Conformational Changes in DNA Gyrase Revealed by Limited Proteolysis*

We have used limited proteolysis to identify conformational changes in DNA gyrase. Gyrase exhibits a proteolytic fingerprint dominated by two fragments, one of ~62 kDa, deriving from the A protein, and another of ~25 kDa from the B protein. Quinolone binding to the enzyme-DNA complex induces a conformational change which is reflected in the protection of the C-terminal 47-kDa domain of the B protein. An active site mutant (Tyr122 to Ser in the A protein) that binds quinolones but cannot cleave DNA still gives the quinolone proteolytic pattern, while stabilization of a cleaved-DNA intermediate by calcium ions does not reveal any protection, suggesting that the quinolone-induced conformational change is different from an “open-gate” state of the enzyme. A quinolone-resistant mutant of gyrase fails to give the characteristic quinolone-associated proteolytic signature. The ATP-induced dimerization of the B subunits is a key step of the gyrase mechanism. The proteolytic fingerprint of this conformation (stabilized by the non-hydrolyzable ATP analog 5’-adenylyl-β,γ-imidodiphosphate (ADPNP)) shows a protection of the 43-kDa N-terminal domain of the B subunit. The presence of quinolones does not prevent dimerization since incubation of the enzyme-DNA complex with both ADPNP and quinolones gives rise to a complex whose proteolytic pattern retains the characteristic signature of dimerization but has lost the quinolone-induced protection. As a result, the quinolone-gyrase complex can still hydrolyze ATP, albeit with different kinetic characteristics. We interpret the proteolytic signatures observed in terms of four complexes of gyrase, each representing a particular conformational state.

DNA gyrase is a type II topoisomerase responsible for the manipulation of the topological state of DNA in bacteria (reviewed in Ref. 1). Catalysis by type II topoisomerases requires the hydrolysis of ATP and involves the passage of a segment of DNA through a double-stranded break in another segment held open by the enzyme. Gyrase is distinct in its catalytic mechanism from the other enzymes of its class in that conventional type II enzymes (such as yeast topoisomerase II) cannot discriminate between DNA segments to be transported, thus favoring DNA relaxation or intermolecular strand passage (catenation/decatenation reactions). By contrast, gyrase is able to dictate the direction of strand passage and, by so doing, is the only topoisomerase able to perform DNA supercoiling.

Gyrase consists of two proteins which combine to form an $A_B$ complex that binds approximately 128 bp of DNA (2) in a positive superhelical sense around its core (3). When the A protein (GyrA, 97 kDa) is treated with either trypsin or chymotrypsin two large fragments are generated with approximate masses of 64 and 33 kDa (4). The C-terminal 33-kDa fragment, comprising the residues from 572 to 875, has been shown to bind DNA in positive superhelical sense but is unable to catalyze any of the reactions of gyrase (5). The N-terminal fragment contains the active site tyrosine and fragments in the size range 58–64 kDa have been shown to possess catalytic activity (6). When complexed with the B protein (GyrB), the N-terminal domain is able to perform ATP-dependent relaxation and increased DNA decatenation compared with the full-length enzyme, thus behaving like a conventional type II topoisomerase (7). Therefore, the positive wrapping of DNA caused by the 33-kDa domains appears to be largely responsible for the unique properties of gyrase.

GyrB (90 kDa) also consists of two domains: a 43-kDa N-terminal and a 47-kDa C-terminal domain (1). In the presence of GyrA, the 47-kDa domain cannot supercoil DNA but is able to perform all the ATP-independent reactions of gyrase (8). The 43-kDa N-terminal domain has been produced as a separate gene product and was found to hydrolyze ATP (9). This domain has been crystallized in the presence of a non-hydrolyzable ATP analog, ADPNP, and the structure has been solved at 2.5-Å resolution (10). As suggested by the crystal structure as well as by biochemical data (9, 11), the protein forms a dimer in the presence of ATP. This dimerization appears to be a key step in the mechanism of supercoiling. Briefly, gyrase binds DNA and wraps it in an interaction mainly supported by the 33-kDa domains of GyrA. This positions the transported segment in the right orientation for strand passage. Binding of ATP causes dimerization of the B proteins which captures the transport segment and directs it through the double-stranded break made by the enzyme on the gate segment. ATP is then hydrolyzed allowing the enzyme-DNA complex to return to its starting conformation.

Gyrase is inhibited by a number of antibacterial agents including the quinolones (12). Addition of a protein denaturant, e.g. sodium dodecyl sulfate (SDS), to a quinolone-arrested gyrase-DNA complex results in DNA cleavage (13, 14). Cleavage does not require ATP, but ATP or ADPNP can change the efficiency of cleavage and alter the pattern of preference between cleavage sites (15, 16). Efficient DNA cleavage by gyrase can also occur in the absence of quinolones if Mg$^{2+}$ is substituted by Ca$^{2+}$ (4).

The application of limited proteolysis can often provide use-
ful information about the conformational changes resulting
from the interaction of a protein with a substrate or effector
molecule. Structured regions of a protein are usually very
resistant to attack by low concentrations of endopeptidases
while cleavage of the peptide backbone can occur in less
structured regions or in loops linking structural domains of
the protein. Using this approach it is often possible to separate
functional domains of a protein and investigate their activity, e.g.,
Refs. 4, 17–19. The susceptibility of a region of a protein to
cleavage can be altered when a conformational change occurs,
resulting in cleavage sites being protected or new sites being
revealed. Comparing the proteolytic pattern of a protein in the
presence or the absence of a ligand it is possible to investigate
conformational changes associated with this interaction (20, 21).
We have used this approach to study the interaction of DNA
gyrase with quinolones and ADPNP, and we have identified
characteristic proteolytic signatures for these complexes.

**EXPERIMENTAL PROCEDURES**

**Enzymes and DNA—**GyrA, GyrB, GyrA<sup>Ser-122</sup>, GyrATrp-83 (gift of C.
Willetts), the 59-kDa N-terminal domain of Gyra59 (gift of C.
Smith), and the 64-kDa N-terminal domain of the same protein (gift of
S. Critchlow) were purified as described previously (6, 22). Negatively
supercoiled and relaxed forms of plasmid pBR322 were provided by
A. J. Howells (University of Leicester). Linear pBR322 was prepared by
digestion of the supercoiled form with EcoRI. A 147-bp fragment con-
taining the major gyrase cleavage site of plasmid pBR322 (23) was
prepared by the polymerase chain reaction using pBR322 as a template.

**Limited Proteolysis—**Samples contained 0.3 mg/ml GyrA and/or
GyrB, 0.4 mg/ml linear pBR322 DNA (where indicated) and various
effector molecules (as described in the figure legends) in 50 mM Tris
HCl (pH 7.5), 50 mM KCl, 4 mM MgCl<sub>2</sub>, 4 mM dithiothreitol, 6.5% (w/v)
glycerol. After incubation for at least 1 h at 25 °C, protease was added
and the reaction was allowed to proceed at 37 °C. Samples were taken
at various times and quenched by adding an equal volume of 62 mM
Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercap-
toethanol, 0.001% (w/v) bromphenol blue, and boiling for up to 15 min.
The products were analyzed by SDS-polyacrylamide gel electrophoresis
(PAGE).

**Enzyme Assays—**Quinolone- or Ca<sup>2+</sup>- induced cleavage assays were
performed as described previously (4). ATPase rates were determined
by a linked enzyme assay (9, 24) in the presence of excess GyrA or
GyrB, and the reaction was allowed to proceed at 37 °C. Samples were taken
at various times and quenched by adding an equal volume of 62 mM
Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (w/v) β-mercap-
toethanol, 0.001% (w/v) bromphenol blue, and boiling for up to 15 min.

**Secondary Structure Prediction**—Secondary structure and solvent
accessibility predictions were performed using the programs PHDsec
and PHDacc (B. Rost, EMBL, Heidelberg, Germany (26, 27)).

**RESULTS**

**The Gyrase-DNA Complex (Complex I)—**To study the confor-
mational changes associated with the interaction of DNA
gyrase with a number of effector molecules a selection of endopep-
tidases was used: trypsin, chymotrypsin, and *Staphylococcus
aureus* V8 protease, giving a wide range of sequence-specific
cleavage sites. Trypsin is a serine protease that cleaves at the
C-terminal side of tyrosine, phenylala-
inone, and tryptophan, while V8 cleaves C-terminal to glutamic
acid residues. Chymotrypsin cleavage sites are located C-terminal side of arginine and lysine residues. Chymotrypsin
cleaves proteins at the C-terminal side of tyrosine, phenylala-
inone, and tryptophan, while V8 cleaves C-terminal to glutamic
acid residues.

- Treatment of the A subunit of DNA gyrase with 10 μg/ml
- Proteolysis treatment of the enzyme-DNA complex (complex I)
did not reveal any significant change in the proteolytic pattern (Fig. 2A), although a low level of resistance to proteolysis was evident in earlier times of the reaction. This resistance can be attributed to the stabilization of the A$_2$B$_2$ complex in the presence of DNA. N-terminal peptide sequencing of the two proteolytic products has identified the ~62 kDa fragment as the N-terminal part of GyrA starting at residue 18 (the sequence SYLDAKQEQY which starts at residue 540 of GyrB).

**Quinolone Binding Induces a Conformational Change Which Protects the 43-kDa Domain of GyrB (Complex III)—**An important step in the catalytic cycle of gyrase is the dimerization of the B subunits in the presence of ATP (9, 11). When the non-hydrolyzable ATP analog ADPNP is used the two B subunits are locked in the dimeric form (10). When GyrB or the A$_2$B$_2$ complex were incubated with ADPNP prior to treatment with 10 μg/ml trypsin, a protection of the N-terminal 43-kDa domain of GyrB was observed (Fig. 1). With time the 43-kDa fragment was slowly converted to a smaller 33-kDa product. This fragment was identified by peptide sequencing as the N-terminal part of the 43-kDa domain (N-terminal sequencing revealed the sequence SNSYDSSSK for both fragments). When chymotrypsin was used instead of trypsin, the 43-kDa domain was very stable to further digestion (data not shown). There was no observable difference in the ADPNP-induced fingerprint between the gyrase-DNA complex and the DNA-free enzyme (Fig. 1). Earlier experiments with the 43-kDa domain alone have shown that in the presence of ADPNP the protein is cleaved by trypsin at lysine 307 to give an N-terminal 33-kDa fragment which is protected from further digestion (data not shown). This effect of quinolones was investigated further using chymotrypsin and Staphylococcus aureus V8 protease. Treatment of the enzyme-DNA complex with chymotrypsin produced a combination of a ~64-kDa product of GyrA and a ~41-kDa fragment of GyrB, to which the 47-kDa domain was added when the complex was incubated with CFX (data not shown). V8 gave a proteolytic pattern very similar to that of trypsin (data not shown). The fact that the protection of the 47-kDa domain was observed with a variety of proteases and quinolones suggests that it is not caused by the drug directly but is a result of a conformational change. When the gyrase-DNA-quinolone complex is disrupted by SDS, the DNA is found to be cleaved and covalently attached to the enzyme (13, 14, 33). It is therefore possible that this fingerprint represents the conformation of the enzyme where the DNA is cleaved and the two segments have been pulled apart. To investigate this possibility an active-site mutant of gyrase (GyrA$_{Ser^{83}}$) was used. This mutant binds quinolones as well as wild-type, but is unable to support DNA cleavage (28). Proteolysis of A$_2$B$_2$ in the presence of CFX showed the characteristic quinolone signature (Fig. 2B). This suggests that the quinolone-induced fingerprint can be denatured by SDS but is a result of a quite different conformational change involving the B subunits.

**Calcium-Induced Cleavage—**When gyrase and DNA are incubated with calcium instead of magnesium ions in the absence of quinolones and the reaction is stopped by the addition of SDS and proteinase K, the DNA is cleaved (4). We have tried to distinguish between calcium- and quinolone-induced cleavage using limited proteolysis. DNA and gyrase were incubated for 2 h under conditions that give calcium cleavage (4 mM Ca$^{2+}$), and then the mixture was treated with 10 μg/ml trypsin at 37 °C. Before the addition of protease, aliquots were removed (data not shown). Although under these conditions the level of Ca$^{2+}$-induced cleavage was comparable to that caused by quinolones, we have been unable to detect any protection of the 47-kDa domain in the presence of either linear or supercoiled DNA (Fig. 4). This suggests that the quinolone-gyrase-DNA complex (complex III) has a different conformation than the calcium-enzyme-DNA complex, in support of the above results suggesting that complex III fingerprint does not reflect a cleaved DNA complex but a quinolone-associated conformational change.
47-kDa domains (Fig. 5). Similarly, preincubation of the gyrase-DNA complex with ADPNP followed by addition of quinolones results in the same tryptic fingerprint. The ability of gyrase to bind quinolones in the presence of ADPNP was tested using rapid-gel filtration in the presence of a 147-bp DNA fragment based around the preferred 990 pBR322 cleavage site (23). The gyrase-DNA-CFX complex was compared with the complex formed when ADPNP was incubated with the enzyme after incubation with CFX and it was found that both of these complexes bound the same amount of CFX (Fig. 6A). The stoichiometry of binding was close to two quinolone molecules per enzyme complex, as observed previously (28). When the gyrase-DNA complex was incubated with ADPNP prior to addition of the quinolone, binding of the drug was slightly reduced (Fig. 6A). When ADPNP was added to the gyrase tetramer in the absence of DNA, to induce closure of the ATP-operated clamp, and DNA and CFX were then added to the complex, it was found that gyrase could still bind the drug, albeit with low efficiency (Fig. 6A).

The ability of gyrase to cause quinolone-directed cleavage of the 147-bp fragment in the presence of ADPNP was investigated. Addition of ADPNP to the gyrase-DNA-quinolone complex resulted in a small decrease in the level of cleavage, while a greater decrease was observed when ADPNP was added before the quinolone (data not shown). When the ATP-operated clamp was dimerized in the absence of DNA and then the 147-bp fragment was added together with CFX, gyrase could still perform DNA cleavage, albeit at a low level (similar results were also obtained by M. O'Dea and M. Gellert (National Institutes of Health, Bethesda, MD) and A. Howells (Leicester University, United Kingdom)²,³). Using rapid gel filtration the effect of quinolones on the binding of ADPNP was investigated (Fig. 6B). Quinolones did not inhibit ADPNP binding; gyrase was able to bind similar amounts of the nucleotide analog irrespective of the presence or absence of DNA or CFX. Addition of the quinolone to the complex prior to or following the addition of ADPNP did not affect the binding of the nucleotide. In each case, the stoichiometry of binding was approximately one ADPNP molecule per GyrB monomer (Fig. 6B).

The fact that ADPNP binding to gyrase is slow (25) can be used to observe the formation of the gyrase-CFX-ADPNP complex. ADPNP (250 µM) was added to the pre-formed quinolone-enzyme complex and samples were removed at certain times and treated with trypsin. Under these conditions, the slow conversion of complex III to this new conformation, complex IV, was evident (data not shown). Similarly, complex III was incubated with increasing low concentrations of ADPNP (0.1–1.0 mM) for the same amount of time and then treated with trypsin. A mixture of complex III and IV fingerprints was obtained with complex III predominating at low ADPNP concentrations and complex IV at higher (data not shown). These results are in-

² M. Gellert, personal communication.
³ A. Howells, personal communication.
Gyrase Can Hydrolyze ATP in the Presence of Quinolones—

Since the gyrase-DNA-quinolone complex has not lost its ability to close the N-terminal clamp in the presence of ADPNP, it is likely that it can still do ATP hydrolysis. Indeed, we found that gyrase that has been preincubated with quinolones and DNA is still able to hydrolyze ATP albeit at a lower rate than that of the drug-free enzyme-DNA complex (Fig. 7). This could be due to either the gyrase-quinolone complex having different kinetic characteristics of ATPase, or the quinolones completely abolishing ATP hydrolysis but trapping only a fraction of the enzyme molecules. This second possibility was explored using the truncated form of the GyrA protein. A592B2 (a version of gyrase deleted for the 33-kDa domains) does not exhibit DNA-stimulated ATPase, probably due to its weaker binding to DNA, but can perform quinolone-induced cleavage almost as efficiently as the full-length enzyme (6, 7). When the A592B2-DNA-quinolone complex is formed it can turn over ATP at a rate similar to that of the full-length enzyme in the presence of the drugs (Fig. 7). These results indicate that the quinolone-arrested gyrase-DNA complex has adopted a conformation which still enables it to hydrolyze ATP but the kinetic characteristics of this reaction are different from those of the reaction of the drug-free enzyme.

**DISCUSSION**

Limited proteolysis has previously been used in gyrase for the identification of functional domains in GyrA (4). Two domains were identified and their properties are now well established (5, 7, 34). In vivo GyrB is found to be proteolyzed into two fragments that have been shown to be different functional domains (1). When applied to eukaryotic topoisomerase II, limited proteolysis revealed a conformational change associated with the binding of ADPNP (20, 21). This result was considered as evidence of the dimerization of the N-terminal domains of topoisomerase II in the presence of ATP. The mechanism of supercoiling by type II topoisomerases involves the translocation of a DNA segment through an enzyme-stabilized DNA gate. This is accomplished by passing the DNA through the enzyme itself (35, 36). This mechanism requires the enzymes to undergo significant conformational changes. Therefore, we decided to use limited proteolysis to identify conformational
states that are involved in the catalytic cycle of gyrase as well as proteolytic fingerprints for inhibitor-trapped enzyme conformations.

Complex I: The Enzyme-DNA Complex—The first step was to establish the proteolytic signatures of the individual subunits and the holoenzyme. Treatment of GyrA with trypsin yielded only one product fragment. Due to the much higher protease concentrations used in these experiments than used previously (4), the 33-kDa domain of GyrA appeared only in earlier times of the reaction and was quickly degraded. Presumably due to the same reason, the N-terminal trypsic site was at lysine 17 rather than arginine 6, as found previously (4). The size of the product fragment was estimated to be 62 kDa, supposing that the C-terminal tryptic site is at arginine 571, as suggested before (4). Nevertheless, it is possible that due to the higher concentration used, trypsin was able to cleave at the stretch of arginine and lysine residues at the region 561–567. This would have resulted in a fragment with size a of 61 kDa. Indeed, in some cases the band corresponding to this fragment appeared as a doublet in SDS-PAGE, possibly representing a mixture of the 62- and 61-kDa fragments (Figs. 1–5). It seems unlikely that cleavage could have occurred at any other Arg or Lys residue because this would have resulted in a significant reduction in the size of this fragment which would have been evident by SDS-PAGE.

Treatment of GyrB produced only one fragment which started at Gly\textsuperscript{540}. Judging by the position of this fragment on SDS-PAGE and comparing with size markers, we estimated the mass of this peptide to be approximately 25 kDa. Examination of the amino acid sequence of GyrB revealed that a fragment of that size could be produced only if cleavage had occurred in a region containing four possible tryptic sites between residues Arg\textsuperscript{760} and Lys\textsuperscript{768}. Cleavage at these sites would result in peptides with sizes between 25 and 26 kDa. We used secondary structure and solvent accessibility prediction for GyrA and GyrB in an attempt to identify which of those residues was more likely to be accessed by proteases. Almost all the above possible cleavage sites appear to be part of loop structures making discrimination between them impossible. Using a recent sequence alignment of type II topoisomerases we identified homologous residues of those possible cleavage sites in yeast topoisomerase II (37). None of these residues is present in the known crystal structure of yeast topoisomerase II (36). For simplicity we decided to call this peptide a 25-kDa fragment and the one produced from GyrA a 62-kDa fragment. When the gyrase subunits were mixed to form the holoenzyme (\(A_2B_2\)) the same proteolytic fingerprint was observed suggesting that the formation of the complex did not cause any changes in the structure of the two proteins that could be detected with the proteases used in these experiments.

Formation of the gyrase-DNA complex results in the positive wrapping of ~130 bp of DNA by the enzyme (2, 3). This mode of binding is believed to direct a contiguous DNA segment to the ATP-operated clamp formed by the B subunits (7). In this first step of the mechanism a DNA cleavage-religation equilibrium is established. DNA cleavage proceeds by the means of a transesterification reaction between a pair of tyrosine residues, one in each A subunit, and a pair of phosphates 4 bp apart. The covalent link is with the 5\textsuperscript{′} ends of the DNA while the 3\textsuperscript{′} ends are left with a free hydroxyl group. Religation is the reversal of this reaction. In the absence of any cofactor this cleavage-religation equilibrium is shifted to the ligated form since treatment of the complex with SDS and proteinase K reveals only minimal levels of cleavage.\textsuperscript{4} This conformation of the gyrase-DNA complex, prior the binding or after the release of any cofactor, we term complex I (Fig. 8). Complex I exhibits a signature identical to that of the DNA-free enzyme, consisting of a combination of the 62- and 25-kDa fragments.

Complex II: A Nucleotide-dependent Conformational Change—An essential subsequent step in the mechanism of supercoiling involves the closure of the ATP-operated clamp and the trapping of the transported segment inside the protein tetramer. This is effected by the dimerization of the B subunits caused by ATP binding. The DNA gate then opens and the DNA segment is transported through the double-stranded break. ATP hydrolysis is not required for this translocation since stoichiometric supercoiling can be observed in the presence of the non-hydrolyzable ATP analog ADPNP (16, 38). ADPNP binding is very tight and results in the trapping of the protein clamp in the dimerized form (10, 11, 25). This conformation of the enzyme, which is a major intermediate in the catalytic cycle, we term

\textsuperscript{4} S. C. Kampranis, unpublished data.
complex II (Fig. 8). ATP hydrolysis is then required to allow the enzyme to return to its native conformation (complex I). The proteolytic fingerprint of complex II is consistent with that seen before with only the 43-kDa domains of GyrB (11). Indeed, dimerization of the 43-kDa proteins gave a resistant 33-kDa fragment which is also present in the case of the holoenzyme (A$_{2}$B$_{2}$). However, in the case of the holoenzyme the whole 43-kDa domain is protected as a result of the dimerization, and the 33-kDa fragment is produced at later times. Presumably the presence of the rest of GyrB restricts access to the tryptic site at residue 307. There was no significant difference in the fingerprint of the gyrase-DNA-ADPNP and the DNA-free gyrase-ADPNP complexes. This is in agreement with previous observations suggesting that DNA is not necessary for the dimerization of the B subunits (9) and that the presence of DNA does not affect the rate or the stoichiometry of ADPNP binding to the gyrase holoenzyme (25). DNA cleavage is not required for the dimerization of the B subunits since the active-site mutant A$_{2}^{Ser-122}$B$_{2}$, that is unable to cleave DNA, can still reveal the complex II characteristic fingerprint.

**Complex III: A Quinolone-induced Conformational Change**—
Trapping of the gyrase-DNA complex by quinolones gives rise to a characteristic proteolytic fingerprint. This could be due to either a conformational change or a direct protection of a proteolytic cleavage site by the drug. The latter seems unlikely since all three proteases used in this study were able to reveal this characteristic protection. Being that a total of six different amino acids could be targeted by all three enzymes it seems quite unlikely that a molecule the size of a quinolone (amino acids could be targeted by all three enzymes it seems this characteristic protection. Being that a total of six different amino acids could be targeted by all three enzymes it seems quite unlikely that a molecule the size of a quinolone (~360 Da) could have such a direct effect. Moreover, this protection was observed with more than one member of this class of compounds (CFX and oxolinic acid). The quinolone-protected fragment seems to comprise the whole C-terminal domain of GyrB since it runs next to purified 47-kDa domain on SDS-PAGE (data not shown). Nevertheless, it is still possible that cleavage could have occurred close to the C terminus, after lysine 798, releasing a 650-Da fragment. A difference of that size would be quite difficult to determine by SDS-PAGE. This characteristic protection of the 47-kDa domain does not appear to be complete. This is evident both by the lower relative intensity of the 47-kDa band and the high proportion of 25-kDa domain fragment still present (Fig. 2, A and B). Since the 25-kDa fragment derives from the 47-kDa domain, protection of this domain would result in decreased intensity of the 25-kDa band. Although significant reduction in the intensity of this band is observed (Fig. 2B), this never completely disappears. It is possible that a proportion of inactive protein exists in the GyrB preparation and does not participate in the formation of the gyrase-DNA-quinolone complex.

To test the specificity of the interaction between the drugs and the enzyme that leads to the protection of the 47-kDa domains, we examined the effect of quinolone-resistant mutations in the proteolytic signature. A mutation in GyrA able to significantly decrease quinolone binding also prevented the appearance of the complex III-characteristic fingerprint. It has been shown previously that the C-terminal truncated version of gyrase (A$_{55}$B$_{2}$ or A$_{64}$B$_{2}$) is almost as efficient as the full enzyme in performing quinolone-induced DNA cleavage (6, 7). We tested the significance of the wrapping of the DNA around the enzyme to the proteolytic signature of the enzyme-quinolone complex. We found that the presence of the 33-kDa domains is not required for the enzyme to undergo the quinolone-associated conformational change.

Denaturation of the quinolone-trapped gyrase-DNA complex reveals covalent linkage of the enzyme to the DNA (13, 14, 33). It is therefore believed that quinolones (as well as other topoisomerase-targeting agents) interfere with the DNA cleavage-religation equilibrium by trapping the complex in a cleaved DNA conformation. To test whether the conformational state observed here reflects the structure of the complex when the DNA is cleaved, an active site mutant was used. It has been shown previously that DNA cleavage is not required for quinolone binding, since active-site mutants of gyrase that have lost their ability to cleave DNA can still bind quinolones as well as wild-type (28). We found that the mutation of Tyr$^{122}$ to Ser does not prevent the appearance of the quinolone characteristic proteolytic signature. Moreover, formation of the gyrase-DNA complex in the presence of calcium ions does not result in the protection of the 47-kDa domains. It is likely that calcium ions shift the cleavage-religation equilibrium to the open form, thus inducing DNA cleavage in the presence of a protein denaturant. It is therefore clear that simply stabilization of the cleaved-DNA conformation cannot account for the conformational change observed here.

**Complex IV: A Strand Passage Incapable Intermediate**—
Quinolone binding does not prevent dimerization of the ATP-operated clamp. When ADPNP is used to trap the gate in the closed form, a fingerprint similar to that of complex II is revealed. The lack of the expected protection of the 47-kDa domain is not due to any weakening of quinolone binding, as manifested by the ability of gyrase to bind the drug in the presence of the nucleotide (Fig. 6A). The small reduction in the level of quinolone binding observed when the complex was preincubated with ADPNP cannot account for the complete disappearance of the protection of the 47-kDa domain. This reduction in binding can be attributed to the trapping of DNA-free gyrase molecules by ADPNP. Such molecules would not be able to bind DNA efficiently, thus reducing the overall number of molecules able to bind quinolones. Indeed, closing of the clamp by ADPNP prior to the addition of DNA to the complex resulted in significant reduction in the level of quinolone binding (Fig. 6A). Similar results were obtained when quinolone-induced DNA cleavage was studied. Cleavage was not affected by addition of ADPNP while little reduction in cleavage efficiency was observed when ADPNP was added to the gyrase-DNA complex before the drug. Closing of the ATP-operated clamp does not abolish binding and cleavage of a linear DNA fragment, although the efficiency of this process is low. Such a result is consistent with the finding of Roca and Wang (39) that topoisomerase II could bind and cleave linear DNA even when preincubated with ADPNP. It has been proposed that in this case the DNA threads through a hole in the enzyme and finds its way to the binding site surrounding the active-site tyrosines (39).

Despite having the same proteolytic signature, complex IV is different from complex II because of the presence of the drug. Although the characteristic protection of the 47-kDa domains has disappeared, the enzyme would still have to adopt a conformation that would prevent catalytic strand passage, which now may not be as resistant to proteases as the one in complex III. Both complexes III and II can give rise to complex IV upon incubation with the appropriate molecule (ADPNP or CFX). The A$_{2}^{Ser-122}$B$_{2}$ complex reveals the characteristic complex II fingerprint when incubated with ADPNP in the absence of the drugs, and can undergo the conformational change required to convert complex III to complex IV, suggesting that DNA cleavage is not required for GyrB dimerization either in the presence or absence of quinolones.

A number of observations suggests that the core of the enzyme-DNA complex is likely to remain associated during the protease treatment. First, when the 43-kDa domain of GyrB was treated with trypsin and the fragments were denatured
with urea, the refolded peptides were still able to assemble and bind coumarin drugs. Second, the results presented here indicate that the disappearance of the 47-kDa domain protection in complex IV would not have been observed had the complex been unstable (Fig. 5). If fragments were dissociating from the gyrase-DNA complex as soon as they were produced, then release of the 43-kDa domains would have resulted in the enzyme reversion back to the complex III conformation. This would have produced a combination of 43- and 47-kDa domain protection (we assume here that the 395 bp site would still be the most sensitive GyrB site in complexes III and IV, as it is in complex I). Moreover, it seems reasonable that the presence of DNA (i.e. the ~130 bp wrap) improves the stability of the complex. However, this overall stability of the enzyme-DNA complex does not seem to extend to the 33-kDa domains of GyrA. These domains are the most susceptible to proteolysis and in time courses of the proteolysis reactions they are the first to be degraded (see “Results” and Ref. 4). The concentration of quinolones that is required to reveal the complex III-characteristic signature is significantly higher than that required to inhibit the enzyme (40). This is probably a manifestation of the degradation of the 33-kDa domains resulting in the destabilization of the enzyme-DNA-quinolone complex. Indeed, the A642B2-DNA-quinolone complex was found to be comparatively unstable, requiring higher concentrations of quinolone for its formation (40).

Quinolones do not abolish ATP hydrolysis, but in their presence gyrase hydrolyses the nucleotide at a slower rate (Fig. 7). Interpretation of this as incomplete binding of the drug is not plausible since under the conditions of these experiments full inhibition of supercoiling occurs. An additional piece of evidence comes from studying the truncated form of the enzyme, A59,B2. In the absence of quinolones, this enzyme does not exhibit DNA-dependent ATPase in the presence of linear DNA (7). In the presence of the quinolones A59,B2 is able to hydrolyze ATP in a rate similar to that of the drug-inhibited full-length enzyme (Fig. 7). The confirmation by proteolysis that dimerization of the B subunits can still occur in the presence of the drugs suggests that the quinolone-trapped complex (complex III) hydrolyzes ATP through the formation of complex IV. This pathway is shown in Fig. 8. The characteristics of this catalytic pathway are studied in the accompanying paper (40).

Mechanistic Implications—The blocking of supercoiling by quinolones contrasts with their ability to induce DNA cleavage. It has been proposed that in mammalian type II topoisomerases, compounds related to quinolones act by inhibiting DNA religation (41), but this does not seem to be the case with quinolones and DNA gyrase. Quinolone-directed DNA cleavage is sequence-specific in that under certain conditions full cleavage occurs only at certain sites while cleavage at other locations takes place with less efficiency (15, 16, 42). It appears that there would be complexes formed between gyrase and certain DNA sites in which the cleavage religation equilibrium in the presence of the quinolones is shifted to the ligated form. If the above proposal was correct, there would be no reason that these complexes could not perform strand passage. The drugs have to fulfill the dual role of blocking strand passage from all these complexes but allowing the DNA to be in the cleaved form in some of them. We believe that the mechanism of inhibition of gyrase by quinolones has its basis on the conformational change seen here. Quinolones could block supercoiling by stabilizing a conformation of the enzyme where, irrespective of whether the DNA is cleaved or not, the DNA gate cannot open sufficiently to allow strand passage. We believe that complexes III and IV reflect such a conformation. It is possible that in the presence of quinolones the 47-kDa domains of GyrB come close together, stabilizing the closed DNA gate conformation and blocking strand passage. Such a conformational change could result in the proteolytic protection of these domains. The fact that DNA cleavage is not required for quinolones to induce this conformational change explains how quinolones block supercoiling from all gyrase-DNA complexes, irrespective of the state of DNA cleavage at those sites.

Conclusions—We have been able to identify a number of proteolytic signatures of DNA gyrase. These fingerprints have been interpreted in terms of four different complexes each representing a different conformation of the enzyme. Two of these complexes are part of the normal catalytic cycle of gyrase while the other two are part of a non-productive cycle undergone by the enzyme in the presence of quinolone drugs (Fig. 8). Quinolone binding stabilizes a conformational state of the enzyme that is responsible for the inhibition of its activity. DNA cleavage is not required for the binding of the drugs or the induction of this conformational change. We suggest that inhibition of supercoiling by quinolones is the consequence of a drug-induced conformational change involving the 47-kDa domains of GyrB and that DNA cleavage is a subsequent slower step. Further evidence for this proposal is presented in the accompanying paper (40).

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