The 43-Kilodalton N-Terminal Fragment of the DNA Gyrase B Protein Hydrolyzes ATP and Binds Coumarin Drugs†

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ABSTRACT: We have cloned and overexpressed a gene encoding a 43-kDa protein corresponding to the N-terminal fragment of the DNA gyrase B subunit. We show that this protein hydrolyzes ATP and binds coumarin drugs. The hydrolysis of ATP shows distinctly non-Michaelis–Menten kinetics and is consistent with a scheme in which the active form of the protein is a dimer, a conclusion supported by molecular weight studies. The coumarin drugs bind very tightly to the 43-kDa fragment, with novobiocin binding to the protein monomer and coumermycin A_1 apparently inducing the formation of a dimer. The implications of these results with respect to the mechanism of supercoiling by DNA gyrase and the inhibition of gyrase by coumarin drugs are discussed.

DNA gyrase is the type II topoisomerase from bacteria which catalyzes the negative supercoiling of DNA [for reviews see Gellert (1985), Wang (1985), and Reece and Maxwell (1991a)]. 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. Mechanistic studies have revealed the principal steps involved in the DNA supercoiling reaction [reviewed in Maxwell and Gellert (1986) and Reece and Maxwell (1991a)]. The enzyme binds to DNA and forms a complex in which ~120 bp are wrapped around the protein core (A_2B_2). This wrapped DNA is then cleaved in both strands with the formation of covalent bonds between the 5′-phosphate groups and Tyr122 of the A subunit. A segment of DNA is then passed through this double-stranded break and probably through part of the enzyme itself. The reaction cycle is completed by the resealing of the broken phosphodiester bonds. This process leads to a decrease in linking number of the DNA by 2 (introduction of two negative supercoils) and normally requires the hydrolysis of ATP. However, the nonhydrolyzable ATP analogue ADPNP[1] (5′-adenylyl β,γ-imidodiphosphate) will support limited supercoiling by gyrase, suggesting that nucleotide binding can promote a single round of supercoiling but that hydrolysis of ATP is required for enzyme turnover (Sugino et al., 1978). In the absence of nucleotide, DNA gyrase can relax negatively supercoiled DNA (Gellert et al., 1977).

DNA gyrase is the target of two groups of antibacterial agents, the quinolones and the coumarins, both of which inhibit the DNA supercoiling reaction. The quinolones (e.g., nalidixic acid and ciprofloxacin) interrupt the DNA breakage and reunion steps of DNA supercoiling and are thought to act at the A subunit of gyrase; the coumarins (e.g., novobiocin and coumermycin A_1) inhibit the ATPase reaction of gyrase and bind to the B subunit [for a review see Reece and Maxwell (1991a)]. It has been reported that coumarin drugs are competitive inhibitors of ATP hydrolysis (Sugino et al., 1978; Sugino & Cozzarelli, 1980).

The A subunit of gyrase is thought to be principally involved in the DNA breakage and reunion aspects of the supercoiling reaction, while the B subunit is responsible for the ATP hydrolysis reaction. There is evidence that both subunits contain distinct functional domains. Cleavage of the gyrase A protein with trypsin yields two fragments: a 64-kDa N-terminal fragment which contains the site of DNA breakage and reunion and the likely site of action of the quinolones drugs, and a C-terminal 33-kDa fragment involved in DNA–protein interactions (Reece & Maxwell, 1989). Genetic constructs have been made which overproduce these fragments, and their properties have been studied (Reece & Maxwell, 1991b,c); the 64-kDa N-terminal fragment has been crystallized (Reece et al., 1990).

It was previously shown that some bacterial strains produce a 47-kDa protein which comprises the C-terminal portion of the gyrase B protein beginning at amino acid Arg394 (Brown et al., 1979; Gellert et al., 1979; Adachi et al., 1987). This protein, when complexed with the A subunit, supports DNA relaxation but not supercoiling. It was inferred that the N-terminal portion of the B protein contains the site of ATP hydrolysis (Brown et al., 1979; Adachi et al., 1987). Indeed, cell extracts from bacterial strains which produce the 47-kDa protein also contain a protein of ~40 kDa which binds tightly to novobiocin affinity columns (Staudenbauer & Orr, 1981; A. Maxwell, unpublished results). Thus it is proposed that the gyrase B protein consists of two domains: an N-terminal domain which hydrolyzes ATP and binds the coumarin drugs, and a C-terminal domain responsible for the interaction with the A subunit and DNA.

In this paper we describe the cloning and purification of a 43-kDa N-terminal fragment of the gyrase B protein and discussion the enzymatic properties of this protein. Elsewhere we have reported the crystallization of this protein (Jackson et al., 1991) and the determination of the structure of the 43-kDa protein complexed with ADPNP to 2.5-Å resolution by X-ray crystallography (Wigley et al., 1991).

EXPERIMENTAL PROCEDURES

Cloning. Plasmid pAJ1 which contains the gene encoding the 43-kDa N-terminal fragment of the DNA gyrase B protein

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[1] ADPNP, 5′-adenylyl β,γ-imidodiphosphate; bp, base pairs; DTT, dithiothreitol; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; IPTG, isopropyl β-D-thiogalactopyranoside; PK/LDH, pyruvate kinase and lactase dehydrogenase.

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was constructed as follows. Plasmid pAG111, which codes for the intact gyrase B protein (Hallett et al., 1990), was digested with *Hin*dIII and *Cla*I (both from Gibco BRL) and the resultant 5635 bp fragment purified from an agarose gel (Maniatis et al., 1982). Two complementary oligonucleotides comprising from bp 1132 to 1179 of the *gyrB* gene (Yamagishi et al., 1986; Adachi et al., 1987), with the addition of a *Cla*I "sticky end" at the 5′ terminus and a TA dinucleotide plus a *Hin*dIII sticky end at the 3′ terminus, were annealed together and ligated to the 5635 bp fragment. The resulting plasmid (pAJ1) contains a *Hin*dIII site which forms part of a TAA stop codon immediately after the codon for Arg393 of *gyrB*, thus leading to the production of a 43-kDa protein. Sequencing of the DNA coding for the 43-kDa protein was carried out using Sequenase (United States Biochemicals).

Enzymes and DNA. The DNA gyrase A and B proteins were purified from strains JMtacA and JMtacB as previously described (Hallett et al., 1990). The 43-kDa B fragment was prepared from *E. coli* strain JM109[pAJ1] by the following procedure based on the purification of GyrB (Hallett et al., 1990).

Cultures of JM109[pAJ1] in Luria–Bertani broth (Maniatis et al., 1982) containing 50 μg/mL ampicillin were incubated at 37 °C until A_{595nm} of 0.5 was reached. Isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 50 μM and the incubation continued for a further 4 h. After harvesting, the cells were resuspended in 50 mM Tris·HCl (pH 7.5) and 10% (w/v) sucrose, rapidly frozen, and stored at −70 °C.

Upon thawing, the cell suspension was adjusted to 2 mM DTT, 20 mM EDTA, and 100 mM KCl, and the cells were disrupted by passing through a French press three times (8000–12 000 psi). The cell debris was removed by centrifugation, and glycerol was added to the supernatant [to a final concentration of 10% (w/v)] which was rapidly frozen and stored at −70 °C. The cell extract was loaded onto a heparin–Sepharose column (Pharmacia), which had been preequilibrated in TED (50 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 5 mM DTT], and circulated around the column for at least 1 h to increase protein binding, prior to washing with 3 volumes of TED. The 43-kDa protein was eluted with TED + 0.2 M NaCl and concentrated by ultrafiltration (Amicon YM30 membrane) to approximately 5 mg/mL, glycerol was then added to a final concentration of 10% (w/v), and the protein solution was rapidly frozen and stored at −70 °C.

The partially purified protein was diluted with TED to give a conductivity of less than 15 mS and loaded onto a FPLC Mono Q 10/10 column (Pharmacia) which had been pre-equilibrated in TED. The 43-kDa protein was eluted from the column with a NaCl gradient; the major peak was the 43-kDa protein which eluted at about 130 mM NaCl (conductivity ~23 mS). The 43-kDa protein was dialyzed exhaustively against enzyme buffer [50 mM Tris·HCl (pH 7.5), 100 mM KCl, 10% (w/v) glycerol, 1 mM EDTA, 5 mM DTT], quickly frozen, and stored at −70 °C.

Relaxed pBR322 plasmid was prepared as described by Reece and Maxwell (1989), a 147 bp DNA fragment was made as described previously (Dobbs et al., 1992), and oligonucleotides were prepared by D. Langton (University of Leicester).

Enzyme Assays. DNA supercoiling and gel retardation assays were performed as described previously (Reece & Maxwell, 1989, 1991b). ATPase assays were performed by two methods:

(*i*) *Pyruvate Kinase/Lactate Dehydrogenase (PK/LDH)-Linked Assay.* ATP hydrolysis by the 43-kDa protein was linked to the oxidation of nicotinamide adenine dinucleotide (NADH) based on the method described by Tamura and Gellert (1990).

ATPase assays (typically 150 μL) were carried out at 25 °C in enzyme buffer plus 5 mM $MgCl_2$ with phosphoenolpyruvate and NADH at 400 and 250 μM, respectively, and 1.5 μL of PK/LDH mix [in 50% (w/v) glycerol, 100 mM KCl, 10 mM HEPES (pH 7.0), 0.1 mM EDTA; Sigma]. ATP was added as a Mg^{2+} complex to ensure that the free [Mg^{2+}] in the assay was maintained at 4 mM. Reactions were initiated by the addition of ATP, and the decrease in A_{340} was measured as a function of time. The change in absorbance was related to ADP production using A^{1mM}_{340} = 6.22 with stoichiometric production of NAD^+ to ADP released.

(*ii*) *Fluorescence Assay.* This assay was adapted from the inorganic phosphate-linked enzyme assay of Banik and Roy (1990) and was used for the ADP inhibition studies. ATPase assays (150 μL) were carried out at 25 °C in enzyme buffer plus 5 mM $MgCl_2$, 80 μM methylguanosine, and 0.2 unit of nucleoside phosphorylase (both Sigma) in a SLM 8000 ratio recording fluorimeter (λ_{ex} 300 nm, λ_{em} 395 nm).

Analytical Ultracentrifugation. Equilibrium sedimentation was carried out on an M.S.E. MK II analytical ultracentrifuge. Centrifugation of the 43-kDa protein (70 μM), in enzyme buffer plus 5 mM $MgCl_2$, was carried out at 12 000 rpm at 25 °C in 20-mm path length cells, where protein movement down the cell was followed by Schlieren optics. Where indicated, ADPNP, ADP, and P_i were included at 2 mM, and coumermycin and novobiocin were at 140 μM.

Gel Filtration. Gel filtration experiments were carried out on an FPLC apparatus with two 10/30 Superose 12 columns (Pharmacia) connected in series and equilibrated in 50 mM Tris (pH 7.5), 100 mM KCl, 1 mM EDTA, 5 mM DTT, and 5 mM $MgCl_5$ and run at 0.25 mL/min. Protein samples (0.2 mL) contained 120 μM 43-kDa protein and, where indicated, nucleotides and phosphate at 2 mM. The eluant was monitored by absorbance at 280 nm. Fractions (0.5 mL) were collected and, where appropriate, assayed for [α-^{32}P]ADPNP (ICN) by scintillation counting and for protein content by SDS gel electrophoresis. The gel filtration column was calibrated using a Sigma gel filtration calibration kit (molecular mass 12 000–200 000 Da).

Other Methods. Western blotting experiments (Harlow & Lane, 1988) were performed by wet electrophoretic transfer; blots were probed with three anti-GyrB mouse monoclonal antibodies (A. Maxwell and M. Gellert, unpublished results) followed by goat anti-mouse antibodies conjugated to alkaline phosphatase (Sigma). N-Terminal sequencing of the 43-kDa fragment was carried out by K. S. Lilley (University of Leicester) as described by Matsudaira (1987). A novobiocin–Sepharose column was prepared as described by Staudenbauer and Orr (1981). Rapid gel filtration (spin column) experiments were carried out as described by Tamura et al. (1992). Computer modeling of kinetic schemes was performed on an IRIX mainframe computer using a nonlinear regression SAS program.

RESULTS

Cloning and Purification of the 43-kDa Protein. Preparations of the DNA gyrase B protein produced by the method of Hallett et al. (1990) often show two principal contaminating proteins of molecular mass 47 and 43 kDa (Figure 1). It was proposed that these were proteolytic fragments of GyrB

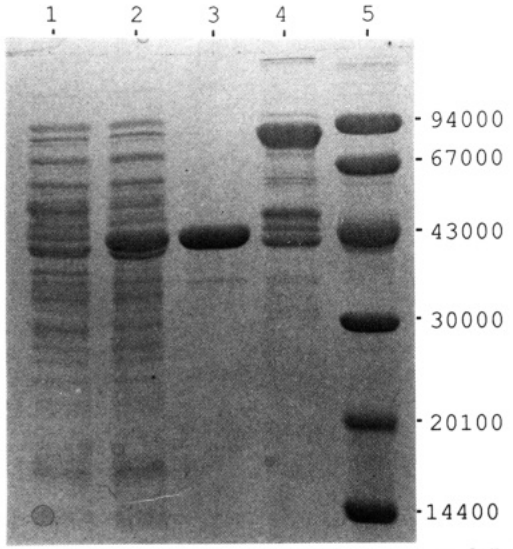

FIGURE 1: An SDS–polyacrylamide gel showing GyrB and 43-kDa protein. Lanes 1 and 2, cell extracts of *E. coli* JM109[pAJ1] with and without IPTG induction respectively; lane 3, purified 43-kDa protein; lane 4, purified GyrB showing contaminating proteins; lane 5, molecular mass markers (sizes in Da).

produced either during overproduction or purification. This was confirmed in two ways. First, Western blot analysis of gels, such as that shown in Figure 1, was performed using three anti-GyrB monoclonal antibodies. Binding to the intact 90-kDa band was detected in each case, and with two of the antibodies, binding to the 47-kDa band was also found, the third antibody bound to the 43-kDa fragment (data not shown). Second, the two contaminating bands were excised from an SDS–polyacrylamide gel and the N-terminal protein sequences determined. The N-terminal residues of the 47-kDa protein were identical to residues 395–403 of GyrB (Lys-Gly-Ala-Leu-Asp-Leu-Ala-Gly-Leu), which is one amino acid shorter than the 47-kDa fragment of GyrB identified by Adachi et al. (1987), Arg394 being missing. The N-terminal residues of the 43-kDa protein were identical to residues 2–6 of GyrB (Ser-Asn-Ser-Tyr-Asp), the N-terminal methionine identified in the DNA sequence (Yamagishi et al., 1986; Adachi et al., 1987) presumably being lost during posttranslational modification. Taken together, these data confirm that the 43- and 47-kDa proteins are respectively the N- and C-terminal fragments of the DNA gyrase B protein.

Production of the 43- and 47-kDa proteins for biochemical analysis by proteolytic digestion of GyrB was not carried out since difficulties in separating the three species were experienced (data not shown) and heterogeneity in the population of the proteins produced in this way is likely. The cloning procedure described in Experimental Procedures involves the introduction of a stop codon after the codon for Arg393, resulting in the deletion of the C-terminal portion of GyrB, corresponding to the 47-kDa protein previously identified (Brown et al., 1979; Gellert et al., 1979; Adachi et al., 1987). The resulting plasmid (pAJ1) was sequenced through the region encoding the 43-kDa protein and found to be identical to the equivalent region of *gyrB* (Yamagishi et al., 1986; Adachi et al., 1987). Expression of the 43-kDa protein was found to be optimal 4 h after the addition of the inducer IPTG. Typically it was expressed to about 30% of the total cell protein and was purified to >98% homogeneity (Figure 1). The N-terminal sequence of the cloned protein was confirmed as Ser-Asn-Ser-Tyr.

Properties of the 43-kDa Protein. The 43-kDa protein was found to be an ATPase, whose activity was inhibited by the coumarin drugs (see below), but no evidence of interaction with DNA or GyrA was found by a variety of methods. Using either a 147 bp DNA fragment or a 54 bp oligonucleotide, gel retardation assays showed no evidence of DNA binding by the 43-kDa protein alone or in the presence of the GyrA protein [see also Reece and Maxwell (1991c)]; the presence of ADPNP did not stimulate DNA binding. Addition of GyrA and/or DNA had no effect on the ATPase activity of the 43-kDa protein (data not shown). The 43-kDa protein did not support supercoiling in the presence of GyrA nor did it inhibit DNA gyrase-catalyzed supercoiling even when a 10-fold molar excess of 43-kDa protein over GyrB was used (data not shown).

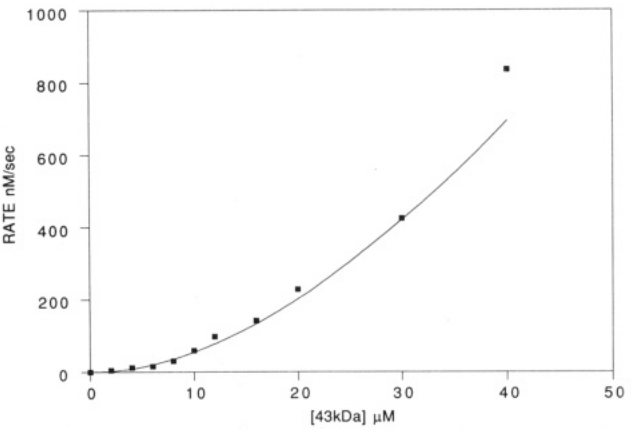

FIGURE 2: ATPase activity of the 43-kDa protein as a function of protein concentration. Rates are initial velocities; the substrate (ATP) concentration was 1.4 mM. The line drawn is a theoretical curve based on the scheme and the rate constants given in Figure 8.

ATPase Kinetics. Using the PK/LDH-linked enzyme assay, the ATPase activity was found not to be linearly dependent upon enzyme concentration over the range 2–40 μM (Figure 2); measurements above 40 μM were not possible due to precipitation of the protein. At a concentration of 40 μM the turnover number of the 43-kDa protein was 0.02 s^{-1}; this compares with turnover numbers of about 1 s^{-1} previously measured for the DNA-dependent ATPase and supercoiling reactions of DNA gyrase and for the ATPase reaction of GyrB alone (Higgins et al., 1978; Staudenbauer & Orr, 1981; Maxwell & Gellert, 1984); however, this value (0.02 s^{-1}) is not the theoretical maximum for the turnover number of the 43-kDa protein (see Discussion). Although the ATPase activity of the 43-kDa protein in Figure 2 is very low, it is not attributable to contaminating proteins, as the activity can be completely inhibited by novobiocin at concentrations equimolar to the amount of protein (see below). In addition, there is no evidence for contaminating GyrB in the preparations of the 43-kDa protein, as judged by silver staining and Western blotting experiments.

The greater than first-order dependence of ATPase activity on enzyme concentration demonstrates that the ATPase reaction of the 43-kDa protein does not follow Michaelis–Menten kinetics and suggests that the active form of the protein may be an oligomer. However, at constant enzyme concentrations (within the range 5–40 μM), it demonstrates the familiar hyperbolic dependence of rate on substrate (ATP) concentration (Figure 3). At each of these enzyme concentrations, values for K_M^{app} and V_{max}^{app} may be determined. However, it must be emphasized that these parameters will not necessarily have their normal meanings (see below).

Inhibition by ADP and ADPNP. It has previously been shown that ADP is an effective inhibitor of the ATPase and supercoiling reactions of DNA gyrase (Sugino & Cozzarelli,

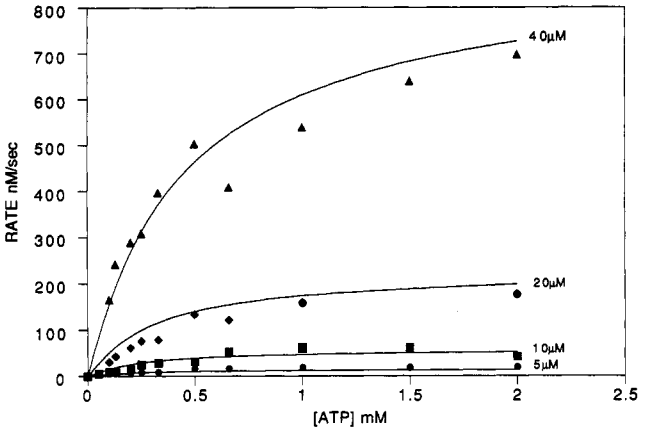

FIGURE 3: ATPase activity of the 43-kDa protein as a function of substrate (ATP) concentration at several enzyme concentrations: 5 μM (●), 10 μM (■), 20 μM (♦), 40 μM (▲). The lines drawn are theoretical curves computed from the scheme shown in Figure 8.

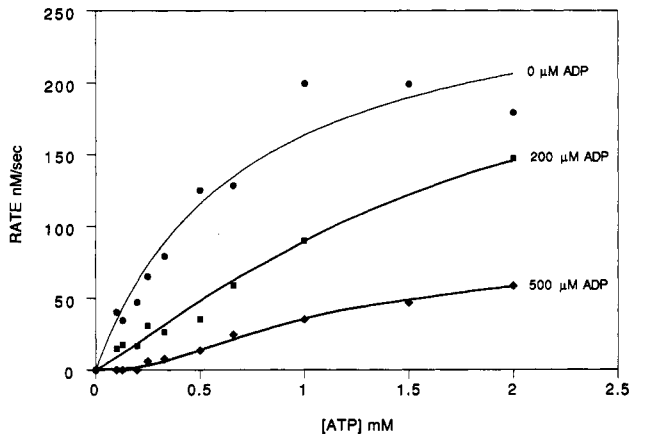

FIGURE 4: ATPase activity of the 43-kDa protein (20 μM) as a function of ATP concentration in the presence of ADP: (●) no ADP; (■) 200 μM; (♦) 500 μM.

1980; Maxwell et al., 1986); this inhibition is proposed to be competitive in nature. Analysis of the steady-state kinetics of the ATPase reaction of gyrase in the presence of ADP showed a sigmoidal dependence of rate upon substrate concentration (Maxwell et al., 1986) and suggested cooperative binding of 2 ATP molecules per gyrase tetramer. Evidence of cooperative binding of ADPNP to gyrase has also been found (Tamura et al., 1992). Using a fluorescence assay, ADP was found to be an effective inhibitor of the ATPase reaction of the 43-kDa protein, which showed a sigmoidal dependence of rate upon substrate concentration (Figure 4). Such a dependence precludes the calculation of apparent K_M and V_{max} values but is once again consistent with a model involving the active form of the 43-kDa protein being an oligomer.

ADPNP does inhibit the 43-kDa ATPase activity, but the interpretation of the kinetics of inhibition is complicated by the slow binding and dissociation of the nucleotide and the ADPNP-induced dimerization of the 43-kDa protein (see Figure 7; J. A. Ali, unpublished observations). Further characterization of these processes is currently underway.

Coumarin Drug Binding. The binding of the 43-kDa protein to coumarin drugs was assessed using a novobiocin affinity column. As found previously with the GyrB protein (Staudenbauer & Orr, 1981), the 43-kDa protein could only be eluted from such a column with 6 M urea (data not shown). Thus novobiocin binds tightly to both GyrB and the 43-kDa protein and with a similar affinity for both proteins. Sugino et al.

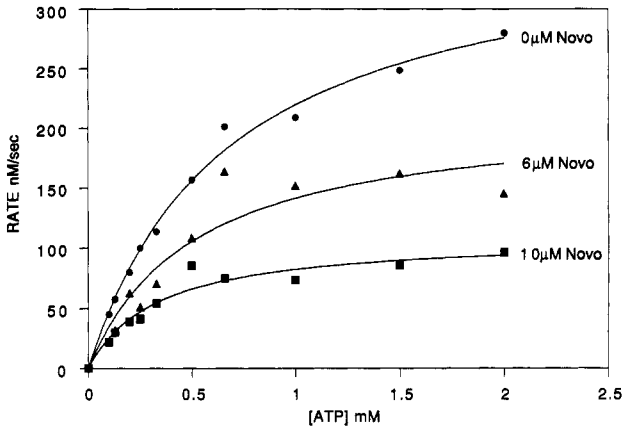

FIGURE 5: ATPase activity of the 43-kDa protein (20 μM) in the presence of novobiocin. For each drug concentration values of K_M^{app} and V_{max}^{app} were determined by fitting the data to rectangular hyperbolae: no novobiocin (●), 0.68 mM and 370 nM s^{-1}; 6 μM novobiocin (▲), 0.51 mM and 210 nM s^{-1}; 10 μM novobiocin (■), 0.33 mM and 110 nM s^{-1}.

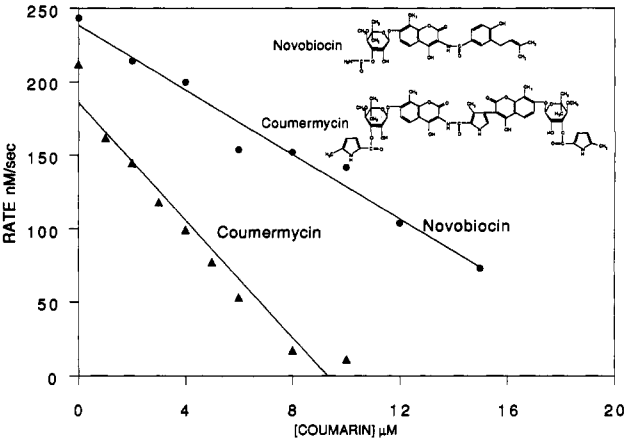

FIGURE 6: ATPase activity of the 43-kDa protein (20 μM) in the presence of coumermycin and novobiocin, at 1 mM ATP. By extrapolation 100% inhibition occurs at 9.3 and 21.8 μM drug for coumermycin and novobiocin, respectively. The inset shows the structures of the two drugs.

(1978) and Sugino and Cozzarelli (1980) proposed that coumarin drugs are competitive inhibitors of the GyrB ATPase activity. Figure 5 demonstrates the effect of a range of concentrations of novobiocin on the 43-kDa ATPase. Novobiocin is an effective inhibitor of the ATPase reaction of the 43-kDa protein, the V_{max}^{app} value decreasing with increasing drug concentration. With reference to the familiar Michaelis–Menten paradigm this behavior would not be predicted for a simple competitive inhibitor. Secondary plots of these data (e.g., Dixon plots) cannot be used to determine the mechanism of inhibition due to the non-Michaelis–Menten behavior of the enzyme and to the very high affinity of the drug for the protein under the conditions used.

Figure 6 shows the effect of increasing novobiocin and coumermycin concentrations at fixed 43-kDa protein and ATP concentrations. In both cases an inverse linear relationship between the rate of hydrolysis and drug concentration was found, indicating a high affinity of the protein for the drugs with an upper limit of 20 μM (the protein concentration) for the equilibrium dissociation constant of the protein/drug complex. From the data in Figure 6, full inhibition of the 43-kDa protein ATPase reaction occurs at 22 μM novobiocin and 9 μM coumermycin, indicating stoichiometries of binding of 1 novobiocin per 43-kDa monomer and 1 coumermycin per two 43-kDa protein monomers.

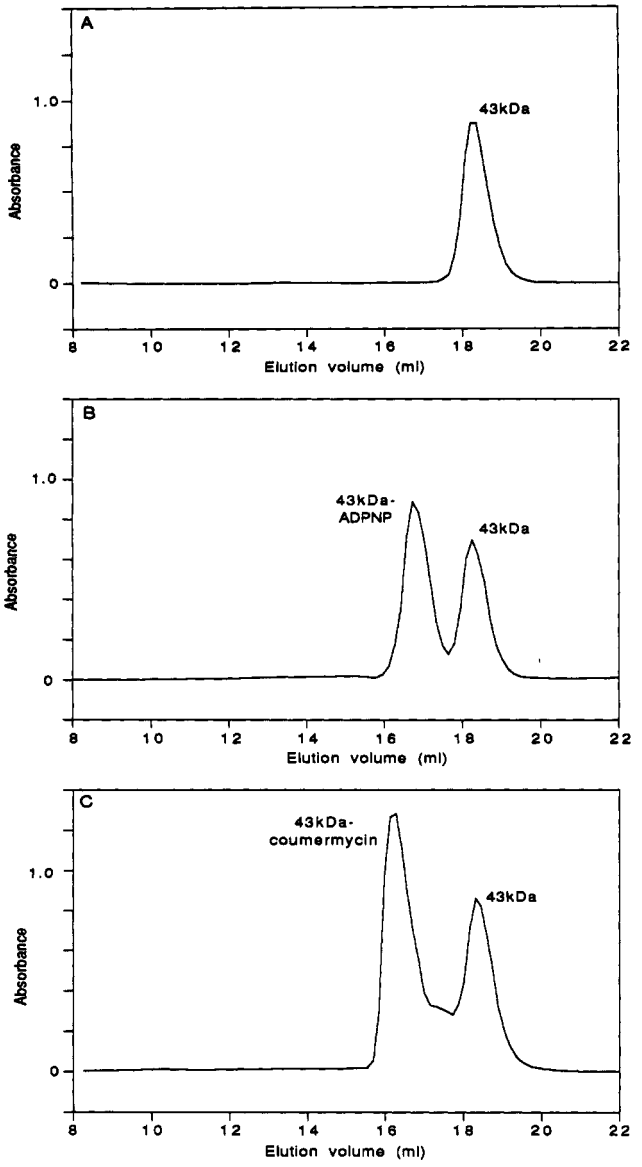

FIGURE 7: Gel filtration of the 43-kDa protein. Panel A shows the 43-kDa protein alone; panel B shows a mixture of 43-kDa protein–[α-^{32}P]ADPNP complex and uncomplexed protein; panel C shows a mixture of 43-kDa protein–coumermycin complex and uncomplexed protein.

Table I: Molecular Mass Studies

sample	apparent molecular mass (kDa)	
	gel filtration	ultra-centrifugation
43-kDa protein	45	43
+ADPNP	79	66
+ATP	50	nd[a]
+ADP/P_i	45	42
+coumermycin	100	80
+novobiocin	45	46

[a] Not determined.

Molecular Mass Studies. The ATPase data reported above suggest that the 43-kDa protein is a dimer under certain conditions. We have used gel filtration and equilibrium ultracentrifugation to examine the oligomeric state of this protein in the presence of various ligands (Figure 7 and Table I). Molecular mass studies of the protein alone suggest that it is a monomer at a wide range of protein concentrations (10–230 μM). In the presence of ADPNP it has a larger molecular mass consistent with the protein being a dimer under these conditions (Table I). A 43-kDa protein–ADPNP complex was made by incubating the 43-kDa protein with a 10-fold molar excess of ^{32}P-labeled ADPNP for 24 h at 25 °C. Unbound nucleotide was then removed using a spin column, an approximately equal amount of 43-kDa protein added to this complex, and the mixture applied to a gel filtration column. Two distinct protein peaks were found in the eluant (Figure 7B). Only the first of these (corresponding to the dimer) contained radioactivity; the second peak (corresponding to the monomer) contained only background levels of radioactivity. This experiment clearly demonstrates that the 43-kDa protein dimerizes in the presence of ADPNP. In addition, it shows that the dissociation rate of ADPNP from the 43-kDa protein is extremely slow, consistent with results reported for the intact gyrase tetramer (Tamura et al., 1992).

In the presence of ADP, the 43-kDa protein is in the monomeric form (Table I); this experiment has been carried out using gel filtration with ADP in the moving phase. With ATP in the moving phase, gel filtration experiments showed that the 43-kDa protein eluted slightly ahead of the monomer position, possibly indicative of a rapid monomer–dimer equilibrium (see Discussion).

Gel filtration experiments were also carried out in the presence of coumarin drugs. With novobiocin the 43-kDa protein eluted as a monomer, but with coumermycin the protein eluted ahead of the 43-kDa protein–ADPNP complex peak (Fiure 7C). This may be explained by coumermycin forming a dimer of the 43-kDa protein of a different conformation from the ADPNP-induced dimer. That novobiocin and coumermycin stabilize respectively monomeric and dimeric forms of the 43-kDa protein is consistent with the stoichiometries for ATPase inhibition described above. It should be noted that gel filtration with or without novobiocin in the moving phase gave identical results. This also suggests that, like ADPNP, the dissociation of the coumarin drugs from the 43-kDa protein is a slow process.

Confirmation of the dimeric state of the 43-kDa protein in the presence of ADPNP has been provided by cross-linking experiments. In the presence of ADPNP, cross-linking of the 43-kDa protein by dimethyl suberimidate generates a dimer as judged by SDS–polyacrylamide gel electrophoresis, whereas no dimer species is evident in the absence of nucleotide (G. Orphanides, personal communication).

DISCUSSION

ATP Hydrolysis. The data presented in this paper show conclusively that the binding and hydrolysis of ATP by DNA gyrase occur within the 43-kDa N-terminal domain of the B subunit. This conclusion is supported by the crystal structure of the 43-kDA protein–ADPNP complex (Wigley et al., 1991). We have found that the kinetics of ATP hydrolysis by the 43-kDa protein are non-Michaelian as evidenced by the greater than first-order dependence of rate on enzyme concentration (Figure 2). However, at constant enzyme concentration, apparent Michaelian kinetics can be observed (Figure 3). These data are suggestive of ATP-promoted dimerization of the 43-kDa protein. Molecular weight studies showed that in the presence of ADPNP the protein behaves as a dimer whereas in the presence of ADP and P_i or in the absence of nucleotide the enzyme is a monomer (Figure 7 and Table I). In the presence of ATP, gel filtration experiments indicated a size intermediate between monomer and dimer. This can be interpreted as reflecting turnover of ATP during gel filtration, leading to a mixture of ATP-bound (dimer) and ADP-bound

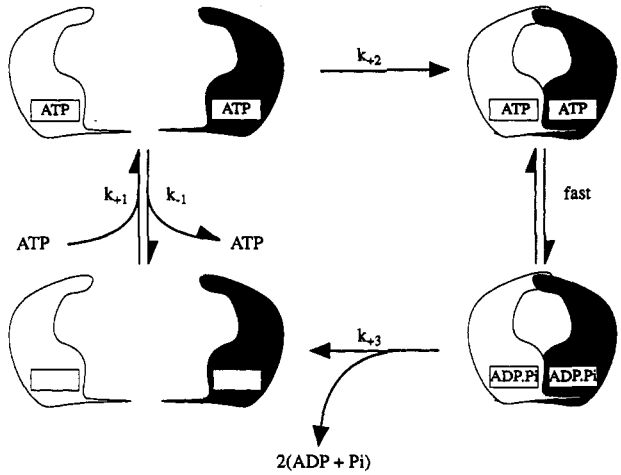

FIGURE 8: Model for the ATPase cycle of the 43-kDa protein. Using the equation derived in the Appendix, the data in Figures 2 and 3 were fitted to this scheme. The following rate constants were found to be consistent with these data: k_{+1}, 121 M^{-1} s^{-1}; k_{-1}, 0.0083 s^{-1}; k_{+2}, 293 M^{-1} s^{-1}; k_{+3}, 1.26 s^{-1}.

(monomer) forms that are in rapid equilibrium, with the monomer being the major species.

A scheme consistent with the ATPase data of Figures 2 and 3 and the molecular weight studies of Figure 7 and Table I is presented in Figure 8. In this scheme ATP can bind to the 43-kDa monomer, but the protein is only active in the dimeric form, $(43\text{-kDa})_2ATP_2$. This complex hydrolyzes ATP which leads to product release and dissociation into the monomeric form. We assume that the hydrolysis step is rapid. A steady-state rate equation based on this scheme has been derived (see Appendix), and the data in Figures 2 and 3 have been fitted to this equation, yielding values for the rate constants in Figure 8. The equation predicts the greater than first-order dependence of rate on enzyme concentration seen under the conditions of Figure 2. At this range of enzyme concentrations dimerization is rate limiting. From this analysis and with the 43-kDa concentrations used, k_{+2} is the rate-determining step. The $S_{0.5}$ value (the substrate concentration at which the rate is $0.5V_{max}$) is affected, under the experimental conditions used, mostly by k_{+1} which affects the enzyme–substrate complex pool available for dimerization; k_{+1} has little effect on V_{max}. The rate constant k_{-1} has a similar effect as k_{+1} but is not as significant. Since k_{+2} is the rate-determining step in this mechanism, k_{+3} has an insignificant effect on V_{max} or $S_{0.5}$. If k_{+2} was not rate limiting, apparent Michaelis–Menten kinetics would be seen. The fact that k_{+2} is limiting explains the observation that in the presence of ATP the monomer is the predominant species (Table I).

The experimental data indicate that increasing the enzyme concentration increases the apparent k_{cat}. Using the above rate constants and by extrapolating the enzyme and substrate concentration to very high levels not possible experimentally, theoretical rates can be obtained. The rate will increase with increasing enzyme concentration to a theoretical maximum of k_{+3}, 1.26 s^{-1}; i.e., k_{+3} becomes the rate-determining step. When the product release step, i.e., k_{+3}, is rate determining for the mechanism, the relationship between the rate and the enzyme concentration is linear, despite the dimerization step.

It is interesting to note that the computed value of k_{+3} (1.26 s^{-1}) is very similar to the previously reported values for the turnover numbers of the gyrase ATPase and supercoiling reactions (about 1 s^{-1}; Higgins et al., 1978; Staudenbauer & Orr, 1981; Maxwell & Gellert, 1984). This suggests that the rate-limiting step in the gyrase reaction occurs after the 43-kDa N-terminal domains of the GyrB protein have come together as a "dimer" such as depicted in the crystal structure of the 43-kDa protein–ADPNP complex (Wigley et al., 1991). Thus we suggest that the rate-determining step in gyrase-catalyzed supercoiling is product release; this could be release of ADP and/or P_i or a conformational change associated with product release. In this scheme ADP would bind to the monomer and prevent the 43-kDa protein from dimerizing. ADP and ATP would compete for the same sites, but because the ATP–monomer complex is inactive (Figure 8), little hydrolysis would occur at low ATP concentrations. However, at higher ATP concentrations, the ATP–dimer complex would form, allowing significant levels of hydrolysis. This would account for the sigmoidal relationship between rate and ATP concentration in the presence of ADP seen in Figure 4.

The ATPase reaction of the 43-kDa protein may be compared to that of the intact gyrase B protein and the A_2B_2–DNA complex. In the case of the B protein, a greater than first-order dependence of rate on protein concentration has also been observed (Staudenbauer & Orr, 1981; Maxwell & Gellert, 1984), again suggestive of the protein being active as a dimer. In the case of the gyrase–DNA complex, the ATPase activity shows a linear dependence upon enzyme activity (A. Maxwell and M. Gellert, unpublished data). These observations may be rationalized as follows. In the gyrase tetramer (A_2B_2) it is likely that the principal interactions between the A and B proteins occur via the C-terminal domain (47-kDa fragment) of the B protein. This is evidenced by the fact that this fragment (B′), in the presence of the A protein, forms a complex capable of relaxing DNA (Brown et al., 1979; Gellert et al., 1979). Thus the 43-kDa N-terminal domain can be viewed as tethered to the gyrase complex via the C-terminal domain, with relatively weak interactions between the two 43-kDa domains in the absence of ATP (or ADPNP). (This situation is analogous to the modeled high concentration of 43-kDa protein which leads to a linear dependence of rate on enzyme concentration and at which the predicted rate of ATP hydrolysis is ~1 s^{-1}.) The binding of nucleoside triphosphate causes a conformational change involving more extensive interaction between the 43-kDa domains, such as those observed in the crystal structure of the 43-kDa protein–ADPNP complex by Wigley et al. (1991). The role of this conformational change is likely to be to trap a segment of DNA which is being passed through the A_2B_2 complex as part of the supercoiling cycle, as suggested by Wigley et al. (1991). The 43-kDa protein alone is normally a monomer in solution as the monomer–monomer interactions are too weak to stabilize a dimer in the absence of nucleoside triphosphate. However, in the presence of ATP or ADPNP, the dimer form is stabilized and is competent to hydrolyze ATP. Although this form might be predicted to bind DNA, we did not detect DNA binding in our experiments. It is possible that stable binding of DNA by the 43-kDa domain only occurs in the context of the A_2B_2 complex.

Interaction with Coumarin Drugs. The experiments in this paper support the notion that the coumarin drug binding site lies within the 43-kDa N-terminal fragment of GyrB. This is demonstrated by the binding of this fragment to a novobiocin column with a similar affinity as the intact B protein and the fact that novobiocin and coumermycin are effective inhibitors of the ATPase reaction of the 43-kDa protein. This conclusion is also supported by the identification of single point mutations in GyrB conferring coumarin resistance (del Castillo et al., 1991; Contreras & Maxwell, 1992), which occur at residues

Arg136 and Gly164, both of which lie within the N-terminal fragment.

Determination of the amount of novobiocin or coumermycin required to inhibit the ATPase reaction of the 43-kDa protein suggests that one molecule of novobiocin binds to the 43-kDa protein monomer whereas one molecule of coumermycin binds to the protein dimer (Figure 6). In addition, molecular mass studies suggest that novobiocin binds to the 43-kDa monomer whereas coumermycin forms a species which elutes ahead of the ADPNP dimer in gel filtration experiments. It is likely that this species is a dimer of different conformation to that found in the presence of ADPNP. It is worth noting that the structure of coumermycin resembles a dimer of novobiocin. Therefore, we suggest that novobiocin binds to the 43-kDa monomer and that coumermycin induces a dimer form of this protein. It is very likely that both drugs bind at the same site on the enzyme as coumermycin-resistance point mutations (e.g., at Arg136 or Gly164) are cross resistant to novobiocin (Contreras & Maxwell, 1992). Computer graphics studies have shown that coumermycin cannot be accommodated in the crystal structure of the 43-kDa protein–ADPNP complex if Arg136 of both monomers is to be contacted by one drug molecule (D. B. Wigley, personal communication). Thus we suggest that the drug induces an alternative dimeric form which is not competent for ATP hydrolysis.

Earlier steady-state kinetic experiments had suggested that the coumarins are competitive inhibitors of ATP hydrolysis by DNA gyrase (Sugino et al., 1978; Sugino & Cozzarelli, 1980). Later steady-state experiments have shed doubt on this idea (Maxwell et al., 1986), and comparison of the structures of the drugs with that of ATP also raises doubts about coumarins acting in a competitive fashion. More recently, the identification of mutations to coumarin resistance lying outside the ATP-binding site (del Castillo et al., 1991; Contreras & Maxwell, 1992) similarly questions the notion of competitive inhibition. Although the steady-state kinetics of the ATPase reaction of the 43-kDa protein do not conform to the Michaelis–Menten paradigm, a steady-state rate equation can be derived (see Appendix) and values of V_{max}^{app} can be determined. In the presence of a competitive inhibitor, the value of V_{max} should not change; the data in Figure 5 show that V_{max}^{app} is reduced with increasing concentrations of novobiocin. However, given the relative complexity of the scheme in Figure 8, it is not straightforward to categorize the type of inhibition caused by the coumarin drugs. Indeed, ADPNP–43-kDa protein complexes do not bind to novobiocin columns and coumarin–43-kDa complexes can be shown to be unable to bind ADPNP (J. A. Ali, unpublished data), observations more consistent with the notion of competitive inhibition. In relation to the scheme in Figure 8 we therefore suggest that coumarins bind to the 43-kDa protein and stabilize a conformation that is unable to bind nucleoside triphosphate. In the case of novobiocin this is a monomeric form whereas for coumermycin it is a dimer which differs from the ADPNP dimer. This effectively reduces the pool of enzyme available for nucleotide binding and thus inhibits ATP hydrolysis. However, an understanding of the molecular details of these interactions awaits further work.

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APPENDIX

A steady-state rate equation for the hydrolysis of ATP by the 43-kDa protein has been derived based on the scheme shown in Figure 8:

$$\begin{array}{ccccccc} E + S & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & ES & & & & \\ & & + & \xrightarrow{k_{+2}} & E_2S_2 & \xrightarrow{k_{+3}} & 2E + 2P \\ E + S & \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} & ES & & & & \\ (e_0 - x - 2y) & s & & x & & y & p \end{array}$$

where e_0, x, y, and p represent the total enzyme concentration, enzyme–substrate complex, dimer complex, and the products, respectively. The rates of formation of x, y, and p are given as follows:

$$dx/dt = k_{+1}(e_0 - x - 2y)s - k_{-1}x - 2k_{+2}x^2$$

$$dy/dt = k_{+2}x^2 - k_{+3}y$$

$$v = dp/dt = 2k_{+3}y$$

At steady state $dx/dt = 0$ and $dy/dt = 0$:

$$\therefore 0 = k_{+1}e_0s - 2k_{+1}ys - k_{+1}xs - k_{-1}x - 2k_{+2}x^2 \quad (1)$$

$$\therefore 0 = k_{+2}x^2 - k_{+3}y \quad (2)$$

$$\therefore k_{+3}y = k_{+2}x^2$$

$$v = 2k_{+3}y = 2k_{+2}x^2$$

Rearranging eq 2:

$$y = k_{+2}x^2/k_{+3} \quad (3)$$

Substituting eq 3 into eq 1 and rearranging:

$$x^2(2k_{+2} + 2k_{+2}k_{+1}s/k_{+3}) + x(k_{+1}s + k_{-1}) - k_{+1}e_0s = 0$$

This is in the form of a quadratic equation with only one positive solution for x:

$$x = \left\{-(k_{+1}s + k_{-1}) + \left[(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0s\left(1 + \frac{k_{+1}s}{k_{+3}}\right)\right]^{1/2}\right\} \Big/ 2\left(2k_{+2} + \frac{2k_{+2}k_{+1}s}{k_{+3}}\right)$$

$$x^2 = \left\{2(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0s\left(1 + \frac{k_{+1}s}{k_{+3}}\right) - 2(k_{+1}s + k_{-1})\left[(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0s\left(1 + \frac{k_{+1}s}{k_{+3}}\right)\right]^{1/2}\right\} \Big/ 4\left(2k_{+2} + \frac{2k_{+2}k_{+1}s}{k_{+3}}\right)^2$$

Since $v = 2k_{+2}x^2$, then

$$v = 2k_{+2}\left\{\left(2(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0s\left(1 + \frac{k_{+1}s}{k_{+3}}\right) - 2(k_{+1}s + k_{-1})\left[(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0s\left(1 + \frac{k_{+1}s}{k_{+3}}\right)\right]^{1/2}\right) \Big/ 4\left(2k_{+2} + \frac{2k_{+2}k_{+1}s}{k_{+3}}\right)^2\right\}$$

$$v = \Big\{ k_{+3}^{2}(k_{+1}s + k_{-1})^2 + 4k_{+1}k_{+2}k_{+3}^{2}e_0 s + 4k_{+1}^{2}k_{+2}k_{+3}s^2 e_0 - k_{+3}^{2}(k_{+1}s + k_{-1})\Big[(k_{+1}s + k_{-1})^2 + 8k_{+2}k_{+1}e_0 s\Big(1 + \frac{k_{+1}s}{k_{+3}}\Big)\Big]^{1/2}\Big\} \Big/ 4k_{+2}(k_{+3}^{2} + 2k_{+1}k_{+3}s + k_{+1}^{2}s^2)$$

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