The Interaction of Coumarin Antibiotics with Fragments of the DNA Gyrase B Protein†

Niall A. Gormley,‡ George Orphanides,‡,§ Andrew Meyer,|| Paul M. Cullis,|| and Anthony Maxwell*,‡

Departments of Biochemistry and Chemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.

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ABSTRACT: DNA gyrase is the target of the coumarin group of antibacterial agents. The drugs are known to inhibit the ATPase activity of gyrase and bind to the 24-kDa N-terminal subdomain of the gyrase B protein. Supercoiling assays with intact DNA gyrase and ATPase assays with a 43-kDa N-terminal fragment of the B protein suggest that the drugs bind tightly, with K_d values $<10^{-7}$ M. In addition, the ATPase data suggest that 1 coumermycin molecule interacts with 2 molecules of the 43-kDa protein while the other coumarins form a 1:1 complex. This result is confirmed by cross-linking experiments. Rapid gel-filtration experiments show that the binding of ADPNP (5′-adenylyl β,γ-imidodiphosphate) and coumarins to the 43-kDa protein is mutually exclusive, consistent with a competitive mode of action for the drugs. Rapid gel-filtration binding experiments using both the 24- and 43-kDa proteins also show that the drugs bind with association rate constants of $>10^5$ $M^{-1}\cdot s^{-1}$, and dissociation rate constants of $\sim 3 \times 10^{-3}$ s^{-1} and $\sim 4 \times 10^{-3}$ s^{-1} for the 43- and 24-kDa proteins, respectively. Titration calorimetry shows that the K_d values for coumarins binding to both proteins are $\sim 10^{-8}$ M and that binding is enthalpy driven.

DNA gyrase is the bacterial type II topoisomerase which can introduce negative supercoils into DNA using the free energy of ATP hydrolysis [for reviews see Reece and Maxwell (1991b) and Wigley (1995)]. 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. All DNA topoisomerases are able to relax negatively supercoiled DNA, but only gyrase can also catalyze the introduction of negative supercoils, in a reaction coupled to ATP hydrolysis. Mechanistic studies have revealed the steps involved in the supercoiling reaction [see Maxwell and Gellert (1986) and Reece and Maxwell (1991b) for reviews]. Briefly, this process involves the wrapping of DNA around the A_2B_2 complex, 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. Resealing of the break results in the introduction of two negative supercoils. Catalytic supercoiling requires the hydrolysis of ATP, but limited supercoiling can be achieved in the presence of the nonhydrolyzable ATP analog ADPNP[1] (5′-adenylyl β,γ-imidodiphosphate) (Sugino et al., 1978). This result suggests that nucleotide binding promotes one round of supercoiling and that hydrolysis is required for the enzyme to turn over.

Both the A and B subunits of gyrase have been shown to contain distinct domains. The A protein (GyrA) 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 (Reece & Maxwell, 1989, 1991a,c). The B protein (GyrB) consists of an N-terminal domain (43 kDa) containing the ATPase activity, and a C-terminal domain (47 kDa) involved in interactions with the A protein and DNA (Brown et al., 1979; Gellert et al., 1979; Adachi et al., 1987; Ali et al., 1993). The relevance of these domains to the structure of the intact protein has been confirmed by differential scanning calorimetry (Blandamer et al., 1994). The structure of the 43-kDa N-terminal domain complexed with ADPNP has been solved to 2.5-Å resolution by X-ray crystallography (Wigley et al., 1991).

The gyrase supercoiling reaction can be inhibited by a number of compounds including the quinolone and coumarin groups of antibacterial agents [for reviews see Drlica and Coughlin (1989), Rádl (1990), Reece and Maxwell (1991b), and Maxwell (1992, 1993)]. The quinolones (e.g., nalidixic acid and ciprofloxacin) interrupt the DNA breakage and resealing reaction of gyrase, while the coumarins (e.g., novobiocin and coumermycin A_1; Figure 1) inhibit the ATPase reaction. Sequencing of mutations in *gyrB* conferring coumarin resistance has suggested that the coumarin-binding site lies in the N-terminal portion of GyrB (del Castillo et al., 1991; Contreras & Maxwell, 1992).

The nature of the inhibition of the ATPase reaction of DNA gyrase by coumarins has been the subject of some debate. Sugino et al. (1978) and Sugino and Cozzarelli (1980) found that novobiocin behaved as a competitive inhibitor in steady-state kinetic studies of the gyrase supercoiling and ATPase reactions, but these authors pointed out that the drug may block ATP access without sharing its

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* Corresponding author.

‡ Department of Biochemistry.

§ Present address: Howard Hughes Medical Institute Research Laboratories, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, Department of Biochemistry, Piscataway, NJ 08854.

|| Department of Chemistry.

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[1] Abbreviations: ADPNP, 5′-adenylyl β,γ-imidodiphosphate; DTT, dithiothreitol; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein.

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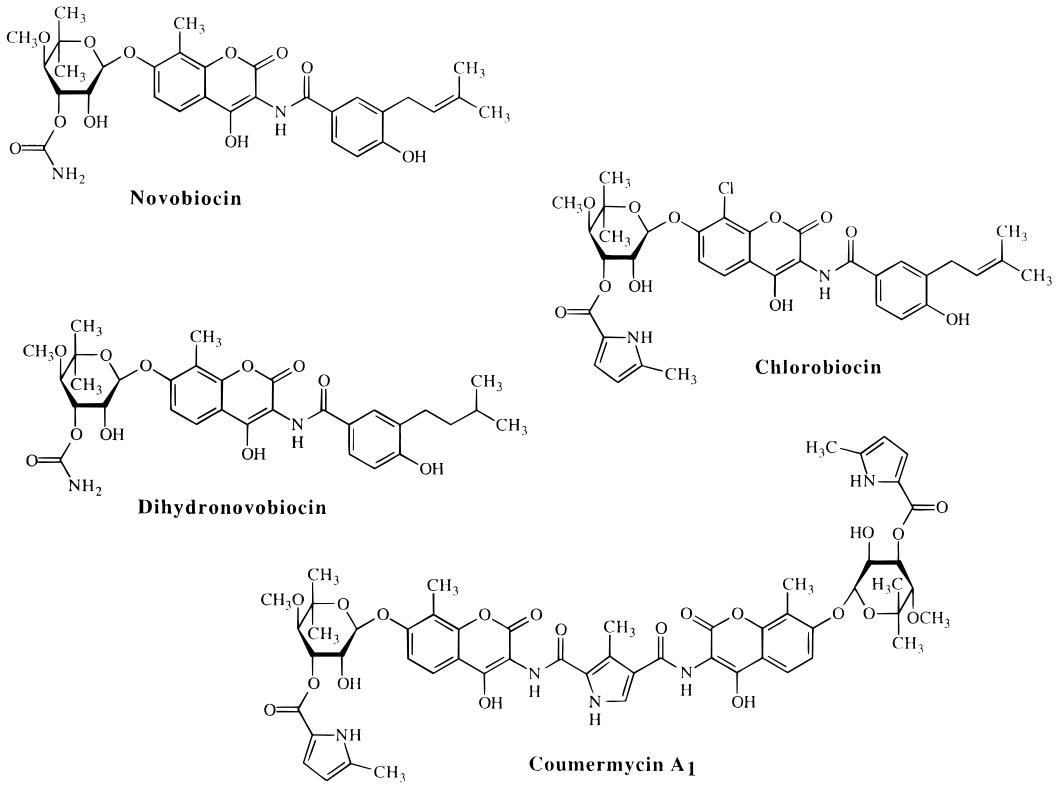

FIGURE 1: The structures of the coumarin drugs used in this work.

binding site, possibly by stabilizing a conformation incompatible with ATP binding. Mizuuchi et al. (1978) found that novobiocin prevents the binding of ATP to GyrB, consistent with the idea that the two ligands share the same binding site. Further steady-state kinetic studies of the gyrase ATPase reaction (Staudenbauer & Orr, 1981) also supported novobiocin being a competitive inhibitor. Later work on the steady-state kinetics of ATP hydrolysis and nucleotide binding to gyrase (Maxwell et al., 1986; Tamura et al., 1992) suggested that the interaction of nucleotides with gyrase is cooperative and that the enzyme does not conform to Michaelis−Menten kinetics. These results cast some doubt on the earlier kinetic analyses and the observations of competitive inhibition by novobiocin. Work on the ATPase reaction of the 43-kDa N-terminal fragment of GyrB has shown that this fragment displays distinctly non-Michaelis−Menten kinetics and that the effect of novobiocin is apparently inconsistent with simple competitive action (Ali et al., 1993). Recent studies on the binding of ADPNP to the 43-kDa GyrB fragment have shown that the nucleotide stabilizes the protein dimer and that protein dimers can contain 1 or 2 molecules of bound ADPNP (Ali et al., 1995). Coumarin drugs were found to inhibit the binding of ADPNP to the 43-kDa fragment, with novobiocin and coumermycin showing apparent stoichiometries of 1:1 and 0.5:1, respectively (Ali et al., 1995).

What emerges from these studies is that the kinetics of ATP hydrolysis by DNA gyrase is not straightforward and that the effects of coumarin drugs are not easy to characterize from kinetic studies. Recently, a 24-kDa N-terminal fragment of GyrB (residues 2−220) has been cloned and expressed and shown to contain the coumarin-binding site (Gilbert & Maxwell, 1994). The complex of this fragment with the coumarin drug novobiocin has been crystallized (Lewis et al., 1994) and the structure solved to 2.7-Å resolution (Lewis et al., 1996). The crystal structure clearly shows that the binding sites for ATP and novobiocin partially overlap, i.e., these ligands are competitive, and coumarin drugs are likely to act by preventing access of ATP to its binding site.

In previous work it has been suggested that the 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 (Sugino et al., 1978; Sugino & Cozzarelli, 1980; Staudenbauer & Orr, 1981) and the tight binding of GyrB and the 43- and 24-kDa fragments to coumarin affinity columns (Staudenbauer & Orr, 1981; Gilbert & Maxwell, 1994; Ali et al., 1995). The stability of the protein−coumarin complex has been suggested as being due to a slow dissociation rate (Maxwell, 1993); however, direct measurement of binding parameters has not been reported. In this paper we have examined the binding of novobiocin and other coumarin drugs to the 24- and 43-kDa fragments of GyrB using the techniques of rapid gel filtration (spin columns) and titration calorimetry.

EXPERIMENTAL PROCEDURES

Drugs. Novobiocin was puchased from Sigma; chlorobiocin and coumermycin were generous gifts from Rhône-Poulenc Rorer and F. Hoffman-La Roche, respectively. Dihydronovobiocin was synthesized by mild catalytic hydrogenation of novobiocin following the method of Hinman et al. (1957). [^{3}H]Dihydronovobiocin (2.07 TBq/mmol) was purchased from Amersham, and [^{32}P]ADPNP (1.85 TBq/mmol) was purchased from ICN.

Enzymes and Assays. The DNA gyrase A and B proteins and the 43- and 24-kDa N-terminal fragments of GyrB were

prepared as described previously (Hallett et al., 1990; Ali et al., 1993; Gilbert & Maxwell, 1994). Protein cross-linking using dimethyl suberimidate was performed as previously described (Ali et al., 1995). DNA supercoiling assays were carried out as described by Reece and Maxwell (1989), and ATPase assays were performed using the pyruvate kinase/lactate dehydrogenase linked assay described by Ali et al. (1993).

Spin Columns. Nucleotide- and drug-binding assays were performed using Sephadex G50 Nick Spin columns (Pharmacia) based on the method described by Tamura et al. (1992). Nucleotide binding was performed as described by Ali et al. (1995). For drug-binding assays, columns were pre-equilibrated with 1 mM novobiocin and 0.5 mg/mL BSA in "Enzyme Buffer" (50 mM Tris·HCl (pH 7.5), 100 mM KCl, 10% (v/v) glycerol, 1 mM EDTA, 2 mM DTT). Before use, columns were drained and spun at 500*g* for 4 min in a bench-top centrifuge. The top surface of the gel bed was moistened with 100 μL of the same buffer immediately prior to the addition of 100 μL of sample. The columns were spun again and the eluates collected. The amount of protein-bound drug was estimated by scintillation counting. For association binding studies, a 5-fold molar excess of [^{3}H]-dihydronovobiocin was added to a solution of either 24- or 43-kDa protein in Enzyme Buffer on ice; 100 μL aliquots were removed at intervals and assayed for drug binding. For drug dissociation studies, protein was incubated with a 5-fold molar excess of [^{3}H]dihydronovobiocin in Enzyme Buffer for 1 h at 25 °C until equilibrium binding was achieved. Samples were then chilled on ice, and a 50-fold excess of unlabeled novobiocin was added; 100 μL aliquots were removed at intervals and assayed for [^{3}H]dihydronovobiocin remaining bound to protein. For studies on the simultaneous binding of [^{3}H]dihydronovobiocin and [^{32}P]ADPNP, 43-kDa protein in Enzyme Buffer plus 5 mM $MgCl_2$ was incubated with a 5-fold molar excess of [^{3}H]dihydronovobiocin for 1 h at 25 °C until equilibrium was achieved. A 30-fold excess of [^{32}P]ADPNP-Mg^{2+} was then added and the incubation continued overnight. Alternatively, 43-kDa protein was incubated overnight at 25 °C with a 30-fold excess of [^{32}P]-ADPNP-Mg^{2+}. A 5-fold molar excess over protein of [^{3}H]-dihydronovobiocin was then added and the mixture incubated for a further 1 h. Both mixtures were then chilled on ice, and 100 μL aliquots were removed and assayed for both [^{32}P]-ADPNP and [^{3}H]dihydronovobiocin binding to the protein using spin columns equilibrated in Enzyme Buffer plus 5 mM $MgCl_2$, 1 mM novobiocin, and 0.5 mg/mL BSA. For competitive binding studies, protein (10 μM) was incubated in Enzyme Buffer with a 5-fold excess of unlabeled drug for 1 h at 25 °C. This was chilled on ice, and then [^{3}H]-dihydronovobiocin was added at a range of concentrations (20−200 μM). At intervals, 100 μL aliquots were removed and assayed for [^{3}H]dihydronovobiocin binding to the protein.

Titration Calorimetry. Calorimetric data were obtained using an Omega titration microcalorimeter (MicroCal Inc., USA) equipped with a Keithley 181 nanovolt preamplifier. The design and operation of this calorimeter has been described in detail by Wiseman et al. (1989). All titration experiments were carried out at 25 °C, and the instrument was calibrated by standard electrical pulses. Ligand and protein samples (in 50 mM Tris·HCl (pH 7.5), 100 mM KCl, 1 mM EDTA) were degassed just prior to the experiment. In individual titrations, up to 50 aliquots (4−11 μL each) of the ligand solution were automatically injected from a microsyringe at 3−4 min intervals into the sample cell (1.4115 mL) containing the solution of the protein (10−65 μM) dissolved in the same buffer. Efficient mixing of the solutions is achieved by a paddle on the end of the syringe rotated at a constant speed (400 rpm).

Analysis of the thermodynamic theory of the titration calorimetry method has shown that accurate binding constants can only be obtained when the experimental design allows for the value of K_aM_{tot} to fall in the range 1−1000 [where K_a is the association constant for the binding process and M_{tot} is the total concentration of the protein in the sample cell; Wiseman et al. (1989)]. Association constants for the coumarins used in this present study were expected to be in the range of 10^7-10^9 M^{-1} (Maxwell, 1993), which fixes the protein concentrations at <30 μM. The consequence of the restriction to relatively low protein concentrations is that the experimental heat signals associated with binding are small relative to the heats of mixing and dilution. Correction for the latter is achieved by performing a series of control injections of the ligand at the same concentration into buffer in the sample cell and using these data to subtract from the ligand-into-protein titration data. To improve the signal-to-noise in these experiments, the control experimental data set was averaged to give a flat baseline for subtraction. The resulting integrated, baseline-corrected data were fitted to theoretical titration curves using the Origin software supplied by MicoCal Inc. From the fitted curve, the molar binding stoichiometry (n), the association constant (K_a), and the enthalpy of binding ($\Delta H°$) were obtained. Poor data sets fail to converge to a satisfactory minimum, providing some additional confidence in the data sets that satisfactorily deconvolute.

RESULTS AND DISCUSSION

Inhibition of the Gyrase Supercoiling and ATPase Reactions by Coumarin Drugs. It is well established that the intracellular target of the coumarin group of antibiotics is DNA gyrase (Gellert et al., 1976) and that *in vitro* these compounds inhibit the supercoiling and ATPase reactions of gyrase (Mizuuchi et al., 1978). For purposes of comparison with the binding studies described below, we have examined the effects of a range of coumarin drugs on the supercoiling reaction of gyrase (data not shown) and the ATPase reaction of the 43-kDa N-terminal domain of GyrB (Figure 2). As regards supercoiling, we found that with 100 nM gyrase all the drugs shown in Figure 1, except coumermycin, gave IC_{50} values (the concentration of drug required to inhibit gyrase-catalyzed supercoiling by 50%) which were approximately the same (∼80 nM); coumermycin yielded an IC_{50} of about half this value. These results are consistent with those obtained previously (Contreras & Maxwell, 1992). As regards the ATPase reaction, there is an inverse relationship between the rate of hydrolysis and the drug concentration for each drug (Figure 2). For novobiocin, dihydronovobiocin, and chlorobiocin, the extrapolated maximal inhibition occurs at ∼20 μM (corresponding to the protein concentration), whereas with coumermycin maximal inhibition occurs at ∼12 μM (i.e., roughly half the protein concentration), consistent with results obtained previously (Ali et al., 1993). These results suggest that these drugs have a high affinity

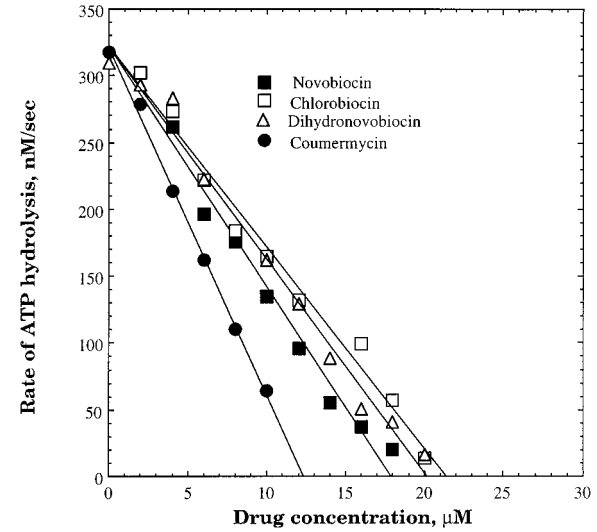

FIGURE 2: The ATPase activity of the 43-kDa protein in the presence of coumarin drugs. Rates are initial velocities; the substrate (ATP) concentration was 2 mM, and the protein concentration was 20 μM.

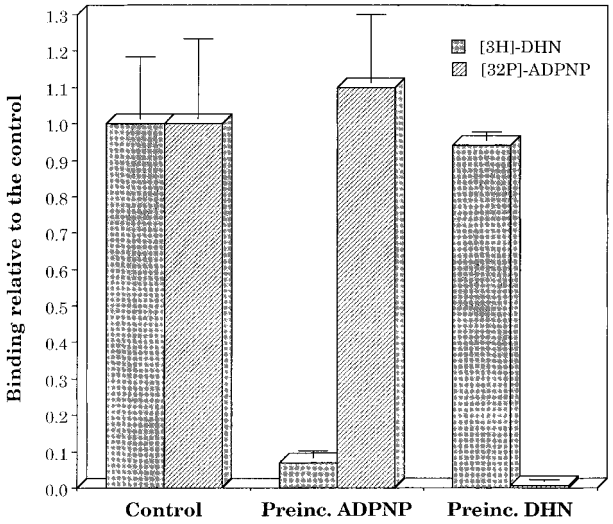

FIGURE 3: The binding of dihydronovobiocin and ADPNP to the 43-kDa protein. The protein (10 μM) was incubated with [^{3}H]-dihydronovobiocin for 1 h and [^{32}P]ADPNP overnight. Amounts of bound ligand were determined by spin columns; the results are averages from 4 determinations.

for gyrase and the 43-kDa fragment and that the K_i values for inhibition by coumarins are likely to be below the enzyme concentrations used here. This situation arises because the enzyme concentrations required to measure supercoiling and ATPase activites are above the K_i values and therefore the amounts of the drugs required to inhibit supercoiling and ATPase are relatively insensitive to differences in K_i values between different drugs. The data do suggest, however, that the stoichiometry of binding of each drug is 1 molecule per 43-kDa monomer, with the exception of coumermycin which binds two 43-kDa proteins, consistent with previous data (Ali et al., 1993, 1995).

To address the question as to whether coumarins bind to gyrase competitively with ATP, we have examined the binding of [^{3}H]dihydronovobiocin and [^{32}P]ADPNP to the 43-kDa protein (Figure 3). Using each ligand on its own, we have determined the time taken for binding to the 43-kDa protein to reach equilibrium. In the case of dihydronovobiocin, using 10 μM 43-kDa protein and 50 μM drug, equilibrium is established within a few minutes (see Figure 5). In the case of ADPNP, using 10 μM 43-kDa protein and 300 μM nucleotide, equilibrium takes at least an hour to be established (Ali et al., 1995). Note that under these conditions 2 molecules of ADPNP will be bound per 43-kDa dimer, whereas at lower nucleotide concentrations a significant amount of the species with 1 ADPNP per 43-kDa protein dimer will be present (Ali et al., 1995). When the 43-kDa protein was preincubated with ADPNP overnight and dihydronovobiocin subsequently added and the incubation continued for 1 h, very little dihydronovobiocin was bound. When the 43-kDa protein was preincubated with dihydronovobiocin for 1 h and ADPNP subsequently added and the incubation continued overnight, little or no ADPNP was bound (Figure 3). These results indicate that, within the limits of experimental error, ADPNP and dihydronovobiocin do not bind to the protein at the same time, indicating overlapping (if not identical) binding sites, supporting the idea that the coumarins are competitive with ATP. This result is consistent with recent X-ray crystallography data on the structure of the complex between the 24-kDa protein and novobiocin (Lewis et al., 1996).

Cross-Linking of Protein−Drug Complexes. The supercoiling and ATPase results discussed above suggest that coumermycin can bind 2 molecules of gyrase or the 43-kDa fragment whereas the other coumarins bind the proteins in a 1:1 complex. This result is consistent with those obtained previously from molecular-weight studies of complexes between coumarins and the 43-kDa fragment (Ali et al., 1993) and studies of the inhibition of ADPNP binding to the 43-kDa protein by coumarins (Ali et al., 1995). We have now used protein cross-linking to directly test this proposition.

Dimethyl suberimidate is a widely used protein cross-linking agent (Davies & Stark, 1970) which can react with primary amino groups (e.g., lysines) in proteins. Incubation of the 43-kDa protein in the presence of dimethyl suberimidate alone generates no cross-linked products, but in the presence of ADPNP a cross-linked species is generated with a molecular mass consistent with a 43-kDa protein dimer (Ali et al., 1995). When such a cross-linking experiment is performed in the presence of novobiocin, no cross-linked products are generated (data not shown). However, at a range of coumermycin concentrations, cross-linked species are seen, but only at concentrations less than the protein concentration (Figure 4). Two cross-linked bands are apparent with molecular masses of ~94 and ~108 kDa. The smaller cross-linked product is likely to be a dimer, but the larger product has an electrophoretic mobility that falls between that of a dimer and a trimer; hence its oligomeric state is unclear. However, it may represent a dimer species cross-linked at an alternative location which yields a conformation of lower electrophoretic mobility. Maximal cross-linking occurs at a coumermycin concentration equal to half that of the protein concentration. This corroborates the results from the coumermycin inhibition of the 43-kDa protein ATPase activity and the gyrase supercoiling activity; i.e., coumermycin binds with a stoichiometry of 1 drug molecule to 2 protein molecules. Cross-linking decreases at coumermycin concentrations greater than half the protein concentration and disappears at concentrations greater than

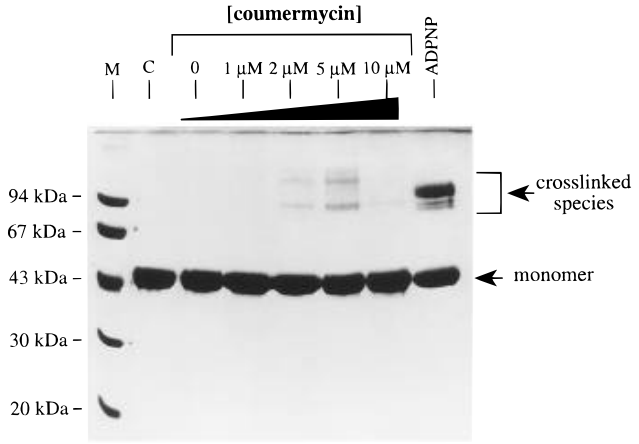

FIGURE 4: Cross-linking of the 43-kDa protein in the presence of coumermycin. The 43-kDa protein (10 μM) was incubated with dimethyl suberimidate in the presence of a range of concentrations of coumermycin, as indicated. Reactions were incubated for 8 h and then analyzed by SDS−polyacrylamide electrophoresis. A sample of protein cross-linked in the presence of ADPNP (1 mM) is also shown.

the protein concentration. Presumably, at higher concentrations of coumermycin, the formation of complexes containing a single molecule each of protein and drug is favored. It appears that the dimer species formed with coumermycin migrate with mobilities distinct from the ADPNP-induced dimers, suggesting that different residues are cross-linked; i.e., it is likely that the protein dimers induced by coumermycin and ADPNP are of different conformations. Evidence from gel-filtration and equilibrium ultracentifugation studies (Ali et al., 1993) would support this conclusion. This would also explain the different efficiencies of cross-linking seen in the presence of ADPNP and coumermycin as presumably different lysines would be involved in the cross-linking of the two dimers. A cross-linking experiment has also been carried out with the 24-kDa protein, which is known to bind coumarins but not ADPNP (Gilbert & Maxwell, 1994). In this case, high molecular weight products are seen at a protein:drug ratio of 2:1 (C. V. Smith, personal communication). This suggests that coumermycin can also bind to 2 molecules of the 24-kDa protein. This suggestion is supported by the titration calorimetry experiments described below.

Coumarin Binding Determined by Spin Columns. We have used the technique of rapid-gel filtration to measure binding of dihydronovobiocin to both the 43- and 24-kDa N-terminal fragments of GyrB. Figure 5 shows that the association of drug with the protein is relatively rapid; i.e., this technique can only yield estimates of the lower limits of the association rate constants for dihydronovobiocin binding to the 43- and 24-kDa proteins, viz., $>10^5$ M^{-1}·s^{-1}. Dissociation rates were measurable by this technique with rate constants of 1.7 ($\pm$0.2) $\times$ 10^{-3} s^{-1} and 3.1 ($\pm$0.2) $\times$ 10^{-3} s^{-1} for dihydronovobiocin dissociation from the 43-kDa and the 24-kDa protein, respectively (Figure 5). Since only dihydronovobiocin carries a radiolabel, direct measurement of rate constants for the other coumarin drugs is not feasible by this method. However, information about the kinetics of binding of these drugs can be obtained indirectly from measurements of their ability to compete with dihydronovobiocin for binding to these proteins. This was achieved by first allowing protein (10 μM) to reach equilibrium with the unlabeled drug (50 μM), then adding [^{3}H]-dihydronovobiocin, and measuring the amount of dihydronovobiocin that binds with time. This experiment was carried out at four [^{3}H]dihydronovobiocin concentrations and with four unlabeled coumarin drugs (Figure 6; data shown for

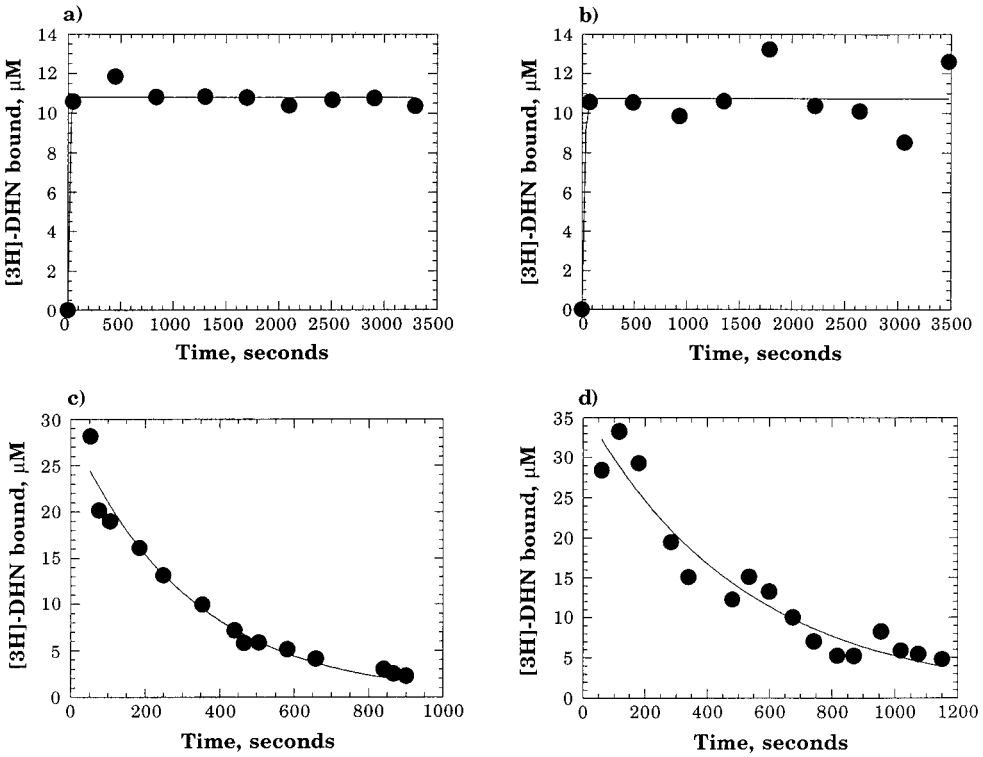

FIGURE 5: The binding of dihydronovobiocin to the 24- and 43-kDa proteins measured by spin columns. Panels are as follows: (a) association of dihydronovobiocin (50 μM) with the 24-kDa protein (10 μM); (b) association of dihydronovobiocin (50 μM) with the 43-kDa protein (10 μM); (c) dissociation from the 24-kDa protein (30 μM complex); (d) dissociation from the 43-kDa protein (30 μM complex).

coumermycin only). The kinetics of these competition experiments can be described as follows:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA$$

$$E + B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EB$$

where $K_A = k_{-1}/k_1$, and $K_B = k_{-2}/k_2$. E, A, and B represent the protein, the unlabeled drug, and [^{3}H]dihydronovobiocin, respectively, and EA and EB represent the drug−protein complexes.

Mathematical analysis of this scheme, based on the work of Jackson et al. (1987), shows that the data in Figure 6 can be fitted to the following single exponential function:

$$[EB] = [EB_\infty](1 - e^{-k't}) \quad (1)$$

where:

$$k' = k_{-1}\left\{\frac{1}{1 + (k_1[A]/k_2[B])}\right\} + k_{-2}\left\{\frac{1}{1 + (k_2[B]/k_1[A])}\right\} \quad (2)$$

and:

$$[EB_\infty] = [E_0]\left\{\frac{1}{1 + (K_B[A]/K_A[B])}\right\} \quad (3)$$

$[EB_\infty]$ corresponds to the equilibrium concentration of the dihydronovobiocin−protein complex and $[E_0]$ to the total concentration of protein. The above derivation assumes that [E] (the concentration of free enzyme) is negligible, which is reasonable given that the experiments are carried out with drug in excess of protein and the affinity of the proteins for the drugs is high. Equation 2 predicts that a plot of k' (the observed first-order rate constant) against [B] (the concentration of [^{3}H]dihydronovobiocin) should yield a value of k_{-1} at infinite [B], and a value for k_{-2} as [B] approximates zero. However, if k_{-1} equals k_{-2}, then the equation predicts that k' will be independent of [B] and a plot of k' against dihydronovobiocin concentration will give a straight line with a slope of zero. Figure 7 shows a plot of k' versus [B] for coumermycin with both the 43- and 24-kDa proteins. In addition to the experimentally-determined values of k', theoretical curves have been plotted using the values for the off rates of [^{3}H]dihydronovobiocin determined from Figure 5 but varying the values of k_{-1} to be twice and half that of k_{-2}, i.e., 2-fold differences in the off rates of the labeled and unlabeled drugs. It can be seen from Figure 7 that for both proteins the difference between k_{-1} and k_{-2} is less than 2-fold, i.e., k' is apparently independent of [B]. Similar data were obtained for the other drugs (not shown). Thus, the values of k' obtained at the four different concentrations of [B] (the concentration of [^{3}H]dihydronovobiocin) represent four determinations of k_{-1} (the off rate of the unlabeled drugs), which approximates to k_{-2} (the off rate of [^{3}H]-dihydronovobiocin). In Table 1 these values have simply been averaged. It can be seen from Table 1 that the dissociation rates for all of the drugs are faster for the 24-kDa protein than for the 43-kDa protein, a difference also reflected in the values derived from Figure 5, which are very similar to those in Table 1. This suggests that the complexes between coumarins and the 24-kDa protein are less stable than those with the 43-kDa protein.

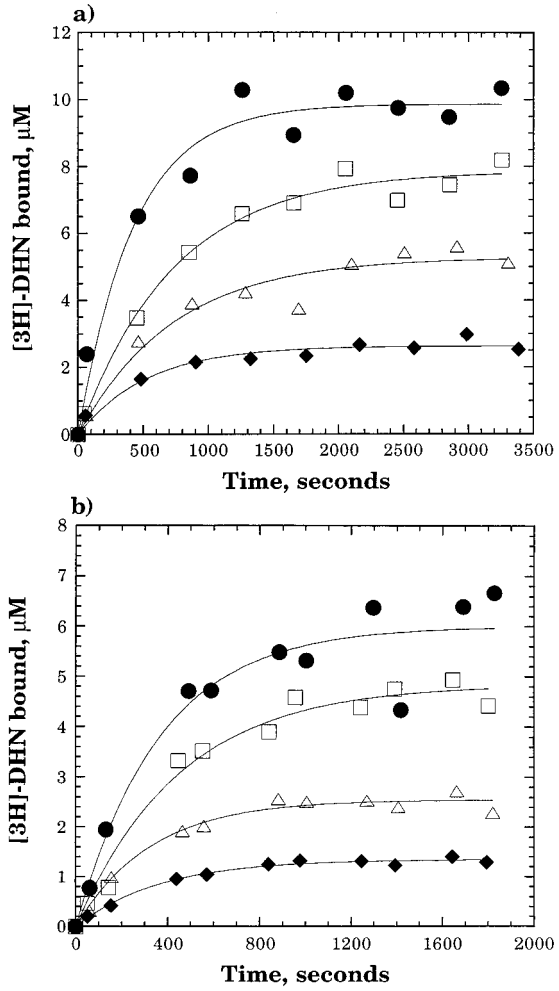

FIGURE 6: The binding of coumarins to the 24- and 43-kDa proteins measured by spin columns. Unlabeled coumermycin was incubated with the 43- (panel a) or the 24- (panel b) kDa proteins for 1 h before the addition of [^{3}H]dihydronovobiocin at the following concentrations: 200 μM (●), 100 μM (□), 50 μM (△), 20 μM (♦). Samples (100 μL) were withdrawn and applied to spin columns.

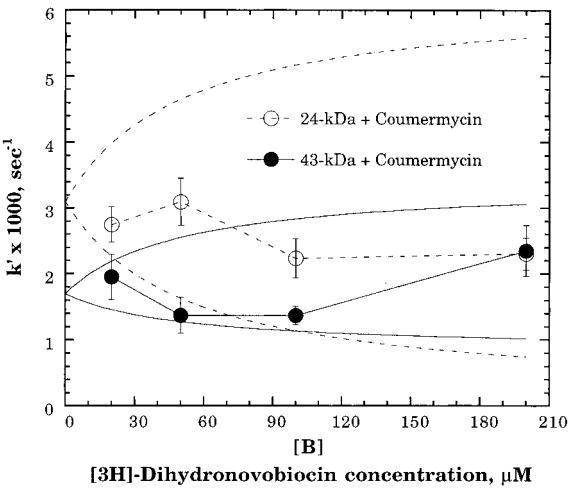

FIGURE 7: Plot of k' (determined from spin-column experiments) versus the concentration of dihydronovobiocin for the 24- and 43-kDa proteins. Theoretical curves based on $k_{-1} = 2k_{-2}$ (upper dashed line for 24-kDa protein + coumermycin, upper solid line for 43-kDa protein + coumermycin) and $k_{-1} = 0.5k_{-2}$ (lower dashed line for 24-kDa protein + coumermycin, lower solid line for 43-kDa protein + coumermycin) are shown.

Table 1: Binding Data Obtained from Spin-Column Experiments

drug	$k' \times 10^3$ (s^{-1})	K_B/K_A
43-kDa Protein		
dihydronovobiocin	3.2 (±0.5)	1.0 ± 0.2
novobiocin	3.4 (±0.7)	1.1 ± 0.1
coumermycin	1.8 (±0.3)	1.6 ± 0.1
chlorobiocin	2.6 (±0.5)	6.5 ± 0.3
24-kDa Protein		
dihydronovobiocin	4.4 (±0.4)	1.2 ± 0.1
novobiocin	4.1 (±0.6)	1.4 ± 0.1
coumermycin	3.5 (±0.3)	2.6 ± 0.2
chlorobiocin	5.3 (±1.2)	9.2 ± 0.4

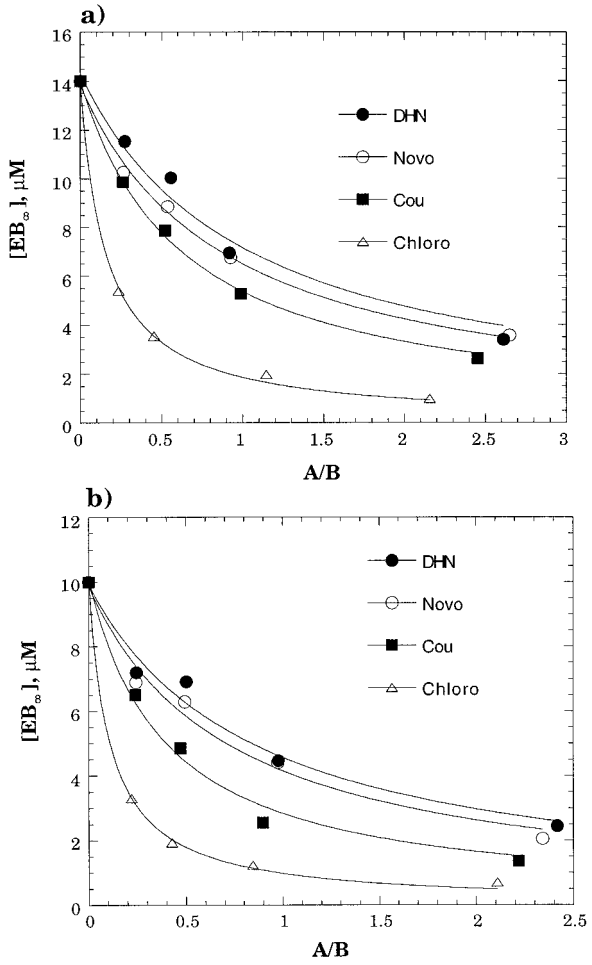

FIGURE 8: Plot of [EB_∞] versus [A]/[B] for the 43- (panel a) and 24- (panel b) kDa proteins. [A] is the concentration of free unlabeled coumarin drug, [B] is the free concentration of [^{3}H]dihydronovobiocin, and [EB_∞] is the extrapolated concentration of enzyme−[^{3}H]dihydronovobiocin complex. See text for details.

Nonlinear regression analysis of a secondary plot of [EB_∞] against [A]/[B] gives a value for the ratio of the equilibrium dissociation binding constants of each drug relative to dihydronovobiocin (K_B/K_A; Figure 8). (Here we have determined [B] from the measured value of [EB], and we have determined [A] assuming that the concentration of free protein, [E], is negligible.) As expected, when the initial equilibrium complex is between protein and unlabeled dihydronovobiocin, this ratio is, within error, equal to 1 (Table 1). The ratio data (Table 1) indicate that the drugs may be arranged in order of affinity for the proteins, with chlorobiocin binding the strongest, followed by coumermycin, then novobiocin, and then dihydronovobiocin. The order

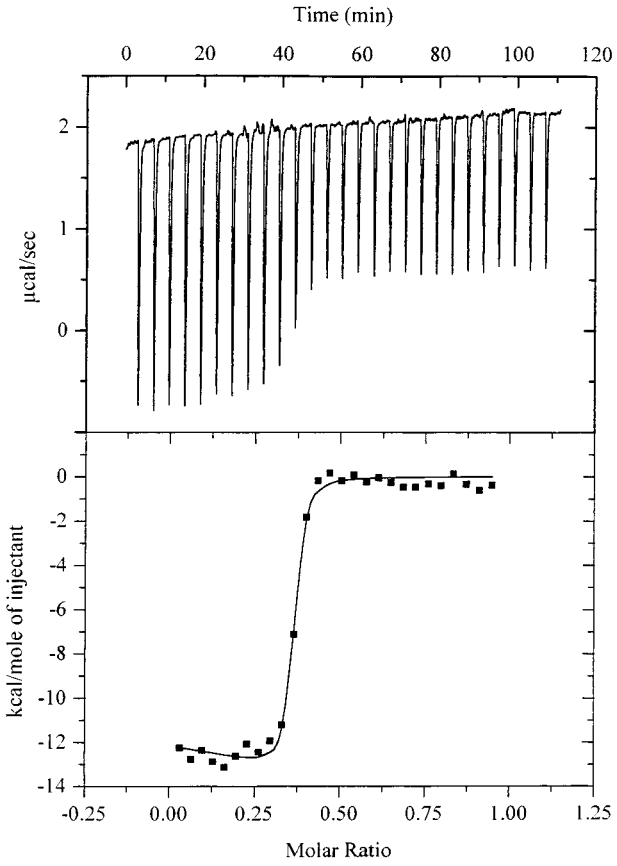

FIGURE 9: Binding of coumermycin to the 43-kDa protein as measured by titration calorimetry. Aliquots (11 μL) of coumermycin (256 μM) were titrated into the 43-kDa protein (1.4 mL at 62.8 μM) in 50 mM Tris·HCl (pH 7.5), 100 mM KCl, and 1 mM EDTA; the raw data are shown in the upper panel, and the integrated, baseline-corrected data are shown in the lower panel. The solid line represents the best fit theoretical curve using the Origin software (MicroCal Ltd).

is the same for both proteins. Furthermore, since the dissociation rate constants are approximately equal, then these values represent ratios of the association rate constants of the drugs.

Coumarin Binding Determined by Titration Calorimetry. Titration methods can be used to measure binding constants directly when there is not an appropriate signal associated with the binding process. For example, the binding of coumarin drugs to fragments of the DNA gyrase B protein does not give rise to a significant change in fluorescence (unpublished data). The recent development of high-sensitivity titration−microcalorimetric techniques has provided a widely-applicable alternative for convenient measurement of binding constants, exploiting the heat signal that is a nearly universal property of binding interactions. Titration calorimetry has the added advantage that it allows the simultaneous determination of all of the binding parameters (K, $\Delta H°$, $\Delta S°$, and n) in a single experiment.

Both the operation of the titration calorimeter and the appropriate thermodynamic theory (and supporting software) have been previously described (Wiseman et al., 1989). It is crucial to appreciate that the extraction of a binding constant requires the fitting of theoretical curves to experimental titration data. For a very tight-binding ligand, effectively all of the ligand becomes bound to the enzyme with each successive addition, and this pattern continues until all of

Table 2: Binding Data from Titration Calorimetry

ligand	[ligand] (μM)	[protein] (μM)	n	$\Delta H°$ (kcal·mol^{-1})	$\Delta S°$ (cal·mol^{-1}·K^{-1})	$K_d/10^{-8}$ (M)
			43-kDa Protein Data			
novobiocin	300	20	0.7	−13.0	6.3	0.7
	300	13.9	1.2	−13.5	8.7	1.0
	302	30.7	0.9	−12.8	8.0	2.3
dihydronovobiocin	300	25.9	1.5	−8.9	−5.3	2.0
	311	40.3	1.3	−7.6	−9.9	1.8
	281	30.7	1.1	−10.0	−1.8	1.8
coumermycin	300	11.4	1.3	−8.2	−7.3	2.4
	256	62.8	0.4	−12.2	7.1	4.0
chlorobiocin	170	14.6	1.6	−9.0	−4.7	2.3
	300	41.6	0.8	−9.5	−3.5	1.8
	242	26	1.1	−6.3	−14.1	2.0
			24-kDa Protein Data			
novobiocin	328	11	0.9	−11.5	4.4	3.3
	300	20	0.7	−13.5	10.6	2.6
dihydronovobiocin	337	42.5	0.9	−7.3	−9.3	4.0
coumermycin	300	15.3	0.3	−9.8	−0.8	4.3
	312	41.7	0.4	−9.6	−1.2	5.0
chlorobiocin	289	38.8	0.5	−8.4	−7.5	1.6
	242	41.1	0.6	−5.8	−14.9	3.0
	331	31.2	0.8	−8.2	−7.3	2.4

the sites are occupied. Therefore, the shape of the binding isotherm is a rectangular curve of height $\Delta H°$, and its appearance is rather insensitive to small changes in the binding constant. For very weak binding, little or none of the added ligand becomes associated with the protein, and the binding isotherm is practically a horizontal line yielding no useful binding information. In between these two extremes, moderately-tight binding interaction leads to the classic sigmoidal titration curve which is extremely sensitive to changes in binding constant and which can be deconvoluted to determine this value. The titration calorimetry experimental conditions can be adjusted to some extent to ensure that one is in the moderate-binding region. However, this adjustment is limited at the weak-binding edge by the availability of protein and its solubility, and on the tight-binding edge (where dilute protein solutions are necessary) by the limits of the sensitivity of the calorimeter. This consideration means that there is effectively a range of binding constants from roughly 10^{-3}−10^{-8} M that are accessible by titration calorimetry. Measurement of the tighter-binding constants is only possible if the enthalpy of binding is large (>10 kcal·mol^{-1}).

The binding of a range of coumarin drugs to both the 43- and 24-kDa fragments of the gyrase B protein has been determined by standard titration calorimetric techniques. A typical set of data for coumermycin binding to the 43-kDa GyrB fragment is shown in Figure 9, with the upper plot showing the raw calorimetric data for a ligand-into-protein titration and the lower plot showing the integrated data corrected for the heat of dilution of the ligand into buffer. The sigmoidal binding isotherm is fitted to the theoretical curve varying K_a (the association constant), $\Delta H°$, and n (the molar binding stoichiometry) to achieve the best fit. It is important to note that since the binding constants are all rather tight ($\sim 10^{-8}$ M), the background heats of dilution are large in comparison with the heat change associated with the binding process. The data obtained for the four coumarin drugs binding to both the 43- and 24-kDa proteins are summarized in Table 2. In most cases, the results of several independent experiments are shown to exemplify the level of variation in the data from this technique under these conditions. The value of the stoichiometry (n) derived from the iterative fitting diverges from integer values either because of the rather poor signal-to-noise or because of errors in the determination of protein concentrations. The titration−calorimetry data provide an estimate of the binding constants for each of the drugs to both the 43- and 24-kDa proteins. All binding constants are in the range of 10^{-8} M, which is consistent with expectations based on the IC_{50} values for inhibition of supercoiling and the corresponding data for the inhibition of the ATPase activity. The apparent rank order of binding of the four coumarins to the 43- and 24-kDa protein fragments deduced from the titration calorimetry differs from that obtained from the spin-column experiments. However, the fact that the ligands are all relatively tight-binding inhibitors required that the titration experiments be conducted at comparatively low protein concentrations (in terms of the calorimetry experiments), which leads to increases in the noise and in the errors on the derived parameters. It is also important to note that, unlike binding constants measured at low enzyme concentrations typical of kinetic experiments, binding constants determined at these comparatively high protein concentrations are not necessarily independent of the protein concentration. For example, if there is any tendency of the protein to adopt higher aggregate states at higher protein concentrations, this will affect the apparent binding constants. Interpretation of small differences in tight-binding constants for different ligands that have been determined at different protein concentration is therefore fraught with difficulties.

Simulation of Coumarin Inhibition of 43-kDa ATPase. There is now substantial evidence to show that coumarins inhibit the ATPase activity of gyrase by directly competing with ATP for binding to the protein. However, this does not agree with the evidence of Ali et al. (1993), who showed that the $V(\max)_{app}$ varies with novobiocin concentration, suggesting that, even at infinite concentrations of ATP, the inhibitory effect of novobiocin cannot be overcome, i.e., inhibition is not competitive. To resolve how a coumarin competing with ATP for binding to the same (partially

overlapping) substrate binding pocket can give rise to apparently noncompetitive behavior, we have simulated the inhibition of 43-kDa ATPase activity by coumarins using the kinetic simulation program KFitSim (D. Thomas, University of Leicester, U.K.). A modification to the kinetic scheme for ATP hydrolysis by the 43-kDa protein, proposed by Ali et al. (1993), to include competitive inhibition by coumarins, yields data for the effects of a range of coumarin concentrations on the ATPase activity. When simulations are performed with substrate (ATP) concentrations less than 2 mM (typical ATP concentration used in actual ATPase assays), a set of data are produced which can be accurately fitted to rectangular hyperbolae, yielding values of $V(\max)_{app}$ which decrease with increasing coumarin concentration, as was observed by Ali et al. (1993). However, if simulations are performed over a much greater substrate range, the data can no longer be fitted to rectangular hyperbolae. Moreover, at very high ATP concentrations (greater than 1 M), the $V(\max)_{app}$ at all coumarin concentrations eventually converge on the same value, indicating that the inhibitory effect of coumarins can be eventually overcome, a fact which cannot be extrapolated from observations of ATPase rates at low ATP concentrations (<10 mM). Thus, the true relationship between the ATPase rate, ATP, and coumarin concentrations is not described by a simple Michaelis−Menten rectangular hyperbola. This is reasonable since the steady-state rate equation given by Ali et al. (1993) does not yield a rectangular hyperbola. Data on the cyclothialidines show that these drugs also inhibit the gyrase ATPase reaction (Nakada et al., 1995; Oram et al., 1996). Nakada et al. (1995) presented experimental data for the inhibition of the 43-kDa protein ATPase activity by cyclothialidines and also determined $V(\max)_{app}$ values by extrapolation, which decreased with increasing drug concentrations. In the light of our simulations, it is likely that this observation is a consequence of the same reasons given above for coumarins. Indeed, the binding site of cyclothialidines overlaps with those of ATP and coumarins (Lewis et al., 1996), and thus their mode of inhibition is competitive as well.

CONCLUSIONS

The inhibition of the supercoiling and ATPase reactions of gyrase suggests that these drugs bind tightly, with K_d values $<10^{-7}$ M. These experiments do not allow a more precise estimate of the K_ds, nor do they distinguish between the different coumarins as regards relative affinities. The ATPase experiments (Figure 2) support the idea that coumermycin binds to 2 molecules of the 43-kDa fragment of GyrB while novobiocin, dihydronovobiocin, and chlorobiocin bind with a stoichiometry of 1:1. This result is consistent with the structures of the drugs (Figure 1); viz., coumermycin resembles a dimer of the other compounds. The ability of coumermycin to stabilize a dimer of the 43-kDa protein was confirmed by cross-linking studies (Figure 4). Rapid gel filtration (spin-column) studies support the notion that coumarins bind competitively with ATP. Kinetic experiments showed that the on rate of drug binding to the 24- and 43-kDa proteins is fast ($k_{on} > 10^5$ $M^{-1}\cdot s^{-1}$) and that off rates were $\sim 3 \times 10^{-3}$ s^{-1} and $\sim 4 \times 10^{-3}$ s^{-1} for the 43- and 24-kDa proteins, respectively. Consequently, K_d values could not be directly measured by this technique, but an order of affinity of the drugs was determined: chlorobiocin > coumermycin > novobiocin > dihydronovobiocin. As the dissociation rates were apparently the same for each drug, this also reflects the order of the association rates.

Titration calorimetry provides a complementary approach to the spin-column work. However, as we are working close to the limits of sensitivity of this technique, the absolute values of constants determined by this method do not closely agree with those determined from the spin columns. However, several interesting and important features emerge from the calorimetry. First, the coumermycin data were consistently best fitted with an n value significantly less than those of the other coumarins, providing direct supporting evidence for a 1:2 ligand:protein binding for coumermycin and a 1:1 stoichiometry for the remaining three coumarins. Second, the binding of each of the coumarins is *enthalpy* driven, with the entropy component being typically small. This is consistent with polar interactions such as hydrogen bonding, rather than hydrophobic interactions which are usually associated with the entropy term. This conclusion is supported by the X-ray structure of the 24-kDa protein complexed with novobiocin, which identified a number of key hydrogen bonds (Lewis et al., 1996). Finally, the K_d values for each of the 4 coumarins binding to the 43-kDa protein are all smaller than the corresponding values for the 24-kDa protein, i.e., all of the coumarins bind more tightly to the 43 kDa fragment, although the differences are relatively small.

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