The potent synthetic fluoroquinolones and the natural CcdB protein encoded by the F plasmid both inhibit bacterial growth by attacking DNA gyrase and by stimulating enzyme-induced breaks in bacterial DNA. The cleavage mechanisms of these structurally diverse compounds were analyzed by purifying and characterizing stable ternary complexes of enoxacin and CcdB protein with gyrase bound to a strong gyrase binding site from bacterial Mu. Three differences between enoxacin- and CcdB-derived complexes were discovered. 1) Enoxacin binds to the DNA active site and alters the breakage/reunion activity of the enzyme. CcdB binds gyrase-DNA complexes but does not influence enzymatic activity directly. 2) Complexes that produce DNA cleavage with enoxacin are irreversible, whereas similar complexes made with CcdB protein are not. 3) Enoxacin stimulates cleavage of both relaxed and supercoiled forms of DNA in the absence of ATP, whereas CcdB induces cleavage only after many cycles of ATP-dependent breakage and reunion. These differences in mechanisms can be explained by a model in which enoxacin induces formation of a novel “cleavable” complex, whereas CcdB protein traps a very rare “cleaved” conformation of the enzyme.

Type II DNA topoisomerases perform essential DNA metabolic functions in both prokaryotic and eukaryotic organisms, and they have become critical drug targets in treatment of disease. Bacterial infections are treated with the fluoroquinolones, and they have become critical drug targets in treatment of a wide range of bacterial infections. Many of these drugs were developed using similarity to the DNA gyrase protein. CcdB binds gyrase-DNA complexes, and this binding renders the enzyme resistant to CcdB toxicity. CcdB protein-mediated DNA cleavage is thought to play a role in cell division and segregation of the F plasmid. The killing process for CcdB protein mirrors that of the fluoroquinolones, and both proteins have been used as drug targets.

**COMPARISON OF STABLE TERNARY COMPLEXES FORMED WITH ENOXACIN AND CcdB PROTEIN**

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Katherine E. Scheier† and N. Patrick Higgins§

From the Department of Biochemistry and Molecular Genetics, University of Alabama, Birmingham, Alabama 35294

The DNA Cleavage Reaction of DNA Gyrase

Protein Purification—GyrA and GyrB proteins were purified independently from cells that express the gyrase subunits from plasmids (pPH3 and pAG111, respectively) following isopropl 1-thio-β-D-galactopyranoside-induced expression (17). The GyrA subunit was purified using a novobiocin affinity column (19), with the elution and refolding conditions of Jackson and Maxwell (17). CcdB protein was isolated as described previously (8). Rabbit anti-CcdB antiserum was made with CcdB protein from complexed DNA gyrase, and this antiserum was used in Western blot analysis to confirm that the purified protein was identical to the native CcdB protein. The GyrA subunit was purified using a combination of affinity chromatography and size-exclusion chromatography, and the purified protein was analyzed by SDS-PAGE and Western blot analysis.

Gyrase Assembly and Sepharose 4B Chromatography—Gyrase was reconstituted from purified subunits by incubation in mixtures containing 35 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 1 mM dithiothreitol, 0.4 mg/ml tRNA (yeast), 0.5 mg/ml bovine serum albumin, 80 μg/ml creatine kinase, 16 mM creatine phosphate, 70 μg/ml of relaxed MPf1000 DNA, and 800 units of purified Escherichia coli GyrA and GyrB subunits. After incubation for 10–30 min at 30 °C, other components (CcdB protein, ATP, or enoxacin) were added, and incubation continued at 30 °C for 1 h. To purify stable complexes, samples were loaded onto a 12-ml Sepharose 4B (Pharmacia Biotech Inc.) column (1 × 16 cm) equilibrated with buffer containing 50 mM Tris-HCl (pH 7.7), 10 mM MgCl₂, 20 mM KCl, 5 mM β-mercaptoethanol, 0.3-ml samples were collected, and portions were assayed all within the day of fractionation.

To stimulate DNA cleavage activity, enoxacin was added to reactions at a final concentration of 12.5 μg/ml, CcdB protein at 100 μg/ml, and ATP at 830 μM; reactions were quenched by the addition of a solution...
that brought the mixture to 0.1% SDS and 0.1 mg/ml of proteinase K. Samples were incubated for 10 min at 30 °C to remove the GyrA protein from cleaved DNA ends and then analyzed by agarose gel electrophoresis. Supercoiling was assayed by the addition of 850 μM ATP and incubation at 37 °C for specified times.

**DNA Substrates and Chemicals—**Plasmid pMP1000 (see Fig. 1) contains a 1.4-kb BamHI-ScaI fragment, which includes the strong gyrase site originally present at the center of bacteriophage Mu (20, 21) cloned between BamHI and HinII sites of pUC18. Plasmid DNA was purified by the alkaline lysis method (22) and relaxed with incubation with DNA topoisomerase I purified either from calf thymus or from wheat germ extract (Sigma). Covalently closed relaxed plasmid DNA was purified by equilibrium centrifugation in a cesium chloride ethidium bromide density gradient.

Westran polyvinylidene difluoride membrane, Rad-Free blocking powder, and Rad-Free Lumi-Phos 530 chemiluminescent substrate sheets used for Western blotting were obtained from Schleicher & Schuell. Agarose (medium EEO) was from Sigma.

**Agarose Gel Electrophoresis—**To separate and quantify the DNA products of gyrase reactions, we used agarose gel electrophoresis conditions that resolved the linear DNA form from the region of gel occupied by relaxed or supercoiled circular molecules with the normal distribution of linking numbers for gyrase reactions (σ = 0 to −0.1). Samples were loaded into the wells of a 15 × 10 × 0.7-cm horizontal 1% agarose slab gel made in 1 × TBE buffer (90 mM Tris borate, 2 mM EDTA (pH 8.0),) and electrophoresis was carried out at room temperature (21 °C) in TBE buffer for 2 h at 50 V. The agar slab was removed from the gel assembly and soaked in ethidium bromide (5 mg/ml) for 20 min. The slab was replaced in the assembly and electrophoresis continued at 20 V for 6 h. DNA was visualized on a Fotodyne UV26 transilluminator and photographed using Polaroid 55 positive/negative film.

Under these conditions, linear DNA migrated above the position of supercoiled or relaxed DNA.

**Mapping Gyrase Cleavage—**To map gyrase cleavage sites on pMP1000 precisely, complexes of gyrase and relaxed DNA were incubated for 15 min at 30 °C followed by the addition of either 850 μM ATP; 12.5 μg/ml exonuclease, or 850 μM ATP and 100 μg/ml CcdB. After incubation at 30 °C for 1 h, reactions were terminated by the addition of SDS and proteinase K (0.1% and 0.1 mg/ml, respectively). The DNA was extracted twice with phenol:chloroform (v/v), ethanol precipitated using SDS and proteinase K (0.1% and 0.1 mg/ml, respectively). The DNA was resuspended in 15 ml of deionized water and 10 μl of Laemmli SDS (pH 8.0), 0.1 mM EDTA. A 2-μl aliquot was used as the substrate for sequence analysis. The sequencing primer (5'-GGAGAAAGAAAGTGAAAG) annealed near an substrate for sequence analysis. The sequencing primer (5'-GGAGAAAGAAAGTGAAAG) annealed near an

**RESULTS**

**Chromatography of GyrA-DNA Complexes—**A pathway for drug-induced cleavage of DNA by gyrase is illustrated by the formula.

\[ \text{Gyr + DNA} \xrightarrow{\text{drug}} \text{Gyr}^*\text{DNA} \xrightarrow{\text{SDS}} \text{Gyr} \sim \text{DNA} \]

where Gyr-DNA is a binary complex formed with gyrase and DNA, Gyr-DNA is a ternary complex in which the drug stabilizes the enzyme close to a transition state, and Gyr-DNA is a covalent complex with both GyrA subunits attached to DNA via phosphotyrosine bonds (25, 26).

How do Gyr-DNA complexes made with a fluorquinolone such as enoxacin compare with complexes made with the CcdB protein? To address this question, we used Sepharose 4B chromatography to isolate stable ternary Gyr-DNA complexes made with each compound and then compared their molecular composition and biochemical properties with those of the binary complex Gyr-DNA (26). An example of the resolving power of Sepharose 4B is shown in Fig. 1B. A reaction mixture containing gyrase, plasmid DNA, CcdB protein, creatine kinase, and ATP was subjected to chromatography followed by SDS-polyacrylamide gel electrophoresis and silver staining. Gyrase complexes (detected by the presence of equivolum amounts of GyrA and GyrB) and plasmid DNA eluted from the Sepharose 4B column in the excluded volume (peak at 6 ml), whereas free GyrA (or GyrB) dimers eluted in a peak at 9 ml, creatine kinase eluted near 10 ml, and the CcdB protein plus small molecular components (like ATP and fluorquinolones) eluted in a peak at 12 ml (Fig. 1, B and C). Using this Sepharose chromatography system, gyrase-DNA complexes can be freed from unbound quinolones and from small non-DNA-binding proteins in a reaction mixture.

Gyrase binds to many different DNA sequences, but its potential to promote supercoiling and its ability to form a drug-induced cleavage product vary in a complex pattern from site to site (27, 28). When gyrase binds to pBR322 or pUC19, it forms an ensemble (complex mixture) because there are many nearly equivalent strength binding sites on these plasmid DNAs (29).

To make biochemical complexes more uniform, we used as the DNA substrate a 3.7-kb plasmid, pMP1000, which contains a 1.5 kb, kilobase(s); SGS, strong gyrase binding site.

The abbreviations used are: kb, kilobase(s); SGS, strong gyrase binding site.
the same as for the reaction mixture that was applied to the column (Fig. 2, lanes 9 and 10, lanes 19 and 20, respectively). Isolated Gyr*A complexes also form cleavage products at low efficiency (2–5% cleaved DNA) when incubated with ATP alone (Fig. 2, lanes 6 and 16). Such ATP-stimulated DNA cleavage is not seen if DNA substrates like pBR322 or pUC19 DNA are substituted for pMP1000 (see 26). Gyrase molecules bound at the SGS of plasmid pMP1000 have a higher propensity to form cleavable complexes compared with enzymes bound at “weaker” sites on pBR322 or pUC19 (see “Discussion”).

We determined (by restriction analysis) that about 70% of the pMP1000 DNA molecules that had been cleaved were broken near the SGS (data not shown). To map cleavage sites at higher resolution, gyrase was incubated with pMP1000 DNA and either enoxacin, CcdB protein plus ATP, or ATP alone. Cleavage was induced with SDS, the residual GyrA protein was removed by proteinase K digestion, and the DNA was purified. Aliquots of cleaved DNA were added as template to Tq polymerase reactions containing a 5'-32P-labeled oligonucleotide primer, and, after thermocycling, samples were analyzed on DNA sequencing gels. Synthesis initiated with the oligonucleotide primer terminates at the cleavage site of molecules cleaved with gyrase and adds a non-template-encoded dAMP, thereby marking sites of cleavage. Profiles of the DNA polymerase-generated bands are shown in Fig. 3. All three methods of inducing gyrase-DNA cleavage produced a band at the position of the third T in the sequence GATTTATG. This position corresponds to the site of enoxacin-stimulated cleavage, which was determined previously in the SGS of phage Mu (20). A second prominent band corresponded to a CG compression near the start of this sequence; this band is caused either by Tq polymerase pausing or by a sequence-directed termination at the C or G residue; it is seen even when substrate DNA has not been incubated with gyrase (Fig. 3, lane 8). A band after the first T in the sequence listed above is a less frequently used cleavage site and is seen in all cleavage reactions. Thus, gyrase must determine the position of DNA cleavage, whereas the stimulants enoxacin, CcdB protein, and ATP influence cleavage efficiency.

*Isolation of Gyr*DNA Complexes Containing Enoxacin*—It has been known for many years that quinolones will stabilize gyrase binding to DNA under conditions of high ionic strength (26). However, isolation of stable quinolone-containing complexes in high yields has not been reported. To see if Gyr*DNA complexes can withstand rigorous isolation, enoxacin was incubated with Gyr*A for 1 h, and the resulting complexes were fractionated on Sepharose to remove unbound drug. The fraction that eluted at the excluded peak (6 ml) retained SDS-
inducible cleavage activity, which shows that enoxacin-containing complexes can be isolated. Moreover, the DNA cleavage efficiency of the isolated complex was indistinguishable from that of the reaction mixture applied to the column (Fig. 4, lanes 5 and 10). Thus, Gyr*DNA complexes are stable at 21 °C (for at least 1 h).

Enoxacin is a good inhibitor of gyrase-catalyzed supercoiling. The effect of the drug can be seen in Fig. 5 where enoxacin retards supercoiling activity. To see if isolated enoxacin-induced Gyr*DNA complexes were inhibited for supercoiling activity, ATP was added to an aliquot and incubation carried out for 1 h. Surprisingly, ATP stimulated full supercoiling of the DNA substrate. What is more, the SDS-induced cleavage activity, which was present before incubation, disappeared; DNA cleavage was reduced from 60% of the DNA substrate to less than 10% (compare Fig. 4, lanes 10 and 12). This cleavage level is only slightly above the level observed in purified complexes incubated with ATP alone (see Fig. 2, lane 16).

Two mutually exclusive interpretations could explain this result. Either enoxacin dissociated from the complex during the incubation, allowing supercoiling activity to be regained. Or isolated complexes became immune to supercoiling inhibition with enoxacin. To decide between these two possibilities, enoxacin was added to the isolated complexes. Fresh enoxacin inhibited ATP-driven supercoiling and stimulated the SDS-induced DNA cleavage reaction (Fig. 4, lane 14). Thus, enoxacin must dissociate from the isolated complexes during the incubation. Drug dissociation could also be seen in a less dramatic fashion when purified complexes were incubated at 30 °C for 1 h in the absence of ATP; SDS-induced cleavage was reduced from 60 to 20% of the total DNA (Fig. 4, lane 11).

Because enoxacin binding is reversible at 30 °C and because dissociation is stimulated by ATP, we tested the possibility that formation of cleavable complexes (at high enoxacin concentration) might be stimulated by ATP. Enoxacin (@12.5 μg/ml) was added at 30 °C to preformed Gyr-DNA complexes, and aliquots of the reaction were quenched after different incubation times by the addition of SDS and proteinase K. DNA cleavage activity appeared relatively slowly after the introduction of enoxacin and reached a plateau in which 60% of the plasmid was cleaved after approximately 10 min (Fig. 5, panel A, lanes 10–14, and panel B). ATP accelerated the process. When ATP was added at the same time as enoxacin, DNA cleavage occurred faster and with a slightly elevated final amount (from 60 to 70% cleaved product Fig. 5, panel A, lanes 15–19, and panel B). Thus, enoxacin converts Gyr-DNA complexes into ternary Gyr*DNA complexes with a slow kinetic step that is stimulated by binding of ATP.

**Isolation of Gyr*DNA Complexes Containing CcdB Protein**—
CcdB protein converts Gyr-DNA complexes into a ternary Gyr*DNA form similar to the one described above for enoxacin (Fig. 2, lane 8). Anticipating that a CcdB-containing complex could be isolated with the strategy described above, a reaction mixture containing pMP1000 DNA, ATP, and CcdB protein was incubated at 30 °C for 1 h and subjected to fractionation on Sepharose. Similar to results found with enoxacin, a complex was obtained in which DNA cleavage was observed after the SDS addition (Fig. 6, lane 9). Also similar to the results with enoxacin, the isolated complex showed the same DNA cleavage effi-
The amount of GyrA protein in the sample was 4 ng which were loaded with different levels of pure protein. The amount of CcdB protein present can be estimated by comparing its band intensity with lanes nearby containing Gyr*DNA complexes. The amount of CcdB protein, this analysis shows that roughly stoichiometric units in the purified complex. CcdB protein is small (11 kDa) and difficult to detect by silver staining. Therefore, we tested for CcdB protein using a Western blot transfer protocol. CcdB protein was seen in the excluded volume fraction (less than 1% found in Fig. 7, data not shown). Thus, CcdB becomes physically associated with Gyr*DNA complexes.

Because the kinetic analysis of enoxacin conversion of Gyr-DNA to Gyr*DNA showed a slow step, we investigated the kinetics of Gyr*DNA formation with CcdB protein. CcdB protein was added to reaction mixtures containing preformed Gyr-DNA complexes, and aliquots were quenched with SDS at different times to monitor cleavage. There were two significant differences from similar experiments done with enoxacin. First, very little cleavage was observed if ATP was omitted from the reaction (Fig. 8); this result confirms our previous experiments showing that ATP hydrolysis is required for the CcdB-mediated cleavage of linear DNA (8). Second, DNA supercoiling was not impeded by CcdB protein, and the rate of cleavable complex formation was exceedingly slow. Most DNA became completely supercoiled 1 min after the ATP addition, whereas the bulk of CcdB-induced cleavage products appeared some time after 10 min of incubation (Fig. 8, lanes 10–14). Because CcdB protein induces cleavage with equal efficiency using linear substrates (8) and cleavage is blocked by the addition of a nonhydrolyzable ATP analog (8), we conclude that the protein requires many
Nevertheless, CcdB can bind to Gyrase and the other half received 100 μg/ml CcdB alone (lanes 5–9), and the other half received 100 μg/ml CcdB plus 830 μM ATP (lanes 10–14). Aliquots were withdrawn and quenched with SDS and proteinase K after incubation at 30 °C for the following times: 5 s, lanes 6 and 11; 1 min, lanes 7 and 12; 10 min, lanes 8 and 13; and 1 h, lanes 9 and 14. Samples loaded in lanes 5 and 9 were quenched prior to the addition of either CcdB protein or ATP. The products were displayed on a 1% agarose gel along with the following marker DNAs: 1-kb ladder, lanes 1 and 15; relaxed pMP1000, lane 2; supercoiled pMP1000, lane 3; EcoRI-linearized pMP1000, lane 4.

rounds of enzyme turnover to form cleavage complexes, and it is a much less efficient toxin than enoxacin.

We also measured the stability of CcdB-induced Gyrase-DNA complexes (Fig. 6, lanes 5–7). Gyrase molecules remained active in this preparation as the fraction of DNA that was not cleavable became relaxed during incubation without ATP and then was supercoiled when ATP was added (Fig. 6, lanes 10 and 11). Significantly, CcdB-generated complexes proved to be much more stable than enoxacin-generated complexes. Extended incubation with (or without) ATP did not change the fraction of cleaved DNA (Fig. 6, lanes 10 and 11). We found no way to reverse cleavage of CcdB-stabilized complexes, including treatment with EDTA, which reverses enoxacin-stabilized complexes rapidly and efficiently (26, 31), or incubation in the presence of 0.5 M NaCl (data not shown).

What is the role(s) of ATP in the CcdB-conversion of Gyrase-DNA to Gyrase-DNA? Is ATP needed for CcdB binding to gyrase? This was tested by mixing gyrase, DNA, and CcdB together in the absence of ATP, incubating the mixture for 1 h, and then fractionating the material on the Sepharose column. Purified gyrase-DNA complexes were tested for supercoiling and cleavage activities; they were very active for supercoiling, and they could be efficiently converted to Gyrase-DNA complexes with enoxacin (Fig. 9, lanes 16 and 7). However, very little cleavage activity was detected following incubation with ATP and SDS (Fig. 9, lanes 6 and 14). To see if CcdB was present, the protein composition was analyzed by Western blot analysis. CcdB protein was detected in these fractions, but the amount of protein was less (by 3–6-fold) than the amount of CcdB protein found in cleavable Gyrase-DNA complexes (Fig. 9, lane 5). Nonetheless, CcdB can bind to Gyrase-DNA complexes in the absence of ATP.

**DISCUSSION**

**Enoxacin and CcdB Protein Stabilize Different Ternary Complexes with Gyrase and DNA**—The cytotoxicity of fluoroquinolones and of CcdB protein is caused by double strand breaks in chromosomal DNA (1, 7). DNA gyrase is the primary target of both agents, and the model that best explains their toxicity is that stable gyrase-DNA complexes cause havoc when they appear in front of a DNA replication fork (14, 32). Two possible outcomes are arrest of replication fork movement or replication fork dismemberment (33). At the ends of the broken forks, the GyraA protomer would remain attached via a covalent 5'-phosphothyrosine bond. Prior to homologous recombination repair of the fragmented DNA, the GyraA remnant would have to be removed either by 5’-3’ DNA excision or by the action of enzymes that hydrolyze the tyrosine-phosphoester bond (34). The question we address is how do two compounds with strikingly different chemical and physical characteristics, a small ring quinolone and the CcdB protein, attack the same gyrase active site? The answer is, they do it differently.

Ternary complexes of gyrase, DNA, and either enoxacin or CcdB protein can be isolated with long half-lives (hours). Evidence for the persistence of quinolone in gyrase complexes has been demonstrated with radioactive drug binding (35) and by DNA cleavage induction with SDS (Fig. 4). The presence CcdB protein in stable ternary complexes is confirmed by Western blot transfer and detection with anti-CcdB antisera, where CcdB was roughly stoichiometric with Gyrase in these complexes (Fig. 7).

Interactions of gyrase with enoxacin differ in three ways from interactions with CcdB protein. First, at 30 °C enoxacin binds to preformed Gyrase-DNA complexes and slowly converts them to cleavable complexes (over a 10-min interval) until approximately 60% of the DNA becomes cleaved after SDS addition (Fig. 5). This is contrasted by CcdB protein, which binds to Gyrase-DNA complexes but forms very few detectable cleavage complexes (in the absence of ATP), even when the incubation time is extended. Second, enoxacin inhibits gyrase supercoiling activity, slowing the approach of substrate to the highest supercoiled state. CcdB does not affect supercoiling; in fact, most CcdB-induced cleavage occurs after many cycles of ATP-driven breakage and reunion when the DNA has become highly supercoiled (Fig. 8). Third, enoxacin-stimulated cleavage is reversible once the drug is reduced to low levels; dissociation is also stimulated by ATP (Fig. 4, lanes 11 and 12). CcdB protein-stimulated cleavage is not reversible with EDTA or NaCl treatment, with or without ATP (Fig. 6).
DNA is bound as a positive supercoil wrapped on the surface of the enzyme, and an equilibrium is established for the breakage/reunion activity of the enzyme. In the absence of ATP, the equilibrium strongly favors the form at the bottom, in which the DNA is intact. Step 2 is binding of ATP, which stimulates breakage/reunion activity and pushes the enzyme through a conformation change that moves DNA through the transient gate created by concerted GyrA protein-DNA strand cleavage (25, 36). In Fig. 10A, the conformation change at step 2 is indicated by a rotation of the B subunits and a movement of the DNA strands. The actual nature of the ATP-driven conformation change in gyrase is unknown and lies at the heart of the supercoiling problem. In step 3, another breakage/reunion equilibrium is established after strand passage, and the DNA phosphodiester form is favored again as in step 1. ADP and Pi are released along with a segment of bound DNA, which changes the substrate linking number by two and allows supercoils to diffuse into the substrate molecule. For processive supercoiling (18), DNA would need to be rewrapped in another positive loop and B subunits repositioned to restart the chain. Note that the breakage/reunion equilibrium at step 3 does not necessarily equal the equilibrium of step 1; at high supercoiling levels, gyrase catalyzes the removal of supercoils (37). The reverse reaction may explain why ATP stimulates a low level cleavage reaction at the SGS. On highly supercoiled molecules the equilibrium favoring the complex in which DNA is rejoined may shift toward the cleaved form.

The CcdB protein appears to attack gyrase using a mechanism that was originally proposed for quinolones (38), i.e. it stabilizes the DNA-cleaved state (forms before and after step 2). This entrapment is inefficient, so many cycles of breakage and reunion must transpire to capture a significant fraction of molecules in the cleaved state. However, once formed, these structures are stable; the only reversal mechanism we know of is extraction of CcdB protein with the CcdA protein (8).

We propose that enoxacin converts the normal four-step cycle into a six-step cycle (Fig. 10B). Step 1 of the enoxacin cycle is drug binding to the DNA active site, creating a DNA conformation that is altered but in which strands are not yet broken. This distortion in Fig. 10 is indicated by a small bubble near two molecules of quinolone (*). Independent evidence for this intermediate has been discovered by two groups. Critchlow and Maxwell (35) showed that quinolones can bind to a gyrase-DNA complex even when the GyrA subunit carries a substitution of phenylalanine for tyrosine 122, which is the critical residue involved in DNA binding. Thus, cleavage is not necessary for stable quinoline binding. Second, Marians and Hiasa (31) showed that DNA near the topoisomerase IV cleavage site (within 1 or 2 base pairs) is distorted after quinolone binding and hyperreactive to oxidation with potassium permanganate. With gyrase, step 2 starts from this new ground state; quinolones stimulate breakage/reunion activity and establish a new equilibrium for the enzyme, which causes accumulation of broken DNA molecules. This hypothesis is in line with the observations of Froelich-Ammon and Osheroff (2), who showed that quinolones can perturb the breakage/religation equilibrium of type II topoisomerases. Note that enoxacin remains bound to the active site even in the broken conformation. In step 3, which is the same as step 2 in Fig. 10A, ATP binding causes a segment of DNA to pass through the gate. Step 4 establishes the second equilibrium between the cleaved and rejoined DNA form of enzyme, with the balance favoring a cleaved intermediate. Step 5 establishes a new drug binding equilibrium with DNA in the rejoined state. Finally, step 6 is release of products.

**Fig. 10. Scheme for enoxacin and CcdB inhibition of gyrase.** Panel A, the gyrase reaction mechanism is illustrated in four steps. Gyrase is shown as a heterotypic tetramer with two GyrA subunits (shaded diamonds), GyrB subunits (circles), and a segment of DNA wrapped over the enzyme surface making a positive supercoil (+SC). In step 1, the "catalytic" tyrosine 122 residues of each GyrA protomer react simultaneously with DNA and Mg\textsuperscript{2+} ions, establishing an equilibrium between the bound colinear phosphodiester DNA substrate at the bottom and the complex with broken strands and covalent phosphotyrosine linkage (GyrB-DNA) on top. The equilibrium (indicated by the thickness of the arrow) strongly favors the lower complex. In step 2, ATP binding induces a conformation change that stimulates the breakage/reunion activity, rearranges gyrase subunits, and changes the topological loop around the enzyme by passing a segment of DNA through the hole formed by the GyrA protomers. In step 3, another breakage/reunion equilibrium is established between the covalent phosphotyrosine-linked form (GyrB-DNA) on top and DNA substrate at the bottom. Again, equilibrium favors the phosphodiester-linked DNA form. By going through steps 1–3, the enzyme converts a positive supercoil into a negative supercoil, a process called the sign inversion mechanism of supercoiling (37). In step 4, the enzyme releases ATP, P\textsubscript{i}, and the segment of DNA which formed the loop, allowing supercoils to diffuse into the DNA substrate. CcdB protein (shown as crossed rectangles) traps gyrase in the cleaved conformation, producing a dead end product. Note that CcdB protein does not influence the breakage/rejoining equilibrium but merely traps the enzyme while it is in the DNA strand-disrupted form. Panel B, quinolones inhibit gyrase by a different mechanism. Enoxacin transforms the four-step gyrase cycle to six steps by creating a new intermediate shown in step 1. Drug binds at the active site (shown by a bubble) and distorts the DNA structure near the cleavage site (31, 35). From this energized state, the quinolone pushes the enzymatic equilibrium (2) in favor of the DNA-cleaved intermediate (step 2). The cleaved intermediate with bound quinolone can continue through the supercoiling cycle, passing DNA through a transient gate formed by GyrA protomers in step 3. But quinolone remains bound at the active site, and the equilibrium of breakage and reunion activity at step 4 still favors the cleaved DNA form. In this model, quinolone remains bound to the enzyme through four of the six steps of the cycle.
In this model, enoxacin is critically bound to four of the six enzyme conformations of the cycle.

**Gyrase Binding Specificity and Regulation**—Conflicting statements have appeared on the role of ATP in gyrase DNA cleavage. Shen et al. (39) reported that ATP or a nonhydrolyzable analog of ATP was necessary for cleavage. Other groups showed that cleavage can occur without ATP (25, 26, 40). The differences may be in the reactivity of target DNA used as the cleavage substrate. Cleavage efficiency varies from site to site (28, 41). With a strong site such as the SGs from the center of Mu (in pMP1000) ATP is not needed (Fig. 8), but it stimulates the rate and increases the fraction of cleaved molecules (Fig. 6 and Ref. 27). For a plasmid having only weak sites or for a substrate such as a synthetic homopolymer, ATP might seem to be a cleavage requirement.

Second, because fluoroquinolones bind DNA weakly in the absence of gyrase (42), cleavage reactions carried out with quinolones might reflect a binding preference of quinolone binding rather than a binding preference of gyrase. Identical cleavage patterns were seen in reactions made with CcdB protein, enoxacin, or simple ATP (Fig. 3). Therefore, at least at the Mu SGs, site selectivity is determined by gyrase, and the other factors only modulate cleavage efficiency.

CcdB-gyrase complexes are stable in two forms that survive Sepharose chromatography. Complexes made in the presence of ATP are ultrastable and can be reversed only by the addition of the antidote CcdA protein (8). Complexes made in the absence of ATP contained less CcdB protein. They were not cleaved or cleavable; the addition of ATP followed by SDS or the absence of ATP contained less CcdB protein. They were not cleaved or cleavable; the addition of ATP followed by SDS or the absence of ATP contained less CcdB protein. They were not cleaved or cleavable; the addition of ATP followed by SDS or the absence of gyrase (42), cleavage reactions carried out with CcdB protein or CcdB protein bound to two different sites on gyrase. Alteration, two or more molecules of CcdB protein may be needed to “catch” gyrase in the stable cleaved conformation. Our stoichiometry measurements do not resolve the issue. There is evidence that binding of quinolines is cooperative (39).

The CcdA-B system is reminiscent of proteins that coordinate orderly progression of cell development and control cell cycling (43). The functions of “checkpoint” proteins include monitoring the successful completion of a cellular task and blocking cell growth, depending on the completion of that task. The CcdA/B proteins already work like a checkpoint that monitors efficient inheritance of the F plasmid. One can imagine that by moving such a system to the bacterial chromosome and modulating CcdA/B expression according to external stimuli (i.e. activation of the Lon protease), one could connect DNA replication and chromosome segregation to other cellular processes with an interlocked system resembling an electronic circuit (44).

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