Probing the Binding of Coumarins and Cyclothialidines to DNA Gyrase[†]

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Received September 25, 1998; Revised Manuscript Received November 30, 1998

ABSTRACT: DNA gyrase is the target of a number of antibacterial agents, including the coumarins and the cyclothialidines. To extend our understanding of the mechanism of action of these compounds, we have examined the previously published crystal structures of the complexes between the 24 kDa fragment of GyrB and coumarin and cyclothialidine drugs and made mutations by site-directed mutagenesis. We used proteolysis as a probe of drug binding to wild-type and mutant proteins. Limited proteolysis of gyrase revealed that binding of these antibiotics is associated with a characteristic proteolytic fingerprint, suggesting a drug-induced conformational change. The ability of the mutants to bind the drugs was studied by testing their ability to induce the coumarin-associated proteolytic signature and to bind to a novobiocin-affinity column. To analyze further the interaction of the drugs with gyrase, we studied the binding using surface plasmon resonance. Mutation of Asn^{46} to Asp has only a modest effect on the binding of coumarins, while an Asn^{46} to Leu mutation results in a 10-fold decrease in the affinity. Mutation of Asp^{73} to Asn completely abolishes binding to both coumarins and cyclothialidines. Mutations at these residues also abolish ATP hydrolysis, explaining the inability of such mutations to occur spontaneously.

DNA gyrase is the target of a number of antibacterial agents (*1*). The most prominent of these are the quinolones, an entirely synthetic class of compounds, which have found wide application in clinical practice (*2*, *3*). The coumarins and the cyclothialidines (Figure 1), which are naturally occurring antibiotics, have failed to become clinically successful due to poor cell penetration, low solubility, and toxicity in eukaryotes (*1*). However, the fact that these compounds are significantly more potent in inhibiting DNA gyrase in vitro than the quinolones has stimulated interest with respect to improving their properties in order to produce structurally related compounds suitable for clinical practice.

DNA gyrase is the bacterial type II topoisomerase which can introduce negative supercoils into DNA using the free energy of ATP hydrolysis (*4*, *5*). Mechanistic studies have revealed the steps involved in the supercoiling reaction. Briefly, this process involves the wrapping of DNA around the enzyme, cleavage of this DNA in both strands (involving the formation of DNA−protein covalent bonds), and passage of a segment of DNA through this double-stranded break. This process is coupled to ATP hydrolysis and results in the introduction of two negative supercoils. The enzyme from *Escherichia coli* consists of two proteins, A and B, of molecular masses 97 and 90 kDa respectively; the active enzyme is an A_2B_2 complex. Both of these proteins have been shown to contain distinct domains. The A protein (GyrA[1]) consists of an N-terminal domain (59−64 kDa) involved in DNA breakage and reunion and a C-terminal domain (33 kDa) involved in DNA-protein interactions (*4*, *5*). The structure of the 59 kDa N-terminal fragment (GyrA59) has been solved to 2.8 Å resolution by X-ray crystallography (*6*). The B protein (GyrB) consists of an N-terminal domain (43 kDa; GyrB43) containing the ATPase activity and a C-terminal domain (47 kDa; GyrB47) involved in interactions with the A protein and DNA. The structure of the 43 kDa N-terminal domain complexed with ADPNP has been solved to 2.5 Å resolution by X-ray crystallography (*7*).

Since gyrase is an essential enzyme in prokaryotes but is not found in eukaryotes, it is an ideal target for antibiotics. The coumarins (e.g., novobiocin and coumermycin A_1; Figure 1) are naturally occurring compounds that have been shown to inhibit the ATPase reaction of gyrase (*8*, *9*). Sequencing of mutations in *gyrB* conferring coumarin resistance has suggested that the coumarin-binding site lies in the N-terminal portion of GyrB (*10*, *11*). Indeed, a 24 kDa N-terminal fragment of GyrB (GyrB24; residues 2−220) has been cloned and expressed and shown to contain the coumarin-binding site (*12*). The cyclothialidines are another family of naturally occurring antibiotics that target DNA gyrase. In vitro studies have shown that cyclothialidines also act by inhibiting the ATPase reaction of gyrase (*13*). Moreover, *Staphylococcus aureus* strains resistant to cyclothialidines carry mutations that map near the ATP-binding

[†] S.C.K. was supported by a CASE studentship funded by the BBSRC and Glaxo-Wellcome, and a grant from the Alexander S. Onassis Public Benefit Foundation, N.A.G. was supported by the Wellcome Trust, G.O. was supported by a CASE studentship funded by the BBSRC and Zeneca Pharmaceuticals, and A.M. was a Lister Institute Jenner Fellow.

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[1] Abbreviations: ADPNP, 5′-adenylyl β,γ-imidodiphosphate; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; SPR, surface plasmon resonance.

10.1021/bi982320p CCC: $18.00
Published on Web 01/28/1999

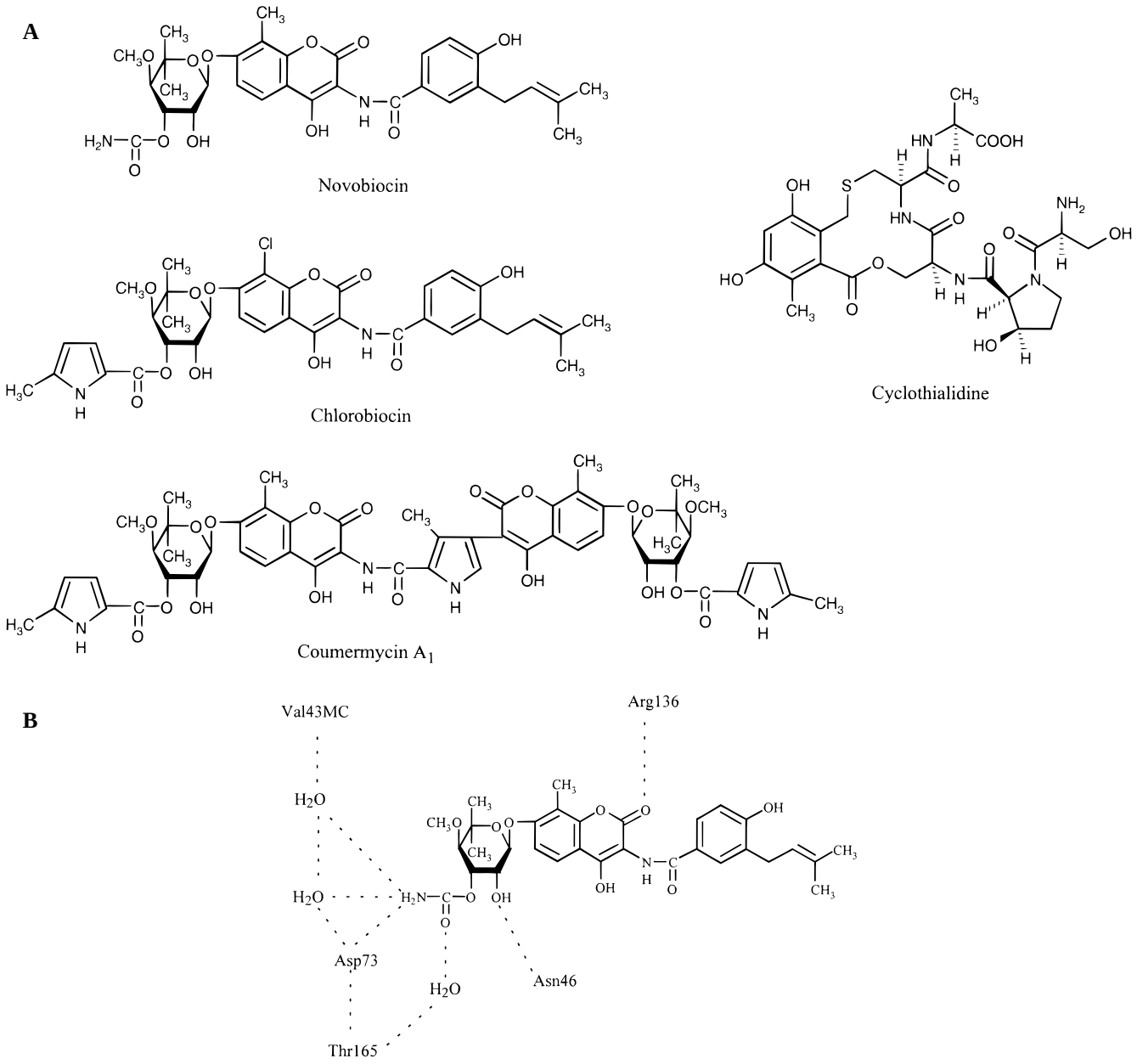

FIGURE 1: (A) The chemical structures of some members of the coumarin and cyclothialidine drug families. (B) Proposed hydrogen bonds (dashed lines) between novobiocin and amino acid residues in GyrB24 (MC = main chain) (*17*).

site in GyrB (*14*), while binding studies suggested that the cyclothialidine-binding site overlaps with those of ATP and coumarins (*15*). However, coumarin-resistant mutants are not necessarily resistant to cyclothialidines (*14*−*16*).

The complexes of the 24 kDa GyrB fragment with the coumarin drugs novobiocin and chlorobiocin and a member of the cyclothialidine family (GR122222X) have been crystallized and the structures solved to high resolution (*17*−*19*). These crystal structures show that the binding sites for ATP and coumarins partially overlap with the sugar ring of the drugs at the binding site of the adenine ring of ATP. The cyclothialidine-binding site is distinct from that of coumarins but also overlaps with that of ATP, with the resorcinol ring of the drug occupying the position of the adenine ring of the nucleotide. Clearly, these drugs act by preventing access of ATP to its binding site.

Although the solution of the crystal structure of the enzyme−drug complexes has improved our understanding of the molecular details of the interaction of coumarins and cyclothialidines with DNA gyrase, a number of outstanding issues remain to be resolved. Spontaneous mutations to coumarin resistance in GyrB have only been found at one residue (Arg^{136} to Cys, His, Leu, and Ser), despite considerable efforts (*10*, *11*, *20*). A temperature-sensitive mutation conferring chlorobiocin resistance has been found at Gly^{164} (*11*, *21*). This mutation is likely to be a folding mutation and has been shown to lie near the pyrrole ring in the GyrB24-chlorobiocin structure (*18*). (Mutations conferring coumarin resistance in *Haloferax* have been found in GyrB at amino acids corresponding to Gly^{81}, Ser^{121}, and Arg^{136} in the *E. coli* sequence (*22*), but it is not clear whether the mutations at 81 and 121 contribute significantly to resistance.) In the crystal structure the drugs appear to be interacting with a number of residues, and it seems surprising that mutation in only one of these amino acids (Arg^{136}) occurs in spontaneous coumarin-resistant mutants (Figure

1B). To corroborate the crystal structure data and to elucidate more details of the drug−protein interaction, we have determined the drug-binding properties of proteins bearing other point mutations in this region.

Coumarins have a high affinity for the gyrase B protein and fragments derived from it. This is manifested by K_i values from ATPase experiments which are in the range of $10^{-7}-10^{-9}$ M (*9*, *23*, *24*) and the tight binding of GyrB and the 43 and 24 kDa fragments to coumarin-affinity columns (*12*, *24*, *25*). Rapid gel filtration binding experiments measured dissociation rate constants for the drugs in the range of 10^{-3} s^{-1}, while titration calorimetry determined the K_d for coumarin binding to be $\sim 10^{-8}$ M. Taken together these results suggest that the drugs bind with association rate constants of $>10^5$ M^{-1} s^{-1}. However, none of the techniques employed so far has been able to generate precise data, so that it has proven difficult to establish definitive values for the binding parameters and to discern differences between the binding of different drugs to the protein. It would therefore be desirable to apply a technique capable of accurately measuring K_d values in the $10^{-7}-10^{-9}$ M range and able to measure fast "on" rates. Moreover, establishing a technique that would allow the fast and accurate determination of the affinity of coumarin and cyclothialidine derivatives for DNA gyrase would greatly facilitate the process of screening for novel inhibitors.

Limited proteolysis has been used successfully in the identification of conformational changes occurring in DNA gyrase in the presence of inhibitors or nucleotide analogues (*26*). In this paper we have applied limited proteolysis in the study of the interaction of coumarins with DNA gyrase in order to identify proteolytic signatures characteristic of drug binding. We have generated novel mutations in the drug-binding site of GyrB and assessed the drug-binding and enzymic properties of proteins bearing these mutations. We have tested drug and nucleotide binding by the wild-type and mutant proteins using proteolysis, and drug binding has also been analyzed by affinity column chromatography and quantitatively using surface plasmon resonance (SPR). In so doing we have established SPR as a technique that can be used to accurately determine binding parameters for the interaction of coumarins and cylothialidine with gyrase.

EXPERIMENTAL PROCEDURES

Materials. Novobiocin was purchased from Sigma; coumermycin and cyclothialidine were generous gifts from F. Hoffman-La Roche; and chlorobiocin was kindly supplied by Rhône-Poulenc Rorer. The 43 kDa N-terminal domain of GyrB was purified from *E. coli* JM109[pAJ1] and derivatives thereof as described by Ali et al. (*27*). The 24 kDa N-terminal subdomain of GyrB and a derivative carrying the mutation Arg^{136} to Cys (*12*) were provided by Dr. G. Walford (University Leicester). DNA gyrase A and B proteins were prepared as described previously (*28*).

Mutagenesis. Mutagenesis was carried out on plasmid pAJ1 (*27*) using Quikchange site-directed mutagenesis (Stratagene). The double-stranded mutagenic oligonucleotides used were Asp^{46}, 5′-AGG TGG TAG ATG ATG CTA TCG ACG A-3′; Leu^{46}, 5′-AGG TGG TAG ATC TTG CTA TCG ACG A-3′; and Asn^{73}, 5′-GTC TCT GTA CAG AAC GAC GGG CGC G-3′ (only the top strands of each pair of oligonucleotide is shown and the mutated codon is underlined). PCR was carried out using Pfu DNA polymerase (Stratagene), and after the end of the reaction, 10 units of Dpn I restriction enzyme (Stratagene) was added directly to each sample. After incubation at 37 °C for 1 h, samples were used to transform Epicurian Coli XL1-Blue supercompetent cells (Stratagene). Positive clones were identified by DNA sequencing.

CD Spectroscopy. To establish whether the mutated proteins were correctly folded, they were analyzed by CD spectroscopy (carried out by Prof. N. C. Price and Dr. S. M. Kelly, University of Stirling). Spectra were recorded using a JASCO J-600 spectropolarimeter at 20 °C. Cell path lengths were 0.01 cm (far UV) and 1 cm (near UV). Molar ellipticity values were calculated assuming a mean residue weight of 110, calculated from the sequence of the protein. Spectra were recorded at a scan rate of 10 nm/min, and the averages of 4 scans were plotted.

Electrospray Mass Spectroscopy. Analysis was carried out by Dr. K. S. Lilley (PNACL, University of Leicester) on a Micromass Platform electrospray mass spectrometer. Prior to analysis, samples were desalted to 1% formic acid using rapid gel filtration through a Sephadex G-50 spin column (Nick spin columns, Pharmacia).

Limited Proteolysis. Samples contained 0.3 mg/mL GyrB, 43 or 24 kDa proteins, and 0.3 mg/mL GyrA (where appropriate), in 50 mM Tris·HCl (pH 7.5), 50 mM KCl, 4 mM $MgCl_2$, 4 mM dithiothreitol, 6.5% (w/v) glycerol. Where indicated, ATP, ADPNP, and/or drug were also added. Samples were incubated for 1 h at 25 °C. Proteolysis was carried out using 10 μg/mL trypsin at 37 °C for 1 h (unless otherwise stated), and the reaction was 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-mercaptoethanol, 0.001% (w/v) bromophenol blue and boiling for 5 min. The products were analyzed by SDS−polyacrylamide gel electrophoresis (PAGE).

Surface Plasmon Resonance. Surface plasmon resonance (SPR) experiments were performed on a BIAcore 2000 system (BIAcore). Protein was immobilized on a CM5 sensor chip (BIAcore) via amine coupling in 10 mM HEPES (pH 7.4), 150 mM NaCl, 3.4 mM EDTA, and 0.05% Tween 20 at a flow rate of 5 μL/min. The immobilization procedure was as follows. Flow cells were activated by a 7 min injection of a solution containing 0.2 M *N*-ethyl-*N*′-(3-(diethylamino) propyl) carbodiimide and 0.05 M *N*-hydroxysuccinimide. Approximately 2000−3000 resonance units (RU) of protein in 10 mM sodium acetate (pH 4.5) was immobilized, and then the surface was blocked by a 7 min injection of 1 M ethanolamine hydrochloride. The buffer was then changed, and the system was primed for the kinetic measurements.

Kinetic experiments were performed at 25 °C in 35 mM Tris·HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 5 mM dithiothreitol, 6.5% (w/v) glycerol, 0.02% Tween 20. The flow rate was set at 50 μL/min and the data collection at 2 Hz. Various concentrations of the drug in the above buffer were injected over the immobilized proteins, and the association phase was observed for 210 s. After the end of the injection the flow was switched back to drug-free buffer and the dissociation phase was observed for 240 s. At the end of the dissociation phase the surface was regenerated by a 10

min injection of 2 M KCl (for novobiocin and cyclothialidine) and the same procedure was repeated for a different drug concentration. In the case of chlorobiocin and coumermycin the surface was regenerated first by competition with novobiocin followed by a 2 M KCl wash. Control experiments showed no loss in binding capacity of the surface after successive regeneration steps, suggesting that there was neither any loss in protein activity nor residual bound drug after the end of each regeneration step.

The kinetic data were analyzed using a global-fitting algorithm in the program BIAevaluation 3.0 (BIAcore) while simulation of mass transport effects was performed using BIAsimulation program (BIAcore) and models of binding described therein.

Other Methods. ATPase reactions were carried out using a pyruvate kinase/lactate dehydrogenase-linked assay as described by Ali et al. (*27*). Drug-binding studies were carried out using a novobiocin-affinity column as described by Staudenbauer and Orr (*24*) and using rapid gel filtration as described previously (*29*).

RESULTS

Site-Directed Mutagenesis. Examination of the crystal structures of the 24 kDa N-terminal fragment of GyrB complexed with the coumarin drug novobiocin and the cyclothialidine analogue GR122222X (*17*) shows that there are certain residues which make key hydrogen bonds to the ligands (Figure 1B). These include Asn^{46}, Asp^{73}, and Arg^{136}. These residues are also important in the interaction of chlorobiocin with this protein (*18*). Arg^{136} has frequently been found to be mutated in bacterial strains which are coumarin-resistant (*10*, *11*, *14*, *22*, *30*), but it is not presently clear why mutations do not arise at other loci. To improve our understanding of the molecular details of drug binding and to corroborate the crystal structure, we have made mutations at Asn^{46} (to Asp and Leu) and Asp^{73} (to Asn). These mutations were introduced into plasmid pAJ1 which carries the gene encoding the 43 kDa domain of GyrB. Wild-type and mutant proteins were purified to >95% purity and were found to behave as expected during the purification procedures. This suggested that the mutant proteins were unlikely to be substantially misfolded. To ascertain whether the mutant proteins were native in structure, they were subjected to CD analysis as described in Experimental Procedures. The far- and near-UV spectra of wild-type and mutant proteins were found to be virtually identical, suggesting that there was no change in secondary structure and that the tertiary structures were essentially the same (data not shown).

Coumarin Binding Results in a Characteristic Proteolytic Fingerprint. To probe coumarin binding to wild-type and mutant gyrase proteins, we have examined drug-dependent changes in proteolysis patterns. Proteolysis of gyrase by trypsin produces two major products, a ~62 kDa fragment belonging to the N-terminal domain of GyrA and a ~25 kDa fragment which is part of the C-terminal 47 kDa domain of GyrB (*26*, *31*). When the enzyme is incubated with novobiocin (100 μM) prior to treatment with trypsin, a major fragment of ~13 kDa and minor fragments of ~10 kDa are produced (Figure 2A). Proteolysis of the complex between

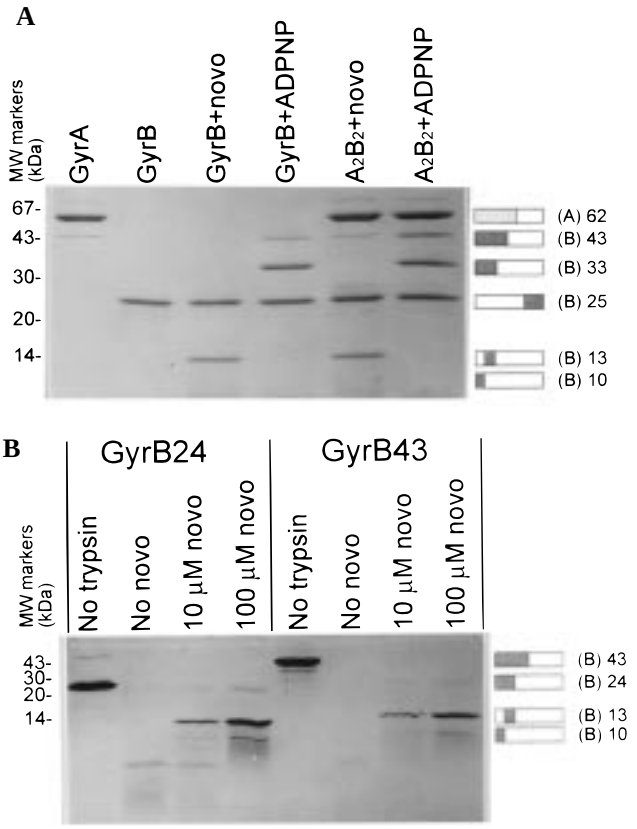

FIGURE 2: The coumarin-characteristic tryptic fingerprint. (A) GyrA, GyrB, or the A_2B_2 complex was incubated for 1 h at 25 °C with novobiocin (100 μM) or ADPNP (2 mM) as indicated. Samples were then treated with 10 μg/mL trypsin for 1 h at 37 °C and the products analyzed by SDS−PAGE. On the right is a diagrammatic representation of the gyrase fragment corresponding to each band. (B) GyrB24 and GyrB43 were incubated as described in A in the presence of the concentrations of novobiocin indicated.

the drug and either GyrB or the 43 kDa domain (GyrB43) alone revealed the same fingerprint, whereas proteolysis of the complex between the 24 kDa subdomain and drug produces a smaller major fragment, most likely a truncated version of the 13 kDa fragment (Figures 2 and 3A). N-terminal sequencing of the 13 kDa peptide produced the sequence VSGGL which corresponds to residues 111−115 of GyrB, identifying it as part of the 24 kDa subdomain of the B protein, in agreement with previous data that map the coumarin-binding site to this domain (*12*, *17*, *18*). N-terminal sequencing of the most prominent ~10 kDa minor fragment has identified this peptide as starting at residue 21 of GyrB (peptide sequencing produced the sequence KRPGMY which corresponds to residues 21−26 of GyrB). To accurately define the identity of these proteolytic fragments, we analyzed the products of the proteolysis reactions by electrospray mass spectroscopy. Analysis of the ~13 kDa fragment determined its size to be 12 780.3 ± 10.2 Da. Trypsin cleaves the peptide backbone C-terminal to a lysine or an arginine residue, and a peptide of this size could be produced if cleavage occurred after Lys^{223}. This peptide (comprising residues 111−223) has a calculated mass of 12 726 Da in reasonable agreement with the results of mass spectrometry. The major ~13 kDa fragment in the case of the 24 kDa domain is slightly smaller (Figures 2B and 3A); given that GyrB24 finishes at residue 220, this fragment is likely to comprise residues 111−220. The exact size of the

minor ~10 kDa fragment could not be determined by mass spectrometry, but this is likely to comprise residues 21−110 since such a peptide would have a calculated molecular weight of 9580 Da. This signature appears to be characteristic of all coumarin drugs since binding of coumermycin also results in the same proteolytic fingerprint (data not shown), and as such presents a means of assessing the formation of enzyme−drug complexes by proteolysis.

To test the ability of novobiocin to stay associated with the protein during and after treatment with trypsin, we removed samples at regular intervals from a proteolysis reaction (containing the 43 kDa fragment) and analyzed them by rapid gel filtration. By using radiolabeled drug, we determined that the protein bound novobiocin equally well throughout the proteolysis experiment (data not shown). Furthermore, when stripped of the bound novobiocin by denaturation in 6 M urea and then refolded by dialysis, the tryptic fragments can once again bind novobiocin and are protected from further degradation by trypsin. Moreover, these refolded peptides can be retained on a novobiocin-affinity column requiring 4 M urea for elution (data not shown).

The Molecular Basis of the Coumarin-Characteristic Fingerprint. At the molecular level, the protection of the 13 kDa fragment (Figures 2 and 3) could be attributed to two mechanisms. One involves direct interaction of the drug with an arginine or lysine residue of the protein so that it cannot be recognized by trypsin. Alternatively, binding of the drug could stabilize a conformational change that results in the protection of a certain tryptic site in the 13 kDa peptide. The fact that the coumarin drugs appear from the crystal structure to be making contact with an arginine residue in the protein (Arg^{136}) makes the first mechanism a possibility. This can be addressed directly using a protein in which Arg^{136} has been mutated to a residue that is not targeted by trypsin and at the same time confers resistance to coumarins. One such protein is the variant $GyrB24^{Cys136}$ (*12*); if the first mechanism is correct then treatment of this mutant with trypsin would reveal the coumarin-characteristic signature in the presence or absence of the drugs. However, this mutant was found to be essentially completely degraded by trypsin irrespective of the presence of novobiocin (Figure 3A), suggesting that the coumarin-characteristic signature is not the result of direct protection of Arg^{136} by the drugs.

However, the possibility still exists that the drugs directly protect another exposed arginine or lysine residue in the proximity of the drug-binding site. Therefore proteolysis experiments were performed using two other proteases, chymotrypsin and *Staphylococcus aureus* V8 protease. With both of these proteases, a characteristic proteolytic signature was apparent in the presence of novobiocin. With chymotrypsin this was very similar to that obtained in the case of trypsin, comprising two major fragments of ~32 and ~13 kDa and a minor fragment of ~10 kDa, the 32 kDa fragment being completely degraded to the 13 kDa peptide at later stages of the reaction (Figure 3B). Peptide sequencing revealed that the 32 and 13 kDa fragments start at Lys^{110} of GyrB, suggesting that they are the result of chymotryptic attack of the peptide backbone after Tyr^{109}. By using electrospray mass spectroscopy, we measured the size of the ~13 kDa peptide to be 13 077 ± 6 Da. Since chymotrypsin cleaves C-terminal to aromatic residues, it appears that this

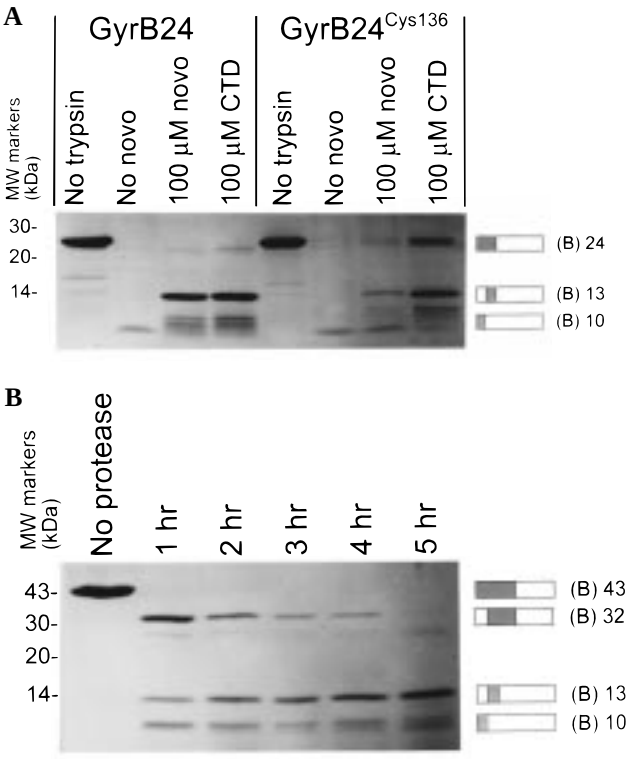

FIGURE 3: Tryptic and chymotryptic digests of GyrB fragments. (A) Wild-type GyrB24 or GyrB24 carrying the Arg^{136} to Cys mutation was incubated with novobiocin or cyclothialidine (CTD), as indicated, and treated with trypsin as described in the legend to Figure 2A. (B) Time course of a digestion of GyrB43 with chymotrypsin (20 μg/mL) in the presence of novobiocin (100 μM).

fragment comprises residues 110−225, that is, cleavage has occurred C-terminal to Phe^{225}. The calculated size of this peptide is 13 072 Da, which agrees well with the mass spectroscopy results. The exact size of the ~32 kDa fragment could not be measured by mass spectroscopy but it is likely to comprise residues 110−393; this peptide has a calculated size of 31 773 Da. Cleavage of this fragment after Phe^{225} would give rise to the ~13 kDa peptide. N-terminal sequencing of the 10 kDa fragment revealed that this starts at Lys^{14}. This fragment probably includes residues 14−109 since the predicted mass of this peptide (10 191 Da) is in good agreement with the size measured with mass spectrometry (10 187 ± 5 Da). To produce this 10 kDa fragment chymotrypsin would have to cleave C-terminal to Leu^{13}. Although Leu is not usually a target for chymotrypsin, it has been noted that under certain conditions this enzyme can cleave next to Leu or Ile residues (K. S. Lilley, personal communication). In the case of V8 protease, drug binding induces an overall protection of the 43 kDa domain (data not shown). These results suggest that the drug-characteristic signature is due to a drug-induced conformational change rather than direct protection of a proteolytic site by the drug.

Cyclothialidine Binding Results in the Same Proteolytic Fingerprint. Cyclothialidines are another class of naturally occurring antibiotics that inhibit the ATPase reaction of gyrase. Solution of the crystal structure of the cyclothialidine−GyrB24 complex has shown that the binding site for these drugs overlaps with that of coumarins (*17*). We addressed the question of whether cyclothialidines stabilize the same conformation of the enzyme by looking at the ability of these drugs to induce a characteristic proteolytic

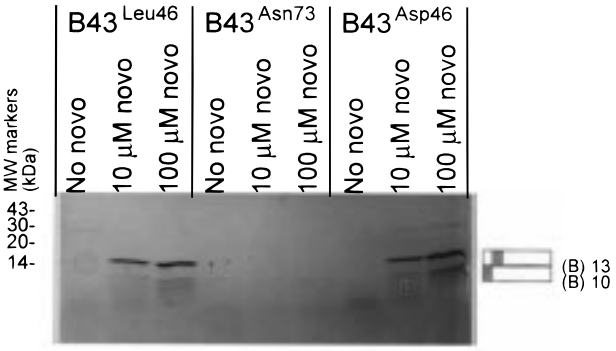

FIGURE 4: Tryptic digests of mutant GyrB43 proteins. Proteins were incubated with the indicated concentrations of novobiocin and digested with trypsin as described in the legend to Figure 2A.

fingerprint. Indeed, cyclothialidine binding to wild-type GyrB24 revealed the same proteolytic signature as novobiocin (Figure 3A). However, mutation of Arg^{136} to Cys did not appear to abolish cyclothialidine binding since incubation of $GyrB24^{Cys136}$ with cyclothialidine revealed low levels of the drug-characteristic footprint (Figure 3A). Since the binding sites for coumarins and cyclothialidine overlap but are not identical, this result adds further support to the notion that the characteristic proteolytic fingerprint is due to a conformational change rather than a direct protection by the drugs.

Probing Coumarin Binding to Mutants of Gyrase. Having established that limited proteolysis can be used as a probe for coumarin binding to gyrase, we have used this technique to test the ability of the mutant 43 kDa proteins to bind novobiocin. The mutants were incubated with 10 or 100 μM novobiocin and then treated with trypsin to reveal the coumarin-characteristic signature. Mutation of asparagine 46 does not seem to affect significantly the binding of the drug since both 43 kDa mutant proteins, Asn^{46} to Leu and Asn^{46} to Asp, can be protected by the drug (Figure 4). By contrast, Asp^{73} appears to be crucial for drug binding. Mutation of this residue to asparagine results in failure of the mutant protein to give the coumarin signature even at 100 μM novobiocin. These results were tested more directly by looking at the retention of the mutants by a novobiocin-affinity column. The wild-type protein was retained on the column and eluted at 4−6 M urea whereas the Asn^{46} mutants were also retained but eluted at somewhat lower urea concentrations. However, the Asp^{73} to Asn mutant did not show any appreciable binding to the column (data not shown). These data indicate that mutations at Asn^{46} have a modest effect on novobiocin binding whereas the Asp^{73} to Asn mutation has a profound effect.

ATP Binding and Hydrolysis. The 43 kDa N-terminal domain of GyrB contains the active site for ATPase activity and on its own shows DNA-independent ATPase activity (*27*). We have measured the ATPase activity of the wild-type and mutant proteins (Figure 5A) and found that the wild-type protein turns over ATP at rates consistent with previous data (*27*), whereas the mutant proteins show very little activity. As the gyrase ATPase reaction is specifically inhibited by novobiocin, the ATPase activity was also measured in the presence of an excess of this drug (Figure 5A). We found that, whereas the wild-type ATPase activity was virtually abolished by novobiocin, the low ATPase activity of the mutants was largely unaffected. As was

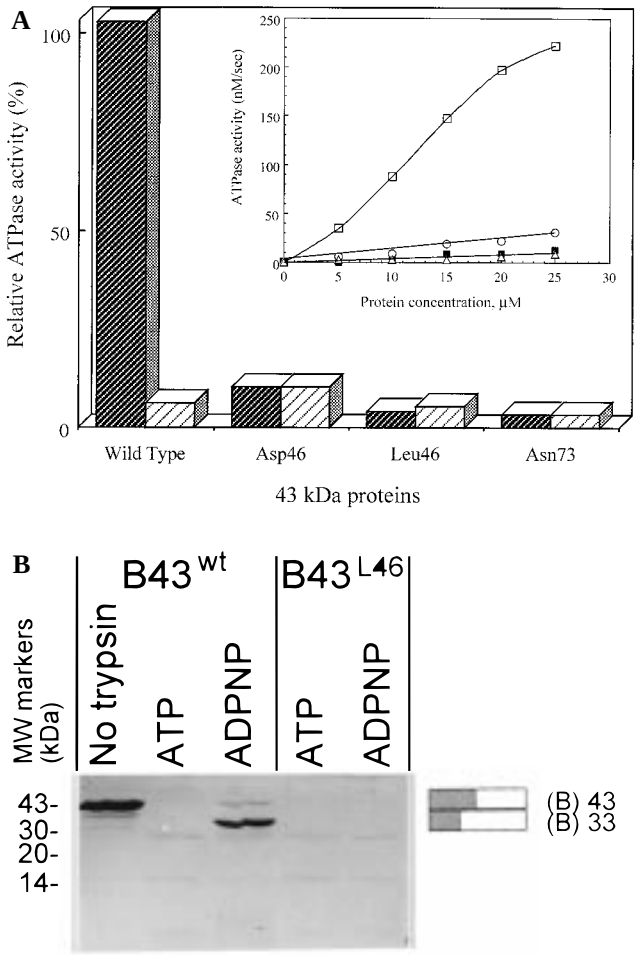

FIGURE 5: Interaction of mutant GyrB43 proteins with ATP and ADPNP. (A) ATPase activity of wild-type and mutant proteins. The bar chart shows the relative rates of ATP hydrolysis by wild-type and mutant proteins at 25 μM protein. Dark shading indicates without, and light shading with, 387 μM novobiocin. The inset shows the ATPase activities as a function of protein concentration (open squares: wild-type, open circles, Asp^{46}; filled squares, Leu^{46}; and open triangles, Asn^{73} mutants). (B) Tryptic digest of mutant proteins in the presence of 2 mM ATP or ADPNP as indicated. Reactions were as described in the legend to Figure 2A.

described earlier, the Asn^{73} protein binds novobiocin very weakly and therefore any intrinsic ATPase activity would not necessarily be affected by the drug. However, the two mutants at Asn^{46} show significant drug binding and would be expected to be inhibitable in the presence of excess drug. The low ATPase activity observed may therefore be due to a contaminating ATPase activity. Taken together, it seems that the mutant proteins have little or no ATPase activity; that is, the mutations in the coumarin-binding site have blocked the ATPase reaction. As CD spectroscopy indicates that the proteins are correctly folded, the loss of activity can be directly attributed to the point mutations.

The inability of the mutant proteins to hydrolyze ATP could be due to either impaired nucleotide binding or failure to perform the actual hydrolysis step. This issue can be addressed using limited proteolysis. It was shown previously that binding of ADPNP results in a conformational change that could be observed by proteolysis (*26*). In the case of A_2B_2, ADPNP-induced dimerization protected the 43 kDa domains, giving rise to a 33 kDa fragment after extensive proteolysis. This 33 kDa fragment was the only product of

trypsin treatment of the 43 kDa domains in the presence of the nucleotide. We confirmed the ability of coumarins to inhibit binding of ADPNP by looking at the appearance of the ADPNP-characteristic fingerprint. ADPNP and novobiocin revealed the respective characteristic proteolytic fingerprint when incubated separately with gyrase (Figure 2A). However, when novobiocin was incubated together with A_2B_2 and ADPNP, only the coumarin-associated protection could be observed (data not shown). Experiments with a GyrB mutant ($Glu42^{Ala}$) that binds ATP but is unable to promote hydrolysis (*32*) revealed that in the presence of ATP this mutant also reveals the dimerization-associated proteolytic protection (*33*). Since with wild-type enzyme ATP cannot induce the dimerization-characteristic fingerprint, proteolysis can be used to identify proteins that can still bind but not hydrolyze ATP. Wild-type and mutant 43 kDa proteins were incubated with ATP and ADPNP and treated with trypsin. As expected, the wild-type protein was locked in the dimerized form by ADPNP but not by ATP, thus protecting the 33 kDa fragment only in the presence of the ATP analogue. However, none of the mutants was able to bind ATP or ADPNP tightly enough to reveal any protection (Figure 5B; some data not shown). It is therefore likely that the inability of these mutants to hydrolyze ATP stems from their deficiency in nucleotide binding. Moreover, when ADPNP was incubated with the mutants for 1 h prior to incubation with novobiocin for another 1 h, and vice-versa, subsequent trypsin treatment revealed that ADPNP was unable to displace bound drug in the case of the asparagine 46 mutants. The Asp^{73} to Asn mutant was unable to bind either of the two molecules. This is in contrast to the wild-type protein where preincubation with ADPNP first, then with novobiocin, yielded a mixture of the ADPNP-protected 33 kDa fragment and the novobiocin-protected 13 kDa fragment, and when the order of incubation is reversed, only the novobiocin-protected 13 kDa fragment was apparent (data not shown).

Kinetic Analysis of Coumarin Binding by Surface Plasmon Resonance (SPR). Coumarin drugs exhibit high affinity for DNA gyrase with equilibrium dissociation constants (K_d) for wild-type protein previously measured by titration calorimetry in the order of $10^{-8}-10^{-9}$ M (*18*, *29*). Dissociation rate constants (k_{off}) for novobiocin of 10^{-3} s^{-1} have also been measured using rapid gel filtration (*29*), but association rate constants (k_{on}) could not be directly measured and were estimated from k_{off} and K_d data to be $>10^5$ M s^{-1}. Neither of these techniques has been able to produce accurate values for the kinetic parameters, nor was the interaction with any drug other than [^{3}H]-labeled dihydronovobiocin measured directly. To accurately measure the binding of coumarin drugs and cyclothialidine to wild-type and mutant gyrase proteins, we have applied the technique of SPR.

Wild-type and mutant proteins (GyrB43 and GyrB24) were coupled to the surface of SPR flow-cell chips, then solutions of novobiocin, chlorobiocin, coumermycin, and cyclothialidine at different concentrations were passed over the immobilized protein. The kinetic traces obtained were analyzed using global fitting to a 1:1 interaction between the drug and the protein (Figure 6). Due to the high levels of immobilized protein these experiments could be subject to mass transport limitations. To accommodate this possibility, global fitting was performed to a 1:1 model accounting

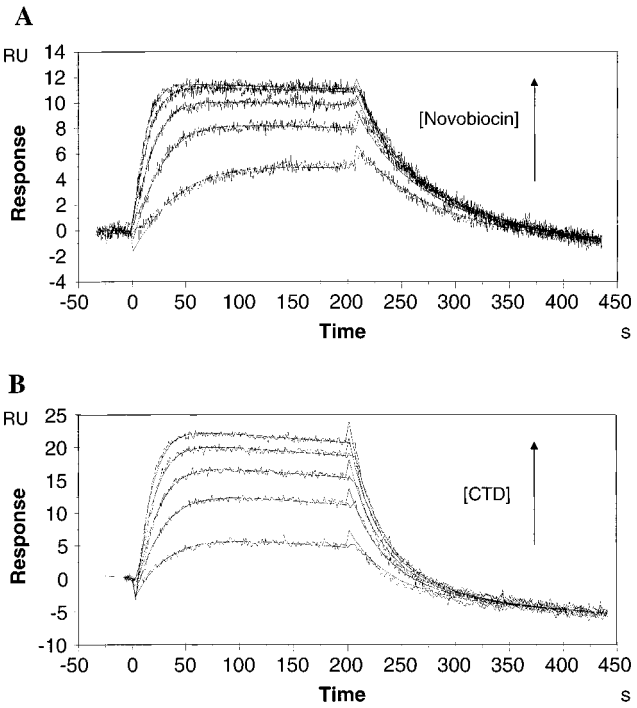

FIGURE 6: Drug binding measured by SPR. (A) Typical SPR experiment with GyrB43 and novobiocin. The sensograms were obtained with the following range of drug concentrations: 20 (lowest curve), 40, 60, 80, and 100 nM (highest curve). (B) Typical SPR experiment with $GyrB^{Asp46}$ and cyclothialidine (CTD). Drug concentrations are as described in A.

for mass transport. The kinetic parameters derived were no more than 20% different from those observed without the mass transport component. Moreover, simulation of the interaction using the BIAsimulation software (BIAcore) revealed that mass transport would have very little effect on the binding curves under the conditions used in these experiments (data not shown). Taking mass transport into account resulted in lower standard error and χ^2 values; therefore the values for the kinetic constants reported here have been determined using this model. The k_{off} values for the coumarins are in the order 10^{-3} s^{-1} while k_{on} values are all $>10^5$ M s^{-1} (Table 1). These yield values for K_d in the range $10^{-8}-10^{-9}$ M. The order of affinity for both wild-type GyrB43 and wild-type GyrB24 is chlorobiocin > cyclothialidine >novobiocin, consistent with previous data (*16*, *29*). Coumermycin binding to coupled proteins did not seem to be a simple interaction. Fitting of the association phase to a model describing a simple 1:1 interaction revealed systematic deviation of residuals, most likely caused by this drug dimerizing two protein molecules (*27*). Thus, only empirical values of k_{on} and k_{off} were determined by fitting to a simple 1:1 binding model. Residual coumermycin binding to protein following the dissociation phase could not be removed with high-salt washes (2 M KCl). A similar effect was observed with chlorobiocin but not with novobiocin or cyclothialidine. As a consequence, an initial competitive elution with novobiocin followed by a high-salt wash was required to regenerate the bound protein. This observation may be a manifestation of the greater hydrophobic character of the binding of these two drugs to the protein. Indeed, examination of the crystal structure of the GyrB24−chlorobiocin complex reveals that the 5-methyl-2-pyrrolylcarbonyl group, which is present in chlorobiocin

Table 1: Binding Parameters from SPR Data[a]

Drug →	Novobiocin			Chlorobiocin			Coumermycin			Cyclothialidine		
↓ Protein	$k_{on}/10^5$ ($M^{-1}\ s^{-1}$)	$k_{off}/10^{-3}$ (s^{-1})	$K_d/10^{-9}$ (M)	$k_{on}/10^5$ ($M^{-1}\ s^{-1}$)	$k_{off}/10^{-3}$ (s^{-1})	$K_d/10^{-9}$ (M)	$k_{on}/10^5$ ($M^{-1}\ s^{-1}$)	$k_{off}/10^{-3}$ (s^{-1})	$K_d/10^{-9}$ (M)	$k_{on}/10^5$ ($M^{-1}\ s^{-1}$)	$k_{off}/10^{-3}$ (s^{-1})	$K_d/10^{-9}$ (M)
GyrB43	9.8 ± 0.2	19 ± 0.3	19.4	19 ± 0.7	2.7 ± 0.4	1.4	8.4*	1.5*	1.8*	17.3 ± 0.4	11.3 ± 0.2	6.5
$GyrB43^{Asp46}$	5.8 ± 0.2	8.0 ± 0.3	13.8	14 ± 0.4	2.7 ± 0.3	2.0	4.1*	0.84*	2.0*	9.8 ± 0.6	29 ± 2.0	29.6
$GyrB43^{Leu46}$	2.0 ± 0.6	32 ± 0.2	160	4.0 ± 0.5	4.4 ± 0.7	10.9	5.7*	4.2*	7.4*	7.5 ± 0.4	57 ± 0.4	75.8
$GyrB43^{Asn73}$	–	–	–	6.2 ± 1.1	118 ± 7.0	191	8.5*	9*	10.6*	–	–	–
GyrB24	10.4 ± 0.4	22 ± 0.3	21.2	12 ± 0.8	3.5 ± 0.6	2.9	4.2*	1.0*	2.3*	23.4 ± 0.7	17.1 ± 0.6	7.3
$GyrB24^{Cys136}$	–	–	–	–	–	–	–	–	–	±	±	±

[a] The symbol - indicates no significant binding observed; * indicates empirical value, did not conform to a simple 1:1 model; ± weak binding observed, no reliable kinetic data could be derived.

and coumermycin but not in novobiocin, sits in a hydrophobic pocket in the protein formed by residues Val^{43}, Val^{71}, Val^{120}, Val^{167}, Ile^{78}, and Ala^{47} (*18*).

The mutation of Asn^{46} to Asp had only a modest effect on the binding of novobiocin and chlorobiocin (Table 1). However, the Asn^{46} to Leu mutation resulted in a ∼10-fold decrease in the affinity of these two drugs. This suggests that it is the carbonyl group and not the amino group of the Asn^{46} side chain that is important in interacting with these drugs. This is in agreement with the crystal structure of Lewis et al. (*17*) which indicated that hydrogen bonds between the carbonyl group of Asn^{46} and the 2′-hydroxyl and 4′-methoxyl of the novobiose moiety of the coumarin drugs. In contrast, both the Asn^{46} to Asp and Asn^{46} to Leu mutants showed reduced affinity for cyclothialidine, by ∼5- and ∼10-fold, respectively (Figure 6B and Table 1), indicating that it is the side chain amino group of Asn^{46} that interacts with cyclothialidine. The mutation of Asp^{73} to Asn completely abolishes binding to both novobiocin and cyclothialidine. In the crystal structures of the complexes of the 24 kDa protein with novobiocin and cyclothialidine, the side chain carboxylate oxygens of Asp^{73} make both direct and water-mediated hydrogen bonds to both drugs (*17*). It would appear that loss of just one of these carboxylate oxygens is sufficient to completely disrupt the binding of novobiocin and cyclothialidine. Weak binding of chlorobiocin was observed to the Asn^{73} mutant ($K_d \sim 2 \times 10^{-7}$ M); this is most likely a result of an additional hydrogen bond between the imino group of the pyrrole ring of chlorobiocin (not present in novobiocin) and one of the carboxylate oxygens of the Asp^{73} side chain (*18*).

As Arg^{136} is the only amino acid residue responsible for naturally occurring coumarin resistance in bacterial strains, an attempt was made to measure drug binding to GyrB24 bearing an Arg^{136} to Cys mutation. No binding of coumarins was detectable and only weak interaction between this mutant and cyclothialidine was detected, but the binding was too small to allow accurate determination of rate constants (Table 1). This reaffirms the important role of this residue in binding these drugs. Previous binding data from calorimetry experiments showed that an Arg^{136} to His mutation resulted in a reduction of the K_d for novobiocin of about 2 orders of magnitude (*19*). The SPR results showed very good reproducibility. In at least three runs performed at the same concentration of drug, there was no significant variation in the kinetic parameters. In addition, there was no loss of activity of the immobilized protein after successive injections.

DISCUSSION

Coumarins and cyclothialidines are excellent inhibitors of DNA gyrase in vitro, but, for a number of reasons, are not successful antibiotics. However, our knowledge of the molecular basis of the action of these compounds on gyrase is considerable, and it is hoped that such knowledge will be beneficial in the design of further antibacterial agents. In this paper we have sought to corroborate the crystal structure information of the complexes of these drugs with gyrase fragments, establish limited proteolysis as a method for probing drug-stabilized protein conformations, and determine definitive binding parameters for these compounds.

Limited proteolysis is a method that has been widely used to obtain information about domain structure in proteins and conformational changes which occur on ligand binding. In the case of DNA gyrase and the related enzyme from yeast, DNA topoisomerase II, it has been used to identify protein conformations stabilized by nucleotides and drugs (*26*, *33*−*35*). In this work we sought to establish a proteolytic signature which is characteristic of the drug-bound state and could be used to assess drug binding to wild-type and mutant proteins. We found that coumarins and cyclothialidines stabilized a conformation of GyrB or its domains (GyrB43 and GyrB24) characterized by the appearance of ∼13 and ∼10 kDa bands on SDS−PAGE. Mass spectroscopy analysis allowed precise mapping of the cleavage sites which generated these fragments. The tryptic cleavage sites are indicated on the structure of GyrB24 complexed with novobiocin and the cyclothialidine analogue GR122222X in Figure 7. Experiments with mutant proteins and with alternative proteases strongly supported the notion that the proteolytic signature resulted from a drug-stabilized protein conformation rather than direct protection of the protease cleavage site by the bound drug. As coumarins and cyclothialidines elicited the same proteolytic signature, it seems that these drugs stabilize a similar conformation of this domain of GyrB. The ability of these drugs to stabilize a conformational state of the protein that is resistant to digestion by proteases is not surprising. The 43 kDa domain of GyrB is responsible for the coupling of the free energy of ATP hydrolysis to the supercoiling reaction performed by gyrase. To achieve this the protein has to undergo a number of conformational changes, the most profound of these being the dimerization of the 43 kDa domain (*7*, *27*). Moreover, recent studies on the ATPase reaction of topoisomerase II have revealed that, in the process of nucleotide hydrolysis and product release, a number of different conformational states are likely to be achieved by the protein (*36*, *37*). It is possible that some of

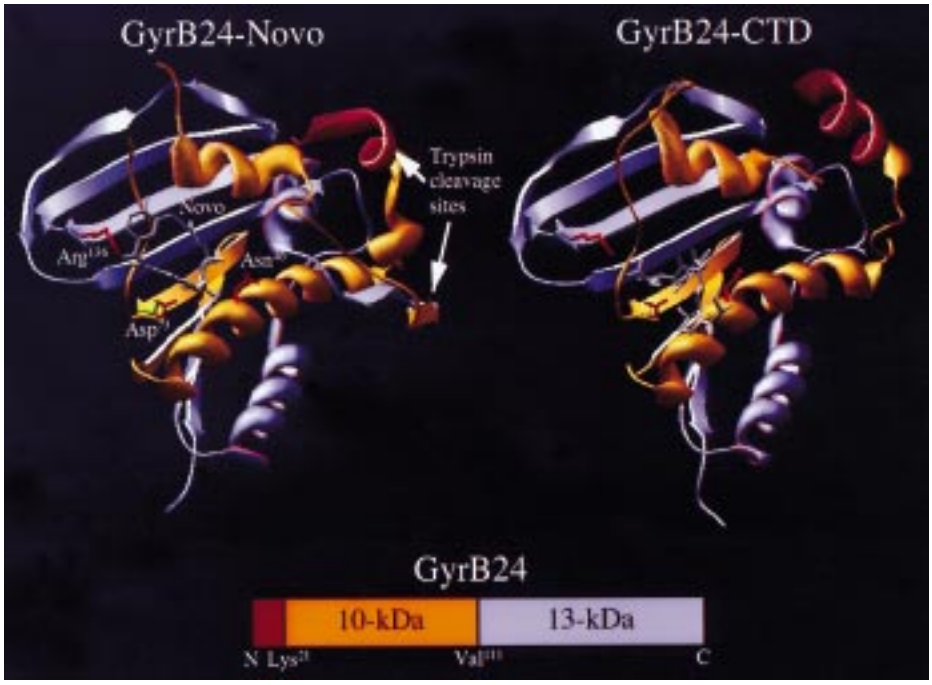

FIGURE 7: Ribbon representations of the crystal structures of GyrB24 (*17*) complexed with novobiocin (left) and GR122222X (right). Amino acid side chains discussed in this paper are shown in red, and the drug molecules are in white. Sites of trypsin digestion are marked. The bar indicates diagrammatically the locations of the sites of trypsin cleavage.

these conformational states are more susceptible to proteases than others. Binding of the drug could therefore stabilize the protein in a conformation that is resistant to proteolysis, while in the absence of the drugs, the protein would be free to achieve the conformations that are more susceptible to degradation. Indeed, this idea is further supported by the solution of the crystal structure of the GyrB24−chlorobiocin complex, where it is found that the conformation of GyrB24 in the chlorobiocin complex is different than that in the complex with ADPNP (*18*).

Examination of the crystal structures of GyrB24 complexed with novobiocin and the cyclothialidine GR122222X (*17*) suggested Asn^{46} and Asp^{73} as residues which contribute to drug binding (Figures 1B and 7). Naturally occurring coumarin-resistance mutations have not been found at either of these positions. Limited proteolysis experiments suggested that the Asp^{73} to Asn mutation in GyrB43 abolished drug binding but that mutations at Asn^{46} (to Asp and Leu) did not significantly affect drug binding. This result was supported by experiments using a novobiocin-affinity column which suggested that the Asn^{46} mutants bound the drug less tightly and that the Asp^{73} mutant showed no binding. ATPase experiments showed that none of the mutants could hydrolyze ATP, and proteolysis experiments suggested that none could bind ATP or ADPNP. As the mutant proteins were shown to be folded by CD spectroscopy, it seems that the mutations had abolished nucleotide binding. Hence such mutations would not occur spontaneously in bacteria as they would render gyrase inactive and the cell inviable. In relation to the crystal structure of the GyrB43−ADPNP complex (*7*), Asn^{46} makes a key contact to the Mg^{2+} ion which is bound to the 3 phosphates of ATP, and Asp^{73} interacts with the N^6 amino group of the adenine ring. Thus it is not unexpected that mutations at these amino acids should have such a deleterious effect on ATPase activity.

To accurately assess the binding of the coumarin and cyclothialidine drugs to the GyrB43 and GyrB24 domains, we used surface plasmon resonance. Previously, we used rapid gel filtration and titration calorimetry to measure these interactions, but we found that neither of these techniques was sufficiently sensitive to yield accurate binding parameters (*29*). SPR is a technique for measuring association and dissociation rate constants of biological macromolecules and their ligands in real time (*38*). It relies on changes in the refractive index on a metal surface to which one of the molecules of interest is covalently coupled. In this case we coupled the proteins (GyrB43 or GyrB24) and passed varying concentrations of drug over the immobilized protein. Although the change in molecular mass upon binding of the drug was as little as ~1% in some cases, reproducible data with a low signal-to-noise ratio were generated (e.g., Figure 6). This allowed accurate determinations of the binding parameters for all drugs (Table 1). Consistent with previous work (*29*), association rate constants (k_{on} values) were found to be around 10^6 M^{-1} s^{-1}, and dissociation rate constants (k_{off} values) were found to be in the 10^{-2}−10^{-3} s^{-1} range, yielding equilibrium dissociation constants (K_d values) of 10^{-8}−10^{-9} M. The order of binding affinity was found to be chlorobiocin > cyclothialidine > novobiocin. The binding data for these drugs could be fitted to a model describing a simple 1:1 complex with the protein, as would be predicted from the crystal structures. In the case of coumermycin the data were not consistent with a simple 1:1 binding model and the binding parameters quoted in Table 1 are empirical. It has previously been found that coumermycin binds the 24 and 43 kDa proteins with a stoichiometry of 1:2 (*27*, *29*), in effect coumermycin cross-links two protein monomers. Due to the potential complexities of dimerization of protein on the chip surface no attempt was made to further interpret the coumermycin data.

As expected from the proteolysis data, the Asn[46] to Asp mutation had only a modest effect on the binding parameters of the coumarin drugs but a more profound effect on the binding of cyclothialidine (Table 1). The Asn[46] to Leu mutation has a more drastic effect on the binding parameters for both coumarins and cyclothialidine, reflecting the more extreme change of the amino acid side chain. The Asp[73] to Asn mutation effectively abolishes binding of novobiocin and cyclothialidine and supports only weak binding of chlorobiocin, reflecting the contribution of the carboxylate group to hydrogen bonding to the drugs. As predicted from the crystal structures and previous work, the Arg[136] to Cys mutation abolishes coumarin binding and supports only weak binding of cyclothialidine, underlining the importance of this residue.

In summary, we have shown that limited proteolysis can be used to probe coumarin interaction with DNA gyrase, and the results support a drug-induced conformational change in the N-terminal domain of GyrB. Proteolysis and coumarin-affinity chromatography showed that point mutations at Asn[46] and Asp[73] disrupt coumarin binding, and ATPase experiments showed that proteins bearing these mutations are inactive. The loss of activity was found by proteolysis to be due to the inability of the mutant proteins to bind ATP. SPR experiments have allowed the accurate determination of kinetic parameters for the binding of wild-type and mutant proteins to several coumarin drugs and cyclothialidine.

ACKNOWLEDGMENT

We thank Dr. R. Loris for assistance with molecular graphics, Prof. N. C. Price and Dr. S. M. Kelly for carrying out the CD spectroscopy, and Dr. K. S. Lilley for performing the peptide sequencing and mass spectroscopy. We acknowledge the Wellcome Trust for funding the purchase of the BIAcore 2000 system.

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BI982320P