portions of kDNA were partially supercoiled (Fig. 6, lanes 6–13). These results coincided well with the results of the supercoiling assay shown in Fig. 4. These results demonstrated that the loss of the GyrA-box did not affect the relaxation and decatenation activities of gyrase. Thus, the GyrA-box was required only for its supercoiling activity.

DISCUSSION

Despite the extensive amino acid sequence similarities between gyrase and Topo IV, the CTDs of the GyrA and ParC subunits were not considered homologous (1, 2). It was long thought that the ability of the non-homologous GyrA CTD to bind to DNA makes gyrase a unique topoisomerase, which is capable of wrapping DNA about itself and catalyzing the supercoiling reaction (9–11). In contrast, footprinting experiments have demonstrated the binding of the ParC NTD, but not the ParC CTD, to DNA (18). However, recent studies have revealed unexpected properties of the ParC CTD. Corbett et al. (12) have identified the significant structural similarities between the GyrA and ParC CTDs, as well as the ability of the ParC CTD to bind and bend DNA. These discoveries required the reexamination of the functional roles of the CTDs and the molecular basis of gyrase-mediated wrapping of DNA.

Although GyrA CTDs possess six full blades and an intact GyrA box sequence, structures of B. burgdorferi and E. coli GyrA CTDs have revealed two distinct types of GyrA CTDs (12, 13). The GyrA-box in the B. burgdorferi GyrA CTD locates in an ordered loop, which closes the β-pinwheel fold into a circular structure. In contrast, the GyrA-box in the E. coli GyrA CTD locates in the disordered β-CA loop, and this CTD adopts an open β-pinwheel fold. The closed β-pinwheel fold of the B. burgdorferi GyrA CTD is likely to be an exception (13, 15). The presence of a Thr residue in the B. burgdorferi GyrA box seems to play a critical role in closing the β-pinwheel fold. It has been shown that, in addition to the full-length GyrA protein as the functional catalytic subunit of gyrase, B. burgdorferi produces the GyrA CTD as an independent and functional 34-kDa DNA-binding protein (27). The closed β-pinwheel fold of the B. burgdorferi GyrA CTD might have evolved to accommodate its function as an independent protein. The majority of GyrA CTDs possess an open β-pinwheel fold, similar to that of the E. coli GyrA CTD (15). It is possible that the entire GyrA CTD, including the β-CA loop, would become ordered when it is docked with the rest of gyrase. Alternatively, the disordered β-CA loop, including the GyrA-box, becomes ordered upon the binding of the GyrA-box to the DNA strand, the G-segment, which stretches out from the catalytic domain. Conformations of two types of gyrase-DNA complexes could be virtually identical.

Because the 7-amino acid-long GyrA-box, QRRGGKG (19), is the only motif unique to the GyrA CTD, we hypothesized that the presence of a GyrA-box alters the interaction of the CTD with DNA and allows gyrase to wrap DNA around itself. Either the deletion or Ala substitution of the GyrA-box abolished the ability of E. coli gyrase to wrap DNA around itself (Fig. 3). These results demonstrated that, in fact, the GyrA-box was required for gyrase-mediated wrapping of DNA. How does the GyrA-box affect the gyrase-DNA interaction to cause the wrapping of DNA? Because the GyrA-box consists of basic amino acid residues, it seems reasonable to assume that the GyrA-box could directly bind to DNA, most likely the G-segment stretching out from the GyrA NTD. The observation that substitution of the GyrA-box with Ala residues abolished gyrase-mediated wrapping of DNA supported the notion that basic amino acid residues in the GyrA-box are essential. It is interesting
Role of the GyrA-box

to speculate that the binding of the GyrA-box to the G-segment coordinates the direction of the bending of the G-segment at both sides of the catalytic domain to ensure the wrapping of DNA. Subtle differences in the amino acid sequences of GyrA-boxes might determine the efficiency of gyrase-mediated DNA wrapping and thus the supercoiling activity of gyrase. Future studies on the structures of a gyrase and a gyrase-DNA complex are necessary to determine the exact nature of the GyrA-box-DNA interaction.

The loss of the GyrA-box almost completely abolished the supercoiling activity (Fig. 4) of gyrase. In contrast, either the relaxation or decatenation activity of gyrase was not affected by the alterations of the GyrA-box (Figs. 5 and 6). Furthermore, a DNA cleavage assay showed that the quinolone sensitivity of either GyrA A-Ala gyrase or GyrA A-del gyrase were similar, if not identical, to those of the wild type gyrase (data not shown). Thus, the wrapping of DNA caused by the GyrA-box is critical only for the supercoiling activity of gyrase. In contrast, either the relaxation or decatenation activities remained intact. Thus, the deletion of a GyrA-box resulted in the generation of a topoisomerase with two important properties. First, this topoisomerase lacked the supercoiling activity, and thus, it did not interfere with the essential function of gyrase. Second, this enzyme could catalyze both the relaxation and decatenation reactions, and thus, it could serve as a prototype of either relaxases or decatenases. E. coli Topo IV, an efficient decatenase (28, 29), may have evolved to specially decatenate daughter chromosomes.

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