Nucleotide Binding to the 43-Kilodalton N-Terminal Fragment of the DNA Gyrase B Protein†

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ABSTRACT: The binding of ADPNP (5′-adenylyl β,γ-imidodiphosphate) to the 43-kDa N-terminal fragment of the DNA gyrase B protein is found to stabilize a dimer of the protein. Analysis of the kinetics of binding of ADPNP to the fragment suggests that protein dimers can contain 1 or 2 molecules of bound nucleotide. ATP, ADP, or coumarin drugs inhibit the binding of ADPNP. The rate of dissociation of ADPNP from the 43-kDa protein is found to be very slow and unaffected by the presence of other nucleotides. These data can be accommodated by a scheme in which the 43-kDa monomer forms a short-lived complex with ADPNP that can be converted into long-lived dimer complexes containing either 1 or 2 molecules of bound ADPNP; dimer formation with 2 bound ADPNPs is strongly favored. Coumarin drugs inhibit the binding of ADPNP to the 43-kDa fragment, with novobiocin binding to the protein with a stoichiometry of 1:1 and coumermycin binding with a stoichiometry of 0.5:1.

DNA gyrase is the bacterial type II topoisomerase which can introduce negative supercoils into DNA using the free energy of ATP hydrolysis [see Reece and Maxwell (1991b) for a recent review]. 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 analogue ADPNP[1] (5′-adenylyl β,γ-imidodiphosphate) (Sugino et al., 1978). This result has been interpreted as suggesting 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 functional domains. The A protein 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 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 structure of the 43-kDa N-terminal fragment 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) inhibit the ATPase reaction. Early studies suggested that the coumarins might be competitive inhibitors of the ATPase reaction (Sugino et al., 1978; Sugino & Cozzarelli, 1980), but more recent work supports the idea that they may act noncompetitively (Contreras & Maxwell, 1992; Ali et al., 1993; Maxwell, 1993). Sequencing of mutations in *gyrB* which confer 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). Subsequently, 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 recently been crystallized (Lewis et al., 1994).

One of the key questions concerning the mechanism of supercoiling by DNA gyrase is the coupling of ATP hydrolysis to the DNA supercoiling reaction. Although some studies of the ATPase reaction of gyrase have been reported

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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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(e.g., Mizuuchi et al., 1978; Sugino et al., 1978; Sugino & Cozzarelli, 1980; Staudenbauer & Orr, 1981; Maxwell & Gellert, 1984; Maxwell et al., 1986), detailed mechanistic studies have been hampered by low specific activities of preparations of the gyrase B protein and evidence of uncoupling of the ATPase and supercoiling reactions (J. A. Ali and A. Maxwell, unpublished data). However, cloning and purification of the 43-kDa N-terminal B fragment has allowed the analysis of the ATPase reaction of this isolated domain (Ali et al., 1993). This has included determination of the mechanism of ATP hydroysis by gyrase (Jackson & Maxwell, 1993). The hydrolysis of ATP by the 43-kDa fragment exhibits non-Michaelis–Menten kinetics and conforms to a scheme in which the active form of the protein is a dimer. Analysis of this scheme suggests that the release of products (ADP and P_i), or a conformational change associated with product release, may be the rate-limiting step in the overall supercoiling reaction (Ali et al., 1993).

Another approach to this problem is to study the binding of nucleotides to the enzyme. Recently the binding of ADPNP to intact gyrase (A_2B_2) has been studied (Tamura et al., 1992). These experiments showed that the binding and dissociation of ADPNP are slow but that these steps may be stimulated by ATP, depending on the presence of DNA. In the absence of DNA, ATP has no effect on the rate of binding of ADPNP, but in its presence ATP can accelerate the rate of binding more than 15-fold. The rate of dissociation of ADPNP is increased by about 100-fold by ATP but only in the presence of supercoiled DNA; linear and nicked-circular DNA are ineffective (Tamura et al., 1992). These results suggest a positively cooperative interaction between the two nucleotide-binding sites in gyrase.

In this paper we analyze the interaction of ADPNP with the 43-kDa fragment and discuss the implications for the hydrolysis of ATP by DNA gyrase and the inhibition of the ATPase reaction by coumarin drugs.

EXPERIMENTAL PROCEDURES

Enzymes and Assays. The 43-kDa N-terminal fragment of GyrB was prepared as described previously (Ali et al., 1993). Protein cross-linking using dimethyl suberimidate was performed as follows. Samples containing the 43-kDa protein (10 μM) in 50 mM Hepes (pH 8.5), 100 mM KCl, 4 mM $MgCl_2$, and 4 mM DTT in the absence and presence of ADPNP (1 mM) were incubated for 1 h at 25 °C. Dimethyl suberimidate was added to 4 mg/mL and the samples were incubated at 30 °C for various times up to 16 h. A second aliquot of dimethyl suberimidate was added after 2 h. Samples were quenched by the addition of glycine (pH 8) to 50 mM and analyzed by SDS–polyacrylamide gel electrophoresis. Tryptic digests were performed under the same conditions with the addition of trypsin (Sigma) to 8.6 μg/mL and incubation for up to 2 h at 25 °C. Samples were quenched by boiling in 2% SDS before analysis by SDS–polyacrylamide gel electrophoresis. N-Terminal sequencing of tryptic fragments was carried out by Miss E. Cavanagh (University of Leicester). ATPase assays were performed using the pyruvate kinase/lactate dehydrogenase linked assay described by Ali et al. (1993). Nucleotide binding was performed using Sephadex G50 spin columns based on the method described by Tamura et al. (1992), using either "homemade" columns in 1-mL syringes (Maniatis et al., 1982) or commercial columns (Pharmacia). Reaction mixtures containing 43-kDa protein and [α-^{32}P]ADPNP (3000 Ci/mmol; ICN) in binding buffer [50 mM Tris·HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA, 10% (w/v) glycerol, and 0.5 mg/mL BSA] were incubated at 25 °C. (When ATP was present, pyruvate kinase and phosphoenolpyruvate (2 mM) were added to maintain the concentration of ATP.) At various times, samples (100 μL) were removed and applied to 1-mL spin columns which had been preequilibrated in binding buffer. Columns were centrifuged at 3000 rpm in a bench-top centrifuge (Maniatis et al., 1982), the eluant was collected, and the amount of protein-bound ADPNP was determined by scintillation counting. Recoveries of 43-kDa protein (in the absence of ADPNP) were estimated by assaying for ATPase activity (Ali et al., 1993).

Dissociation rates were determined by incubating 43-kDa protein (10 μM) with an excess of radiolabeled ADPNP (300 μM) at 25 °C for 1 h. Unbound nucleotide was removed by passing 100 μL of the reaction mixture through a spin column. The eluted protein–ADPNP complex was diluted 10-fold in binding buffer in the presence of a >50-fold excess of cold ADPNP, and samples (100 μL) were removed at various times up to 5 h and applied to spin columns as described above.

Novobiocin–Sepharose Chromatography. Novobiocin–Sepharose columns were made as described by Staudenbauer and Orr (1981) and protein samples were applied and eluted as described by Gilbert and Maxwell (1994).

Modeling. Computer modeling of potential kinetic schemes was performed using the kinetic simulation programs KSIM (Neil Millar, Kings College London) and by KINSIM (Carl Freiden, Washington University, St. Louis, MO). Global fitting of the data was performed by the OS/2 version of the numerical fitting procedure FITSIM (Barshop et al., 1983; Zimmerle et al., 1987; Zimmerle & Frieden, 1989). Each data set had eight time points, from 15 to 240 min, but as the fitting process requires evenly spaced time points, a further eight were interpolated by fitting each data set to a single exponential and determining the required interpolated points from the fit. The fitting was scaled by a factor of 1.2 to account for the difference between the maximum amplitude of ADPNP binding and the concentration of the 43-kDa protein determined by protein assay (Bradford, 1976). ADPNP binding data for 5, 10, and 20 μM protein and the ADP inhibition data were globally fitted.

RESULTS

Interaction of ADPNP with the 43-kDa Protein. The 43-kDa N-terminal fragment of the DNA gyrase B protein has been crystallized in the presence of ADPNP and its structure solved to high resolution by X-ray crystallography (Wigley et al., 1991). In this structure the 43-kDa protein is a dimer and there is evidence from ATPase kinetics that the nucleoside triphosphate stimulates dimerization (Ali et al., 1993). In addition, gel filtration and equilibrium sedimentation experiments suggested that the 43-kDa protein is a dimer in the presence of ADPNP (Ali et al., 1993). We have now obtained independent evidence for the existence of this nucleotide-induced dimer in solution.

Dimethyl suberimidate is a widely used protein cross-linking agent (Davies & Stark, 1970) which can react with

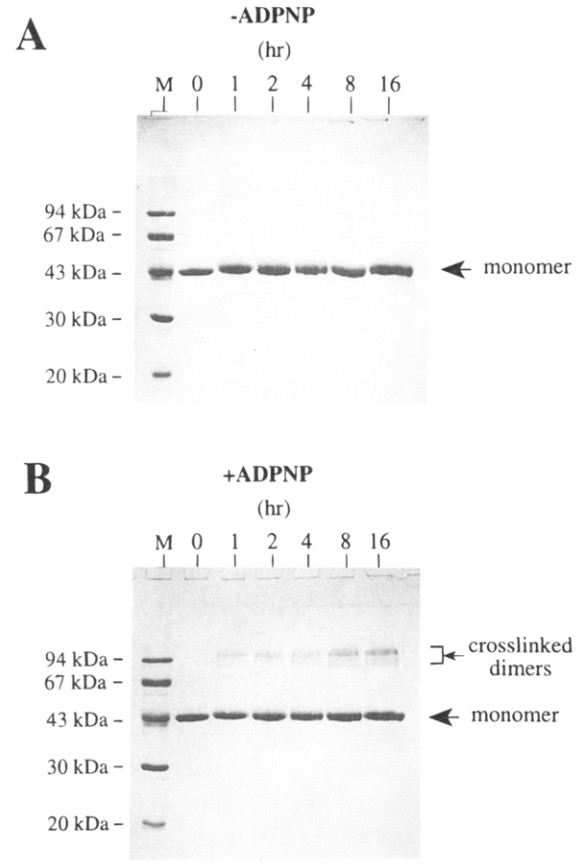


FIGURE 1: Cross-linking of the 43-kDa protein with dimethyl suberimidate. The 43-kDa protein (10 μM) was incubated with dimethyl suberimidate (4 mg/mL) either alone (A) or in the presence of 1 mM ADPNP (B) for the times indicated. Samples were analyzed by SDS−polyacrylamide gel electrophoresis.

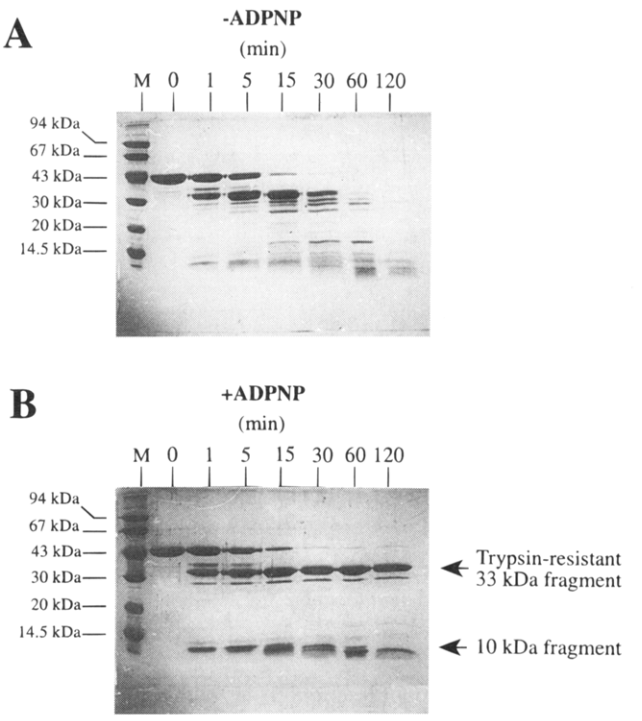


FIGURE 2: Limited proteolysis of the 43-kDa protein. The 43-kDa protein (10 μM) was incubated with trypsin (8.6 μg/mL) either alone (A) or in the presence of 1 mM ADPNP (B) for the times indicated. Samples were analyzed by SDS−polyacrylamide gel electrophoresis.

primary amino groups (e.g., lysines) in proteins. Incubation of the 43-kDa protein in the presence of dimethyl suberimidate generates no cross-linked products (Figure 1A). However, in the presence of ADPNP (1 mM) the 43-kDa protein can be cross-linked, generating species of molecular masses consistent with dimers (Figure 1B). The existence of several cross-linked products of similar electrophoretic mobility suggests that cross-linking can occur at several different positions. In a similar experiment in the presence of 50 μM ADPNP, results identical to those shown in Figure 1B were obtained (data not shown). In other work using this method we have examined the abilities of coumarin drugs to induce dimer formation. We have found that novobiocin stabilizes the monomer form of the 43-kDa protein, whereas coumermycin can induce dimer formation under certain conditions (Gormley et al., manuscript in preparation).

Limited proteolysis of proteins can be used as a probe of their conformational states. When digested with trypsin at 1/50 (w/w), the 43-kDa protein is degraded to small peptides in ~2 h (Figure 2A). Under the same conditions, the 43-kDa protein complexed with ADPNP (1 mM) is relatively resistant to digestion, yielding a 33-kDa fragment, which is stable to further digestion for at least 2 h, and a 10-kDa fragment, which is produced within 1 min and gradually further digested with time (Figure 2B). In a similar experiment in the presence of 50 μM ADPNP, results identical to those shown in Figure 2B were obtained (data not shown). N-Terminal sequencing of the 33-kDa fragment revealed the sequence SNSYDS, corresponding to residues 2−7 of the 43-kDa protein (the N-terminal methionine is removed following translation). N-Terminal sequencing of the 10-kDa fragment gave the sequence XXATGD, corresponding to residues 308−313. This suggests that the protein is cleaved at lysine 307 to yield a 33-kDa N-terminal fragment which is protected from further tryptic digestion by the presence of the nucleotide. Such protection is consistent with the proposed structure of the 43-kDa protein−ADPNP complex from X-ray crystallography (Wigley et al., 1991).

Kinetics of ADPNP Binding to the 43-kDa Protein. The kinetics of binding of ADPNP to the 43-kDa protein was measured at a range of protein and nucleotide concentrations using the technique of rapid gel filtration (spin columns). Figure 3 shows the data obtained at 5, 10, and 20 μM 43-kDa protein at a range of ADPNP concentrations. The amplitudes of the binding curves were found to vary from approximately 0.5 to 1 mol of ADPNP/mol of protein, the amplitudes tending to increase with increasing ADPNP concentration (Figure 3). This observation suggests the existence of protein dimers containing either 1 or 2 bound ADPNP molecules (see Discussion).

Effect of ADP and ATP. ADP is known to be an inhibitor of the ATPase reaction of gyrase and the 43-kDa protein (Sugino & Cozzarelli, 1980; Maxwell et al., 1986; Ali et al., 1993) and also of the binding of ADPNP to gyrase (Tamura et al., 1992). Figure 4 shows the effect of ADP on the binding of ADPNP to the 43-kDa protein. ADP decreases both the amplitude and the apparent rate of binding.

Under certain conditions ATP has been found to stimulate the rate of binding of ADPNP to intact gyrase (Tamura et al., 1992). In Figure 5 we show the effect of ATP on the binding of ADPNP to the 43-kDa protein at a range of ATP concentrations. Increasing concentrations of ATP lead to a

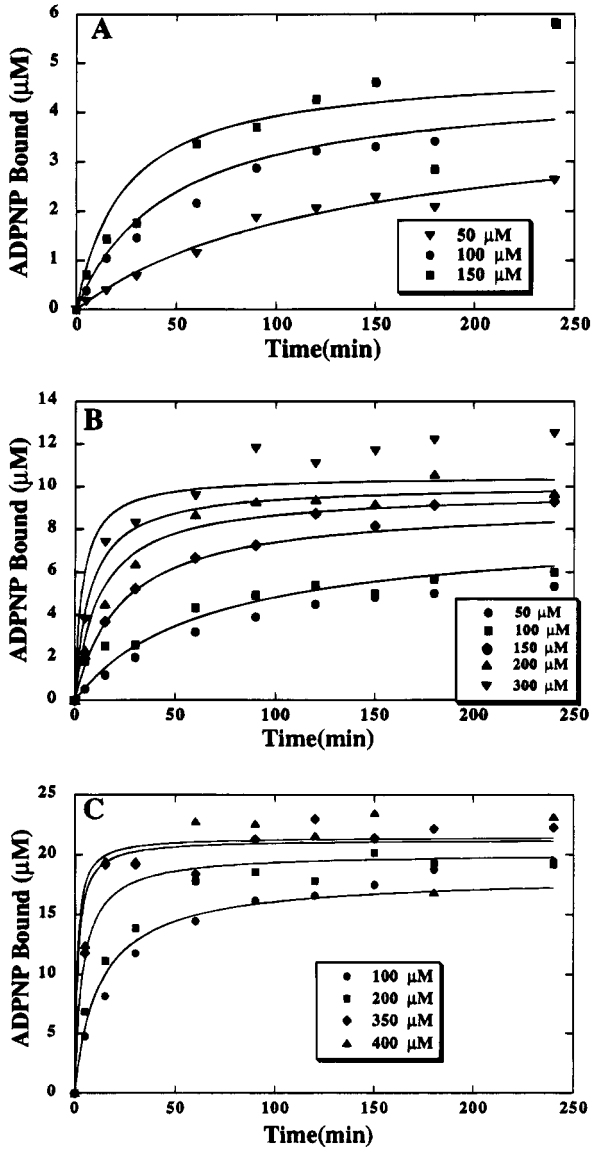


FIGURE 3: ADPNP binding to the 43-kDa protein. Binding experiments were carried out at 5 μM (A), 10 μM (B), and 20 μM (C) protein at the indicated concentrations of ADPNP. Curves were fitted using FITSIM and the scheme and rate constants given in the text.

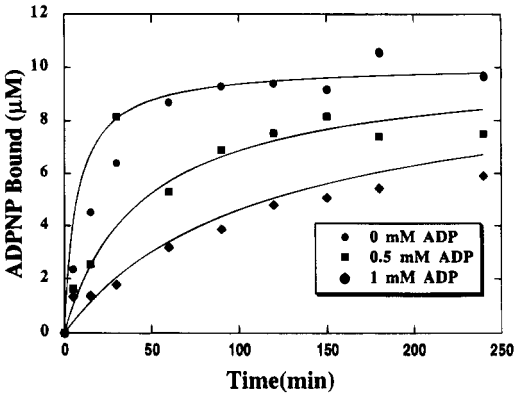


FIGURE 4: Effect of ADP on the binding of ADPNP to the 43-kDa protein. Protein and ADPNP concentrations were 10 and 200 μM, respectively; the ADP concentrations are indicated. Curves were fitted using FITSIM and the scheme and rate constants given in the text.

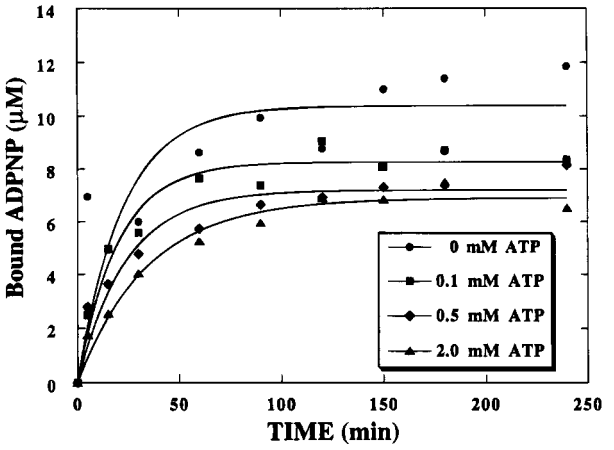


FIGURE 5: Effect of ATP on the binding of ADPNP to the 43-kDa protein. Protein and ADPNP concentrations were 10 and 200 μM, respectively; the ATP concentrations are indicated. The data have been fitted to single exponentials.

decrease in the amplitudes of ADPNP binding to the 43-kDa protein with little effect on apparent "on" rates.

Effect of Coumarin Drugs. Coumarin drugs are known to be potent inhibitors of the gyrase ATPase reaction with reported K_i values in the $10^{-7}-10^{-9}$ M range (Sugino et al., 1978; Sugino & Cozzarelli, 1980; Staudenbauer & Orr, 1981). In addition, both novobiocin and coumermycin have been shown to inhibit the ATPase activity of the 43-kDa fragment (Ali et al., 1993). Figure 6 shows that both novobiocin and coumermycin inhibit the binding of ADPNP to the 43-kDa protein. With 10 μM protein the amplitude of ADPNP binding was reduced to 50% in the presence of 5 μM novobiocin (Figure 6A) or 2.5 μM coumermycin (Figure 6B). At 10 μM novobiocin or 5 μM coumermycin, the binding was reduced essentially to background levels. These data show that coumarins inhibit nucleotide binding to the 43-kDa protein and are consistent with recent ATPase data (Ali et al., 1993) which suggest that coumarins bind very tightly to the 43-kDa protein and that novobiocin binds to the 43-kDa protein monomer while coumermycin stabilizes a dimer form.

The binding of novobiocin and ADPNP to the 43-kDa protein was also assessed using a novobiocin affinity column. GyrB and the 43-kDa protein have been shown to bind tightly to such a column and can only be eluted with high concentrations of denaturant such as urea (≥5 M) (Staudenbauer & Orr, 1981; Ali et al., 1993). A sample of 43-kDa protein (20 nmol) was bound to a 1-mL novobiocin column, and radiolabeled ADPNP (180 nmol) was subsequently added and allowed to remain on the column for 1.5 h. All the radioactivity was found to be eluted in a subsequent low-salt wash while the 43-kDa protein could only be eluted with 6 M urea (data not shown). This result suggests that ADPNP has a low affinity for the protein−novobiocin complex.

ADPNP Dissociation from the 43-kDa Protein. The spin-column method was used to determine the rate of dissociation of ADPNP from the 43-kDa protein at a range of protein concentrations (data not shown). The dissociation rate was found to be slow with a rate constant of $\sim 1.3 \times 10^{-6}$ s^{-1}. The addition of ATP (0.5 mM), ADP (0.5 mM), or novobiocin (0.1−1 mM) had little or no effect on this rate. However, at higher novobiocin concentrations (10 or 100 mM), the rate of dissociation was significantly increased (Figure 7). This suggests that the protein−ADPNP complex

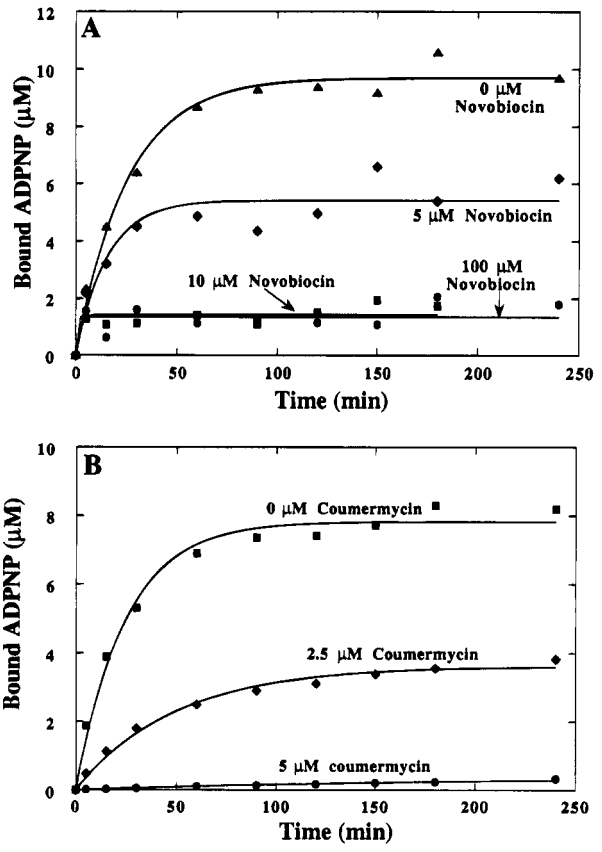


FIGURE 6: Effect of novobiocin (A) and coumermycin (B) on the binding of ADPNP to the 43-kDa protein. Protein and ADPNP concentrations were 10 and 200 μM, respectively; the drug concentrations are indicated. The data have been fitted to single exponentials.

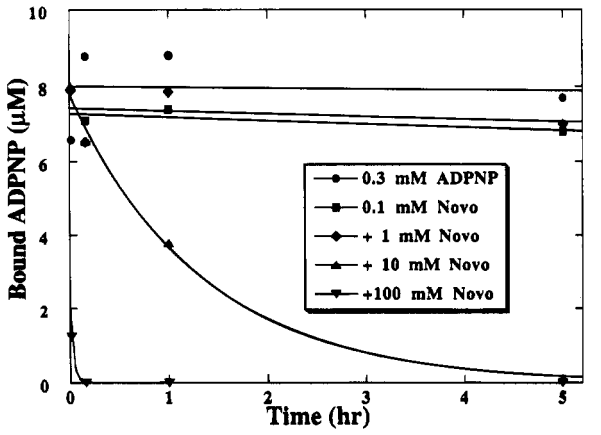


FIGURE 7: Dissociation of ADPNP from the 43-kDa protein, in the presence of ADPNP and novobiocin at the concentrations indicated. The data have been fitted to single exponentials.

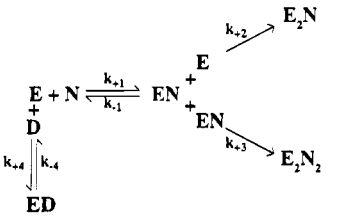
is able to bind novobiocin, albeit with low affinity, and that the resulting ternary complex rapidly releases the bound ADPNP.

In a related experiment, a 43-kDa protein−ADPNP complex was formed with radiolabeled ADPNP and any unbound nucleotide removed using a spin column. The eluted protein−ADPNP complex was applied to a novobiocin affinity column. In this case all the radioactivity eluted in a low-salt wash with some 43-kDa protein, but most of the protein was bound to the column and could only be removed with 6 M urea (data not shown). This again suggests that novobiocin can bind to the 43-kDa protein−ADPNP complex, resulting in the release of ADPNP from the complex.

DISCUSSION

The 43-kDa N-terminal fragment of the DNA gyrase B protein constitutes the ATPase domain of this enzyme. Protein cross-linking and proteolysis experiments presented here confirm earlier work (Wigley et al., 1991; Ali et al., 1993) demonstrating that in the presence of nucleoside triphosphate (ATP or ADPNP) this fragment forms a dimer, whereas the free protein is a monomer. We have proposed previously (Ali et al., 1993) that the dimer is the active form of this fragment, and in the context of the intact gyrase enzyme (A_2B_2), the 43-kDa domains undergo cycles of association−dissociation during the ATPase reaction. Proteolysis has also been used to probe nucleotide-induced conformational changes in yeast topoisomerase II (Lindsley & Wang, 1991). In this case different sites in the enzyme are susceptible to SV8 endoprotease in the absence and presence of ADPNP, indicating allosteric interdomainal movements in response to ATP binding.

We have studied the binding of ADPNP to the 43-kDa protein using rapid gel filtration. It was found that binding occurred with stoichiometries of ~0.5−1, depending on the nucleotide and protein concentrations (Figure 3). In the model for ATP hydrolysis presented by Ali et al. (1993), the monomer−ATP complex is converted to a dimer complex (43_2ATP_2), which can hydrolyze the bound ATP. The dimer complex with ADPNP was found to be very stable. Therefore the stoichiometries found in this study may be attributed to 43-kDa protein dimers with either one or two bound ADPNP molecules formed by the pathways shown in the following scheme:



In this scheme ADPNP (N) binds to the 43-kDa protein (E) to form a monomer−ADPNP complex (EN). This complex is assumed to be short-lived and cannot be detected by the spin-column method. The monomer complex can then form a dimer with either a free enzyme monomer or another monomer complex (E_2N or E_2N_2). Support for the existence of the E_2N species comes from the cross-linking and proteolysis experiments, which suggest that the 43-kDa protein is in the dimer form at both low (50 μM) and high (1 mM) ADPNP concentrations. With reference to Figure 3, we would expect stoichiometries of binding of ADPNP of ~0.5 and ~1, respectively, at these ADPNP concentrations. The formation of the dimer forms (E_2N and E_2N_2) is essentially irreversible, as shown by the value of the determined off rate ($\sim 1.3 \times 10^{-6}$ s^{-1}). In addition we have included the binding of ADP (D), which competes for the protein with ADPNP.

The kinetics for the formation of the dimer species E_2N and E_2N_2 is complex and there is no analytical solution to the reaction scheme given above. Therefore the ADPNP binding data (Figure 3) and the ADP inhibition data (Figure 4) were globally fitted to the above scheme. The data were

simulated by KSIM and KINSIM and were globally fitted using the numerical fitting routine of KINSIM, which is FITSIM. A total of 14 data sets were globally fitted (Figures 3 and 4). The following parameters were obtained from this global analysis: $k_{+1} = 1.1 \times 10^6\ M^{-1}\ s^{-1}$; $k_{-1} = 1.48 \times 10^4\ s^{-1}$; $k_{+2} = 2.1 \times 10^3\ M^{-1}\ s^{-1}$; $k_{+3} = 4.31 \times 10^5\ M^{-1}\ s^{-1}$; $k_{+4} = 2.1 \times 10^3\ M^{-1}\ s^{-1}$; $k_{-4} = 0.67\ s^{-1}$. The errors when all the parameters were floated were found to be large, in some cases as large as the rate constants. However, simulations show that the kinetics are dependent not on the absolute value of k_{+1} and k_{-1} but on their ratio, i.e., the K_d, so long as the equilibrium is rapid relative to the time course of ADPNP binding. A similar result was obtained for k_{+4} and k_{-4}, where the kinetics were dependent not on the absolute values of these rate constants but on their ratio, i.e., the K_i of ADP inhibition, so long as a rapid equilibrium relative to the time course of ADPNP binding was established. This yielded values for K_d and K_i of ~13 mM and ~320 μM, respectively. The kinetics of ADPNP binding are very sensitive to the dimerization rate constants, i.e., k_{+2} and k_{+3}. When k_{+1}, k_{-1}, k_{+4}, and k_{-4} were fixed at the rate constants shown above and the dimerization rate constants k_{+2} and k_{+3} were floated in a global fit by FITSIM, values of $k_{+2} = 2.1 \times 10^3\ M^{-1}\ s^{-1} \pm 37.8$ and $k_{+3} = 4.3 \times 10^5\ M^{-1}\ s^{-1} \pm 3.8 \times 10^3$ were obtained, i.e., small errors. Thus we can say with confidence that dimerization is considerably favored for the doubly occupied dimer (~200-fold greater). The values of these rate constants are not necessarily unique but show that the above scheme can indeed accommodate the data shown in Figures 3 and 4. Further work will be required to measure individual rate constants using other methods.

In this scheme and with these rate constants, ADPNP binding to the 43-kDa monomer is very weak (K_d ~13 mM). The concentrations of protein and ADPNP used in all the studies were significantly below this, so the equilibrium of ADPNP binding to the 43-kDa monomer ($E + N \rightleftharpoons EN$) would lie well over to the left. (Due to the weak binding, no 43-kDa protein−ADPNP monomer complexes would be detected by the spin-column method.) ADPNP binding to the 43-kDa protein is in sharp contrast to the binding of ATP to the 43-kDa monomer, which has a K_d of ~70 μM (Ali et al., 1993). The weaker binding of ADPNP could be due to a different conformation adopted by the Mg·ADPNP complex (Yount, 1975). Furthermore, an apparent slow initial binding of ADPNP to intact gyrase was found (Tamura et al., 1992), which was attributed to a slow conformational change, but it could also be due to the GyrB subunits having a much lower affinity for ADPNP, possibly due to a different conformation of the Mg·ADPNP complex.

The binding of ADPNP to the 43-kDa protein is driven forward by the irreversible dimerization reactions $E + EN \rightarrow E_2N$ and $EN + EN \rightarrow E_2N_2$. ADPNP binding has already been shown to promote the dimerization of the 43-kDa protein, with this dimer being a stable and long-lived species (Wigley et al., 1991; Ali et al., 1993). The off rate of ADPNP has been measured over a period of 3 days and found to by extremely slow, ~10^{-6} s^{-1} (data not shown). The assumption of irreversibility of the dimerization reaction is therefore valid. Although the rate constant for the formation of E_2N_2 was found to be ~200-fold greater than that for the formation of the singly occupied dimer E_2N, the formation of E_2N is significant due to the weak binding of ADPNP to the 43-kDa monomer, and the consequent very large excess of E with respect to EN that exists in solution. With ATP, very little of the singly occupied dimer would be likely to exist because ATP binding to the 43-kDa monomer is significantly tighter than ADPNP (~70 μM; Ali et al., 1993). Since the ATP concentration was above the K_d in this case and the dimerization reaction is rate-limiting, the major steady-state species would be the 43-kDa protein−ATP monomer complex. If the rate of formation of E_2ATP_2 is 200-fold greater than E_2ATP, then very little E_2ATP would exist.

Molecular weight studies have shown that ADP does not promote the dimerization of the 43-kDa protein (Ali et al., 1993). The ADP inhibition data were fitted so that ADP could reversibly bind to the 43-kDa monomer, yielding a K_i of ~320 μM. The off rate of ADP from the 43-kDa protein−ADP complex was computed to be 0.67 s^{-1}, which is very similar to the k_{cat} of gyrase (~1 s^{-1}; Higgins et al., 1978; Staudenbauer & Orr, 1981; Maxwell & Gellert, 1984; Ali et al., 1993), suggesting that product release is rate-limiting in the gyrase mechanism. However, given the potential uncertainties in the values of individual rate constants in the above scheme, definitive proof of product release being rate-limiting awaits the demonstration of a burst of ADP formation on-enzyme.

When ATP was included in the reaction mixture, the amplitudes were found to decrease with increasing ATP concentrations. Note that pyruvate kinase and phosphoenolpyruvate were included in the reaction mixture to convert any ADP produced back into ATP. No simple model was found to be able to account for these data. However, we can say that ATP would compete with ADPNP for binding to the 43-kDa protein monomer. E_2ATP_2 dimers would form in this mixture (Ali et al., 1993) and we cannot discount the possibility of E_2ADPNP·ATP complexes being formed, although we could find no evidence of this complex by the spin-column method (data not shown); i.e., if this species existed it would have to be short-lived. Hence the inhibition of ADPNP binding by ATP is likely to be complex. Single exponentials were fitted to these data for visual purposes (Figure 5). At a minimum we can say that ATP does not stimulate ADPNP binding to the 43-kDa protein. This is quite different for the case with gyrase, where ATP was found to stimulate ADPNP binding and this was explained in terms of positive cooperativity existing between the two ATPase sites in an intact gyrase molecule in the presence of DNA (Tamura et al., 1992). Clearly this type of positive cooperativity cannot occur for the 43-kDa protein.

The scheme described above is in agreement with steady-state data for the hydrolysis of ATP by DNA gyrase (Maxwell et al., 1986). In these experiments the hydrolysis of ATP by gyrase in the absence and presence of ADP was found to show a sigmoidal dependence on ATP concentration. These data were found to fit a model where two molecules of ATP were required to be bound to gyrase for turnover to occur. The molecular basis of this can now be described. ATP turnover cannot occur unless the 43-kDa domains of gyrase are in a dimer form (Wigley et al., 1991; Tamura et al., 1992; Ali et al., 1993). Dimerization of these domains is highly favored if both sites are occupied by ATP; this is likely to occur due to the positive cooperativity between the two ATPase sites within gyrase (Maxwell et al., 1986; Tamura et al., 1992). The ADPNP-binding studies with the 43-kDa protein show that, even in the absence of

this kind of positive cooperativity, dimerization of the 43-kDa protein with ADPNP bound is favored; the formation of E_2N_2 is favored over E_2N by ~200-fold. ATPase data for DNA gyrase in the presence of ADP showed a marked sigmoidal dependence of the rate on ATP concentration (Maxwell et al., 1986). These data were modeled such that ADP bound at either of the nucleotide-binding sites would prevent ATP hydrolysis; i.e., ADP would prevent the 43-kDa N-terminal domains of GyrB dimerizing within the intact gyrase enzyme, this dimerization being necessary for ATP hydrolysis to occur (Wigley et al., 1991; Ali et al., 1993).

It is interesting that in the above scheme the binding of ADPNP to only one monomer of the 43-kDa protein is sufficient to promote dimerization to an E_2N complex. Studies on yeast topo II (Lindsley & Wang, 1993) show that binding of ADPNP to one nucleotide-binding site is sufficient to drive allosteric interdomainal movements within the enzyme which lead to different proteolysis patterns detected in the N-terminal region of the enzyme, this region being equivalent to the B protein of gyrase. The different proteolysis patterns obtained by Lindsley and Wang in the presence of ADPNP for yeast topo II are likely to be due to the dimerization of the 43-kDa equivalent; indeed, this has been suggested to occur in yeast topo II in the presence of ADPNP (Roca & Wang, 1992, 1994). There is however, presently no evidence for nucleotide binding at one site in gyrase being sufficient to promote protein conformational changes. Thermodynamically, there is no reason why the binding of one molecule of ATP cannot drive the initial rounds of supercoiling in relaxed pBR322. However, since there is suspected to be strong positive cooperativity between the two ATP-binding sites in gyrase (Maxwell et al., 1986; Tamura et al., 1992), it is likely that two ATP molecules bind, since the binding of ATP to one site facilitates the binding of ATP to the other site of the intact gyrase molecule. In addition, we have shown here that dimerization of the 43-kDa protein strongly favours the species with nucleotide bound to both monomers, E_2N_2.

Coumarins were found to decrease ADPNP binding (Figure 6), consistent with their previously noted high affinity for the 43-kDa B fragment and stoichiometries of binding of 1 molecule of novobiocin per 43-kDa monomer and 1 molecule of coumermycin per dimer (Ali et al., 1993). When the 43-kDa protein was bound to a novobiocin affinity column, binding of ADPNP to the protein was not detected. Conversely, a preformed 43-kDa protein−ADPNP complex was found to bind to a novobiocin column with the release of ADPNP. In addition, high concentrations of novobiocin (10−100 mM) were found to accelerate the rate of dissociation of ADPNP from the 43-kDa protein (Figure 7). ATP, ADP, or low concentrations of novobiocin (up to 1 mM) had little or no effect.

The novobiocin results can be rationalized as follows. Novobiocin binds to the 43-kDa monomer with very high affinity, forming a complex which demonstrates no binding of ADPNP as measured by the spin-column method, i.e., the dimer complex is not detected. The 43-kDa dimer−ADPNP complex (E_2N_2) can bind novobiocin but with low affinity. This leads to the formation of a transient dimer complex containing both ADPNP and novobiocin, which leads to rapid dissociation of ADPNP. This result suggests that the coumarin drug and nucleotide-binding sites on the gyrase B protein may be at least partially distinct. However, given the high concentrations of novobiocin required to stimulate ADPNP dissociation, it is possible that this effect is nonspecific. It has been demonstrated that at high concentrations novobiocin can interact with several proteins, including histones and transcription factors (Cotten et al., 1986; Van Dyke & Roeder, 1987); i.e., the effects observed on the 43-kDa-ADPNP complex may reflect nonspecific binding of the drug.

In summary, we have shown that the 43-kDa N-terminal fragment of the DNA gyrase B protein forms dimers in the presence of ADPNP, containing either 1 or 2 molecules of bound nucleotide. These dimers are very stable, having very slow dissociation rates, with the formation of the dimer with 2 ADPNPs bound being strongly favored over the species with 1 ADPNP bound. ADP binds to the monomer form of the protein and prevents the dimerization reaction. Coumarin drugs inhibit the binding of ADPNP to the 43-kDa fragment, with novobiocin binding to the protein with a stoichiometry of 1:1 and coumermycin binding with a stoichiometry of 0.5:1, in agreement with previous observations.

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