Exploiting Nucleotide Thiophosphates To Probe Mechanistic Aspects of *Escherichia coli* DNA Gyrase†

Paul M. Cullis,*,‡ Anthony Maxwell,§ and David P. Weiner‡,§,||

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

Received October 31, 1996; Revised Manuscript Received January 30, 1997⊗

ABSTRACT: The interaction of DNA gyrase with ATP has been probed using a range of thiophosphate ATP analogs. ATPγS is not detectably hydrolyzed by gyrase but can support limited, probably catalytic, DNA supercoiling. ATPγS is a good inhibitor of both ATP hydrolysis and ATP-supported supercoiling. In contrast, both ATPαS(R_p) and ATPβS(R_p) have been shown to be good substrates for the ATPase reaction of gyrase and to support catalytic DNA supercoiling. The corresponding S_p diastereoisomers do not support significant levels of supercoiling and are not readily hydrolyzed, but are shown to be reasonable inhibitors of gyrase. For ATPαS(R_p), the supercoiling and ATPase activities appear to be tightly coupled with the thionucleotide being apparently a better substrate than ATP in terms of both DNA supercoiling and nucleotide hydrolysis. In the case of ATPβS(R_p), DNA supercoiling and nucleotide hydrolysis appear to be uncoupled in that ATPβS(R_p) is almost as good a substrate as ATP for the ATPase reaction of both intact gyrase and the 43 kDa GyrB fragment, whereas it only supports slow DNA supercoiling; the mechanistic consequences of these observations are discussed in terms of a new model for energy coupling in gyrase. DNA gyrase has been shown to be capable of catalyzing DNA supercoiling in the presence of Mg^{2+}, Ca^{2+}, and Mn^{2+} but not Zn^{2+}, Co^{2+}, Ni^{2+}, or Cd^{2+}. The pronounced diastereoselectivity seen in both the DNA supercoiling and ATPase activity with ATPαS and ATPβS together with evidence from the X-ray structure of the 43 kDa GyrB−ADPNP−Mg complex is consistent with metal ion coordination at both of these sites, and probably to the γ-phosphoryl center during turnover. Thus, the absolute configuration of the catalytically active Mg^{2+}−ATP complex is likely to involve coordination to the *pro-S* oxygens at both Pα and Pβ, leading to the α,β,γ-tridentate Mg−ATP complex with the Λ-exo configuration.

DNA gyrase catalyzes the introduction of negative supercoils into closed-circular DNA using the Gibbs energy derived from ATP hydrolysis [for reviews, see Reece and Maxwell (1991) and Wigley (1995)]. The enzyme from *Escherichia coli* consists of two proteins, GyrA and GyrB, encoded by the *gyrA* and *gyrB* genes. The molecular masses of the A and B proteins are 97 and 90 kDa, respectively, and the active enzyme is an A_2B_2 complex.

The mechanism of DNA supercoiling by gyrase involves the following steps: (i) wrapping of a segment of DNA (∼130 bp) around the enzyme in a positive superhelical sense, (ii) cleavage of the wrapped DNA in both strands with the formation of covalent bonds between the newly formed 5′-phosphates and Tyr122 of the A subunits, (iii) passage of another segment of DNA through this double-strand break, and (iv) resealing of the broken DNA, leading to a change in the linking number of −2 (ΔLk = −2). Catalytic supercoiling is coupled energetically to the hydrolysis of ATP. In the presence of the nonhydrolyzable ATP analog ADPNP (5′-adenylyl β,γ-imidodiphosphate), limited supercoiling can be achieved, suggesting that nucleotide binding promotes one cycle of supercoiling and that hydrolysis is required for enzyme turnover [reviewed in Maxwell and Gellert (1986), Reece and Maxwell (1991), Roca (1995), and Wigley (1995)].

Gyrase is an essential enzyme in bacteria but is not present in eukaryotes. It is therefore an ideal target for antibacterial agents. Two major groups of gyrase-specific drugs have been identified, the quinolones and the coumarins, both of which inhibit the DNA supercoiling reaction *in vitro* (Drlica & Coughlin, 1989; Rádl, 1990; Reece & Maxwell, 1991; Maxwell, 1992, 1993). The quinolones (e.g. nalidixic acid and ciprofloxacin) are thought to act at the A subunit and interfere with the DNA breakage and reunion process. Incubation of gyrase with DNA in the presence of a quinolone drug and termination of the reaction by the addition of SDS result in the irreversible covalent attachment of the cleaved DNA to the enzyme. The coumarins (e.g. novobiocin and coumermycin A_1) act at the B subunit and inhibit the hydrolysis of ATP by gyrase.

There is good evidence that both subunits of gyrase are organized as distinct functional domains (Reece & Maxwell, 1991; Wigley, 1995). The N-terminal domain of GyrA (59−64 kDa) contains the DNA breakage and reunion activities and is the likely site of action of the quinolone drugs. The C-terminal 33 kDa domain has no intrinsic catalytic activity but can bind DNA and is thought to be involved in DNA

† This work was supported by the BBSRC and is a publication from the Leicester Biomolecular Sciences Centre. A.M. is a Lister Institute Jenner Fellow.

* Address for correspondence: Department of Chemistry, Leicester University, Leicester LE1 7RH, U.K.

‡ Department of Chemistry.

§ Department of Biochemistry.

|| Present address: Department of Molecular Biology and Chemistry, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037.

⊗ Abstract published in *Advance ACS Abstracts,* May 1, 1997.

S0006-2960(96)02725-0 CCC: $14.00

wrapping. The N-terminal 43 kDa domain of GyrB contains the ATPase site and the binding site for coumarin drugs. The structure of this 43 kDa protein complexed with ADPNP has been solved by X-ray crystallography to 2.5 Å resolution (Wigley et al., 1991), and the structure of a 24 kDa subdomain of this protein complexed with novobiocin and a cyclothialidine analog has recently been solved (Lewis et al., 1996). The C-terminal 47 kDa domain of GyrB is thought to be involved in the binding to GyrA and DNA. The existence of these domains within the structure of the intact proteins has been confirmed by differential scanning microcalorimetry (Blandamer et al., 1994).

In common with all energy-coupled enzyme-catalyzed processes, the crucial problem in understanding the gyrase mechanism concerns the nature of the coupling between the hydrolysis of the nucleotide and the energy-requiring process, namely DNA supercoiling. Recent work from this laboratory has focused on the mechanism of the gyrase ATPase reaction. This has involved kinetic analysis of ATP hydrolysis and nucleotide binding by the 43 kDa fragment (Ali et al., 1993, 1995), identification of the active site residues (Jackson & Maxwell, 1993), and analysis of the energy coupling (Cullis et al., 1992). Nucleotide thiophosphate analogs in which one or more of the nonbridging phosphoryl oxygens is substituted by sulfur have been widely used to probe enzyme-catalyzed reactions at phosphorus, and these studies have been extensively reviewed (Cohn, 1982; Eckstein, 1985; Frey, 1989). In the present study, we have exploited the thiophosphoryl ATP analogs (i) to probe the mechanism of the ATPase reaction of DNA gyrase and the 43 kDa domain of GyrB, (ii) to probe the nature of the coupling between nucleotide hydrolysis and DNA supercoiling, and (iii) to define the structure of the active metal−nucleotide complex. The metal ion coordination is of particular interest since, for a number of nucleoside triphosphate-handling enzymes, for example the Ha-Ras p21 protein (Pai et al., 1990; Schlichting et al., 1990), important changes in protein conformation associated with regulation appear to be triggered through interactions with the coordinated metal ion. Therefore, in the case of DNA gyrase, interactions with the metal ion might play a crucial role in mediating interactions between the various domains and hence in energy transduction itself.

EXPERIMENTAL PROCEDURES

Synthesis of Thiophosphate ATP Analogs. ATPαS was synthesised as a racemic mixture by the method of Ludwig and Eckstein (1989). The R_p and S_p epimers of ATPαS were isolated from the racemic mixture by preparative reverse phase HPLC using a linear gradient of acetonitrile (0 to 20%) in 100 mM triethylammonium bicarbonate (TEAB) with the S_p epimer eluting before the R_p epimer. Generally, HPLC resolution of the thionucleotides used in this study was achieved using conditions based on those of Ludwig and Eckstein (1989), Lazewska and Granowski (1990), and Marquetant and Goody (1983). Fractions corresponding to single peaks on the chromatogram were pooled, concentrated repeatedly with additions of methanol to remove TEAB, and stored at −20 °C as aqueous solutions. The isomeric purity of the samples was confirmed by analytical reverse phase HPLC and by ^{31}P NMR.

The triethylammonium salt of adenosine 5′-*O*-(2-thiodiphosphate) (ADPβS) was synthesised following the procedure of Goody and Eckstein (1971). The pure R_p and S_p epimers of ATPβS were prepared from ADPβS by exploiting the stereospecificity of kinase action at prochiral centers using high-energy phosphorylating agents. Procedures were based on those reported by Eckstein and Goody (1976). Pyruvate kinase was used to give predominantly the S_p epimer of ATPβS, while hexokinase was used to degrade contaminating amounts of the R_p epimer. The reaction conditions for the pyruvate kinase-catalyzed reaction were as follows: 50 mM Tris-HCl (pH 8.0), 200 mM KCl, 1 mM DTT, 4 mM $MgCl_2$, 2 mM ADPβS, 2.2 mM phosphoenolpyruvate, and 0.7 mg of pyruvate kinase (350 units, Sigma), in a final volume of 27 mL. The reaction mixture was incubated at 25 °C for 4 h, and the reaction was stopped by extraction with an equal volume of chloroform/isoamyl alcohol (24:1) and the aqueous layer loaded onto a DEAE-Sephadex (Pharmacia) column and eluted with a linear gradient of TEAB (100 to 600 mM). The isolated ATPβS contained significant amounts of the R_p epimer which was degraded to ADPβS using the following conditions: 50 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM DTT, 4 mM $MgCl_2$, 1 mM ATPβS(S_p), 1 mM glucose, and hexokinase (250 units, Sigma), in a final volume of 25 mL. The reaction was stopped by passage through Chelex 100 (Sigma), and the pure ATPβS(S_p) was purified by ion-exchange chromatography on DEAE-Sephadex, eluting with a linear gradient of TEAB (100 to 600 mM). After removal of TEAB, the triethylammonium salt of ATPβS(S_p) was stored at −20 °C as a frozen aqueous solution.

Acetate kinase was used to catalyze the phosphorylation of ADPβS to give almost entirely the R_p epimer of ATPβS, while any S_p contaminant was degraded with myosin S1 fragment (gift of R. Ankrett, Department of Biochemistry, Leicester University). The reaction conditions for the acetate kinase-catalyzed reaction were as follows: 50 mM Tris-HCl (pH 8.0), 7.2 mM $MgCl_2$, 1 mM DTT, 3.6 mM ADPβS, 30 mM acetyl phosphate, and acetate kinase (400 units, Sigma). The reaction mixture was incubated at 25 °C for 4 h and stopped by extracting with an equal volume of chloroform/isoamyl alcohol. The ATPβS was purified by ion-exchange chromatography as described for the S_p epimer. Any contaminating ATPβS(S_p) was removed using the following conditions: 50 mM Tris-HCl (pH 8.0), 4 mM $MgCl_2$, 1 mM DTT, 1 mM ATPβS(R_p), and myosin S1 (4 units). The reaction was stopped by passage through Chelex 100 (Sigma), and the pure ATPβS(R_p) was purified by ion-exchange chromatography. After removal of TEAB, an aqueous solution of the triethylammonium salt of ATPβS-(R_p) was stored at −20 °C. The isomeric purity of both R_p and S_p ATPβS was confirmed by analytical reverse phase HPLC using a linear gradient of 0 to 5% acetonitrile in 100 mM TEAB containing 20 mM $MgCl_2$.

NMR Data. ^{31}P NMR proton-decoupled spectra were recorded at 121.5 MHz on a Bruker AM300 instrument. For ATPαS, samples consisted of 2 mM nucleotide dissolved in 100 mM CAPS buffer (pH 9.6) containing 10% D_2O and 10 mM EDTA, in a final volume of 0.5 mL. ATPαS(S_p): ^{31}P NMR (121.5 MHz, D_2O) δ + 42.75 (d, J = 28 Hz; Pα), −23.11 (overlapping dd, Pβ), −6.20 (d, J = 20 Hz, Pγ). ATPαS(R_p): ^{31}P NMR (121.5 MHz, D_2O) δ +42.44 (d, J = 28 Hz, Pα), −23.13 (overlapping dd, Pβ), −6.19 (d, J = 20 Hz, Pγ). For ATPβS, samples consisted of 11.9 mM nucleotide dissolved in 200 mM Tris-HCl (pH 8.8) containing 10% D_2O and 10 mM EDTA, in a total volume of 0.5

mL. ATPβS(S_p): ^{31}P NMR (121.5 MHz, D_2O) δ −12.20 (d, J = 27 Hz, Pα), +28.59 (overlapping dd, Pβ), −6.89 (d, J = 28 Hz, Pγ). ATPβS(R_p): ^{31}P NMR (121.5 MHz, D_2O) δ −12.19 (d, J = 26 Hz, Pα), +28.54 (overlapping dd, Pβ), −6.93 (d, J = 28 Hz, Pγ).

Enzymes and DNA. The DNA gyrase A and B proteins were prepared as described by Hallett et al. (1990), and the A_2B_2 complex was reconstituted as described by Bates and Maxwell (1989). The N-terminal 43 kDa fragment of GyrB was purified as described by Ali et al. (1993). Protein concentrations were determined by the method of Bradford (1976). Relaxed plasmid pBR322 DNA was prepared as described previously (Bates & Maxwell, 1989).

Enzyme Assays. DNA supercoiling assays were carried out as described by Cullis et al. (1992). Three ATPase assays were used. (i) A pyruvate kinase/lactate dehydrogenase (PK/LDH)-linked assay was carried out as described previously (Tamura & Gellert, 1990; Ali et al., 1993). The reaction conditions were the same as for DNA supercoiling assays except that in addition 250 μM NADH, 400 mM phosphoenolpyruvate, pyruvate kinase (1.1 units, Sigma), and lactate dehydrogenase (1.4 units, Sigma) were present and the total assay volume was 150 μL. (ii) A continuous enzyme-linked phosphate assay coupling the release of phosphate to the nucleoside phosphorylase-catalyzed phosphorolysis of 7-methylguanosine (m^7Guo) to 7-methylguanine (m^7Gua) based on the method of Banik and Roy (1990) was carried out as described by Ali et al. (1993). (iii) A single-time point malachite green assay involving the formation of a phosphomolybdate complex with P_i which can be detected spectrophotometrically at $A_{600}-A_{650}$ (Lanzetta et al., 1979; Chan et al., 1986) was used. For most experiments, a scaled down version of the assay in microtiter plates was used. Thirty microliters of ATPase reaction mixture was incubated for various times (usually 5 h), and successive 2-fold dilutions were made in a microtiter plate. One hundred and fifty microliters of malachite green reagent was added to each well. The plate was incubated on ice for 30 min, and 20 μL of 0.5% Tween 20 was added to each sample. After 15 min at room temperature, the plate was read at 630 nm in a microtiter plate reader. An internal calibration of phosphate standards was incorporated on each plate.

The hydrolysis of ATPγS was investigated using ^{35}S-labeled ATPγS (Amersham International). In these experiments, gyrase (75 nM) was incubated with ATPγ^{35}S (1 mM, ca. 100 000 cpm) and DNA (linear pBR322, 10 μg/mL) and the reaction progress monitored by separation of the product by thin layer chromatography on PEI−cellulose plates and quantitation by scintillation counting of the spots corresponding to ATPγ^{35}S and $P^{35}S_i$.

ATPase data for the 43 kDa GyrB fragment were fitted to a steady state rate equation (Ali et al., 1993) using MacCurveFit 1.2 (Kevin Raner Software, Australia). Gyrase ATPase data were fitted to the Michaelis−Menten equation using Kaleidagraph (Abelbeck Software).

RESULTS AND DISCUSSION

The various nucleotide thiophosphate analogs that have been prepared and investigated are shown in Scheme 1. In the case of both ATPαS and ATPβS (but not ATPγS), the substitution of one of the nonbridging oxygens by sulfur generates a new chiral center, leading in each case to two diastereoisomers that are distinguished in the following discussion by reference to their absolute configurations at the thiophosphoryl center.

Scheme 1

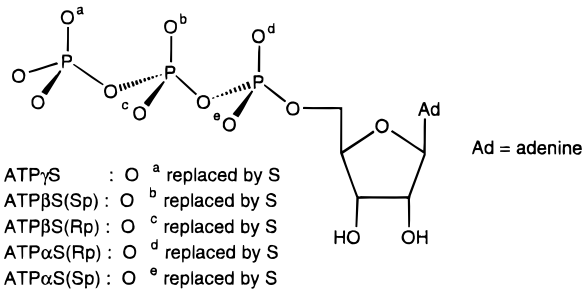


Table 1: Inhibition of ATP-Dependent Supercoiling by ATP Analogs

	concentration giving 50% inhibition of supercoiling (mM)	
nucleotide	0.3 mM ATP	0.03 mM ATP
ATPγS	0.02	−
ATPαS(S_p)	−	1
ATPβS(R_p)	0.3	−
ATPβS(S_p)	0.6	−

DNA Supercoiling

ATPγS. Under the standard supercoiling assay conditions with ATPγS at 1 mM replacing ATP, a gyrase concentration of 25 nM, and a DNA concentration of 3.4 nM, a change in the linking number of −10 was observed in 120 min. Because gyrase catalyzes a change in the linking number of DNA in steps of two, and the enzyme to DNA ratio was approximately 7, the maximum ΔLk achievable by stoichiometric supercoiling would be approximately −14. In practice, the real limit is likely to be below this because of the fraction of the enzyme that is known to be inactive in supercoiling (Ali et al., 1995). In addition, recent work by Bates et al. (1996) would suggest that the coupling probability with relaxed DNA is ∼0.25; i.e. stoichiometric supercoiling would introduce a ΔLk of approximately −4. The fact that the observed ΔLk of −10 is above this level suggests that the supercoiling supported by ATPγS is likely to be catalytic rather than stoichiometric.

To provide evidence that ATPγS does indeed bind to gyrase, we have also looked at the inhibition of ATP-dependent DNA supercoiling. At an ATP concentration of 0.3 mM, 50% inhibition of supercoiling by ATPγS was observed at a concentration of ∼0.02 mM (Table 1), i.e. at an approximately 10-fold lower concentration, confirming that ATPγS binds comparatively tightly to gyrase.

ATPαS. DNA gyrase supercoiling assays were carried out in the presence of ATP and the R_p and S_p diastereoisomers of ATPαS. Under the standard assay conditions, the nucleotide concentration was kept constant at 1 mM while the enzyme concentration was varied. The concentration of enzyme required to convert approximately 50% of the relaxed plasmid pBR322 to the supercoiled form in 1 h at 25 °C was compared for each nucleotide. The results show a striking preference for the R_p diastereoisomer over the S_p diastereoisomer of ATPαS (Table 2); indeed, under these conditions, ATPαS(R_p)-supported supercoiling was indistinguishable from that of ATP, while no activity with the S_p

Table 2: Comparison of the Epimers of ATPαS *versus* ATP as Substrates for Gyrase

nucleotide (1 mM)	gyrase concentration for 50% supercoiling (nM)	relative effectiveness as substrate
ATP	1.5	1
ATPαS(R_p)	1.5	1
ATPαS(S_p)	≫150	≪0.01

epimer of ATPαS was evident. At 2 mM ATPαS(S_p), a very low level of supercoiling was apparent with a change in Lk of approximately −2, but this was observable only at very high gyrase concentrations (150 nM) which could represent a stoichiometric reaction (data not shown).

The nucleotide dependence of the rate of supercoiling can be estimated from laser densitometry of the supercoiled DNA band resolved by agarose gel electrophoresis. The rate of the ATPαS(R_p)-supported DNA supercoiling reaction was investigated by determining the effects of nucleotide concentration at a fixed enzyme concentration and directly comparing this with ATP. At nucleotide concentrations below 1 mM, ATPαS(R_p) appeared to be a better substrate than ATP. For example, the progress of the supercoiling reaction was very similar for ATP at 1 mM and ATPαS(R_p) at 0.1 mM. With ATP at 0.1 mM or ATPαS(R_p) at 0.01 mM, the supercoiling rates were again very similar but greatly reduced from reactions at the higher nucleotide concentrations. Significantly, above 1 mM, the rates of supercoiling supported by ATP and by ATPαS(R_p) were very similar. These data are suggestive of a difference in binding affinity of approximately 1 order of magnitude between ATP and ATPαS(R_p), with the latter having the higher affinity.

A comparison of the apparent rate of supercoiling for ATP and the analog at 0.1 mM reveals that ATPαS(R_p)-supported supercoiling is ∼4−5 times faster than that with ATP. These apparent rates are not linear but curve off with increasing time. This reflects the difficulties in measuring the kinetics of supercoiling and the fact that the supercoiled band on an agarose gel consists of many topoisomers with specific linking differences of roughly −0.04 or more. Thus, the experiment described here only monitors the formation of moderately supercoiled topoisomers and cannot follow further supercoiling; i.e. the quantitation of supercoiling by this method is not accurate.

The S_p epimer of ATPαS supports very limited supercoiling even at high concentrations. Given that ADPNP has been shown not to be hydrolyzed by gyrase (Tamura et al., 1992) and to promote stoichiometric supercoiling (Sugino et al., 1978; Bates et al., 1996), in principle, ATPαS(S_p) could support either stoichiometric or catalytic supercoiling. Attempts to distinguish these two possibilities were inconclusive. For stoichiometric supercoiling, ΔLk should reach a maximum, after sufficient time, which is proportional to the gyrase concentration. This was not observed for ATPαS(S_p), and instead, a slow but steady increase in ΔLk up to about −8 was seen in 12 h with 150 nM gyrase and 3.4 nM DNA (data not shown). Because of the high levels of enzyme required to detect activity with ATPαS(S_p), this level of supercoiling is still below the level that could in principle arise from stoichiometric supercoiling [even allowing for a proportion of inactive GyrB in the preparation, see Ali et al. (1995); and for low coupling efficiencies, see Bates et al. (1996)]. Hence, it does not unequivocally indicate enzyme turnover. However, the observation of slow, but apparently catalytic, hydrolysis of ATPαS(S_p) (see below) supports the possibility that the supercoiling reaction is also catalytic but very slow.

The failure to support efficient supercoiling may arise from poor binding of ATPαS(S_p) to DNA gyrase or from inefficient hydrolysis or energy coupling. In order to explore the binding of this analog to the enzyme, its properties as an inhibitor of the ATP-dependent supercoiling reaction were investigated. The concentration of ATPαS(S_p) was varied at a constant ATP concentration of 0.03 mM in a gyrase-catalyzed supercoiling reaction. Approximately 50% inhibition occurred at a concentration of 1.0 mM ATPαS(S_p), i.e. ∼35-fold higher than the ATP concentration (Table 1). This suggests that ATPαS(S_p) is a comparatively poor competitive inhibitor of ATP and that this will contribute to its failure to support significant DNA supercoiling.

ATPβS. With ATPβS, gyrase again exhibited a marked preference for the R_p diastereoisomer over the S_p diastereoisomer. In a standard 1 h supercoiling assay (1 mM nucleotide), no activity was observed for ATPβS(S_p) while a slow introduction of superhelical turns was noted with ATPβS(R_p). At a high enzyme concentration (75 nM), a ΔLk of about −6 was produced with the R_p diastereoisomer under these conditions. As a rough indication of the efficacy of ATPβS(R_p) in supporting DNA supercoiling, at a gyrase concentration of 75 nM, ATPβS(R_p) gave less supercoiling than ATP in the presence of a gyrase concentration of 0.75 nM; i.e. over 100-fold more enzyme was required for ATPβS(R_p). Thus, the effectiveness of ATPβS(R_p) was <0.01 compared to ATP under these conditions.

The low level of supercoiling with ATPβS(R_p) was further investigated by monitoring the progress of the supercoiling reaction over a long time course. After an 8 h incubation, a ΔLk of approximately −8 was seen which corresponds to four rounds of supercoiling. Since the proportion of enzyme to DNA was roughly 7:1 (a DNA concentration of 3.4 nM and an enzyme concentration of 25 nM), it would seem that the low level of supercoiling observed could in principle be ascribed to a stoichiometric mechanism. However, the hydrolysis of ATPβS(R_p) by gyrase is comparatively efficient (see below), which would be consistent with a catalytic supercoiling reaction, albeit at a very slow rate.

Both the modest supercoiling supported by ATPβS(R_p) and the lack of activity with ATPβS(S_p) could again arise because of poor binding of the nucleotide to the enzyme. This was addressed by investigating the ability of both diastereoisomers of ATPβS(S_p) to inhibit ATP-dependent supercoiling (Table 1). The results show that under these conditions both diastereoisomers of ATPβS are moderate inhibitors and suggest that ATPβS(R_p) is bound by gyrase approximately as tightly as ATP while ATPβS(S_p) was bound roughly 2-fold more weakly than ATP. Clearly, the poor supercoiling seen with ATPβS(R_p) and the lack of supercoiling with ATPβS(S_p) must arise from factors other than weak binding. Of course, it is possible that when inhibiting the ATP-induced supercoiling reaction the two ATPβS diastereoisomers could in each case be bound in a nonproductive binding orientation.

ATPase Activity

ATPγS. The ability of gyrase to hydrolyze ATPγS was investigated using both a malachite green assay (relying on

the conversion of inorganic thiophosphate to phosphate under acidic conditions) and ^{35}S-labeled ATPγS. Using both methods, no significant hydrolysis could be detected. Under the conditions of these assays, it would have been possible to detect $\geq$1% hydrolysis, from which it can be concluded that if ATPγS is hydrolyzed by gyrase the rate must be $\leq 10^{-3}$ the rate of ATP hydrolysis. However, it is clear that the level of hydrolysis of ATPγS that would be required to account for the DNA supercoiling discussed above falls well below the limits of detection in these assays.

To confirm that ATPγS is capable of binding to gyrase, the inhibition of ATP hydrolysis by ATPγS was briefly investigated. At 0.3 mM ATP, the concentration of ATPγS required to inhibit P_i formation by 50% was roughly 0.03 mM, in good agreement with the IC_{50} values determined in the DNA supercoiling assays (see above).

ATPαS and ATPβS. Monitoring the ATPase reaction with thiophosphate analogs is not entirely straightforward. The pyruvate/lactate dehydrogenase-coupled ATPase assay has been previously used with DNA gyrase for ATP itself (Tamura & Gellert, 1990; Ali et al., 1993) and relies on the easy monitoring of the regeneration of ATP by the coupled enzymes. This convenient assay is not appropriate for monitoring the hydrolysis of ATPαS and ATPβS. With ATPβS, both the R_p and S_p diastereoisomers are hydrolyzed to ADPβS which is now prochiral at Pβ. Since the stereospecificity of pyruvate kinase is not absolute (Cohn, 1982; Eckstein, 1985), both the R_p and S_p diastereoisomers of ATPβS will be regenerated with a progressive loss of stereochemical integrity, thus altering the kinetics in a complex manner. With ATPαS, hydrolysis yields ADPαS which still retains its chirality and therefore, in principle, the PK/LDH assay should be suitable. However, the fact that the sulfur analogs of ADP [especially ADPαS(R_p)] are much worse substrates for pyruvate kinase than ADP would require that large amounts of coupling enzyme be used so as not to be rate-limiting.

A coupled assay measuring phosphate release that utilizes nucleoside phosphorylase to catalyze the phosphorolysis of 7-methylguanosine to give 7-methylguanine and ribose 1-phosphate has been developed (Banik & Roy, 1990). This reaction is associated with a convenient change in fluorescence. We have used this assay to monitor the hydrolysis of ATPαS and ATPβS by gyrase for comparison with ATP. Control experiments indicated that the coupling system was active in the gyrase supercoiling buffer, giving a linear decrease in fluorescence with increasing phosphate concentrations up to about 20 μM (data not shown). It was found to be necessary to omit BSA from the reaction as this gave a large fluorescence quenching effect. Control supercoiling and PK/LDH ATPase experiments with gyrase in the presence and absence of BSA gave identical results. When ATPase activity was measured using the fluorescence assay, it was found that the rate was proportional to the gyrase concentration over the range tested (up to 150 nM).

The steady state kinetics of ATP hydrolysis by DNA gyrase in the presence of DNA, investigated by both the PK/LDH assay and the fluorimetric phosphate assay, are shown in Figure 1A. A hyperbolic curve of the form of the Michaelis−Menten equation was fitted to the data, and a least-squares nonlinear regression program was used to obtain values for V_{max}^{app} and K_M^{app}. In this analysis, the presence of two ATP sites and the probable cooperativity between the two sites were not considered (Maxwell et al., 1986; Tamura et al., 1992). It can be seen that there was a

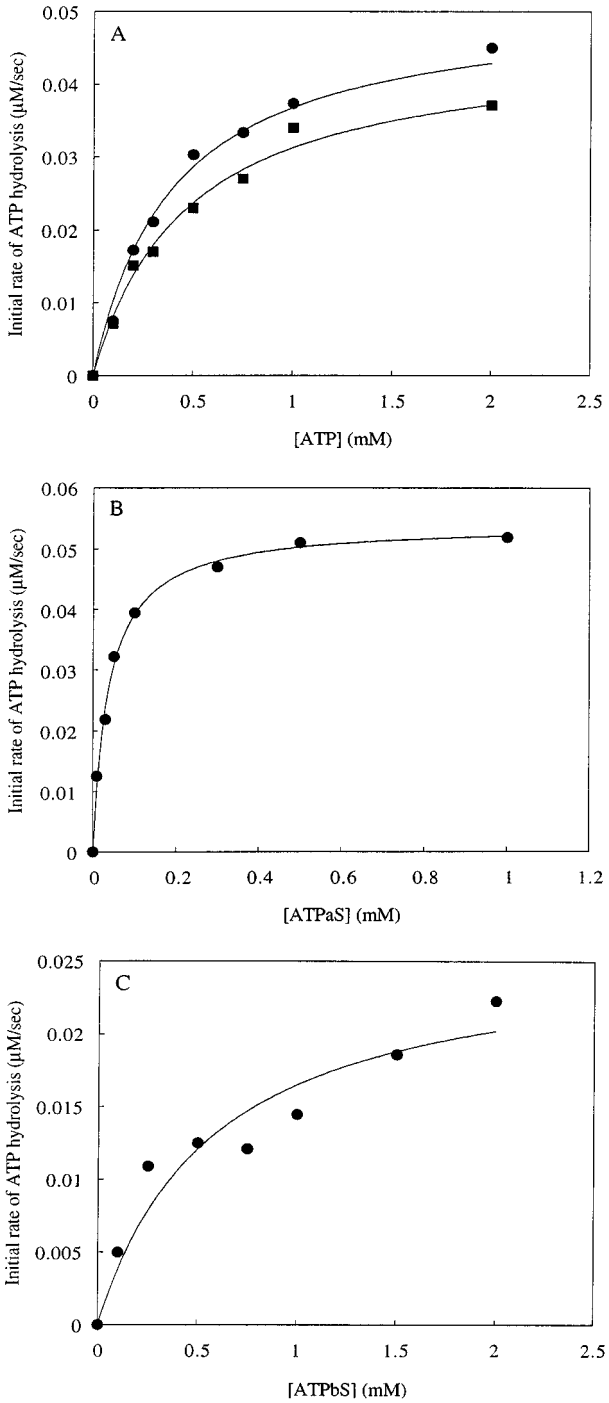


FIGURE 1: Hydrolysis of ATP and the thiophosphate analogs by DNA gyrase. ATPase assays were carried out in the presence of excess linear pBR322 DNA using the fluorescence assay (Banik & Ray, 1990) or the PK/LDH assay (Ali et al., 1993) as indicated. Panel A shows the hydrolysis of ATP by gyrase as measured by the fluorescence assay (filled circles) and the PK/LDH assay (filled squares). Curves were fitted to the Michaelis−Menten equation and the following kinetic parameters derived: fluorescence assay, K_m^{app} = 0.40 mM and V_{max}^{app} = 0.051 μM s^{-1}; PK/LDH assay, K_m^{app} = 0.47 mM and V_{max}^{app} = 0.046 μM s^{-1}. Panel B shows the hydrolysis of ATPαS(R_p) by gyrase as measured by the fluorescence assay: K_m^{app} = 0.038 mM and V_{max}^{app} = 0.054 μM s^{-1}. Panel C shows the hydrolysis of ATPβS(R_p) by gyrase as measured by the fluorescence assay: K_m^{app} = 0.58 mM and V_{max}^{app} = 0.026 μM s^{-1}. In all experiments, the gyrase concentration was 50 nM, yielding k_{cat}^{app} values of 1 and 0.9 s^{-1} for ATP and 1 and 0.5 s^{-1} for ATPαS(R_p) and ATPβS(R_p), respectively.

reasonable comparison between the two assays, giving similar values for K_M^{app} and k_{cat}^{app} which compared favorably with those reported in the literature, e.g. Maxwell and Gellert (1984). Panels B and C of Figure 1 show the initial rates of hydrolysis plotted against nucleotide concentration for ATPαS(R_p) and ATPβS(R_p); no hydrolysis was detected for the S_p epimers which explains their inability to substitute for ATP in the supercoiling reaction. ATPαS(R_p) was hydrolyzed well by gyrase and gave a k_{cat}^{app} similar to that of ATP of ~1 s^{-1}, while the K_M^{app} was almost 11-fold lower than that of ATP. These kinetic properties of ATPαS(R_p) are reflected in its effectiveness as a substrate for supercoiling, where ATPαS(R_p) had an activity equivalent to that of ATP at a concentration roughly 10-fold lower. Furthermore, at high concentrations (1 mM) of ATP and ATPαS(R_p), well above their respective K_M^{app}s, their effectiveness in the supercoiling reaction was roughly equivalent, which corresponds to the similar rates of hydrolysis at saturating levels of nucleotide. An additional correlation between hydrolysis and supercoiling with these nucleotides is available from a comparison of the rates of supercoiling at different nucleotide concentrations and the rates of hydrolysis; for example, in the supercoiling experiments, the rate with 0.1 mM ATPαS-(R_p) is between 4 and 5-fold faster than that with 0.1 mM ATP, and correspondingly, under these conditions, ATPαS-(R_p) is hydrolyzed ~4-fold faster than ATP. Thus, in this respect, the hydrolysis of this analog appears to be well coupled to the supercoiling reaction.

To confirm the stereoselectivity of gyrase with ATPαS, the hydrolysis of a racemic mixture of ATPαS was analyzed over a long time course by reverse phase HPLC (data not shown). Reaction mixtures were comprised of 150 nM gyrase and 1 mM nucleotide in a total volume of 30 μL. After 112 min, almost all of the R_p epimer of ATPαS was hydrolyzed to the corresponding epimer of ADPαS with virtually no hydrolysis of ATPαS(S_p). This gives an average turnover rate over this period of about 0.5 s^{-1}. After 1320 min, a low level of hydrolysis of ATPαS(S_p) was detected, up to about 15%. This corresponds to about 75 μM ATPαS-(S_p) hydrolyzed and an average turnover rate of 6.3×10^{-3} s^{-1}. Thus, hydrolysis of ATPαS(S_p) is slow but clearly catalytic.

The detection limit for formation of P_i by either the malachite green or the nucleoside phosphorylase single-time point assay is ~5 μM. No hydrolysis of ATPβS(S_p) was detected by either assay after a 5 h incubation with 75 nM gyrase and 1 mM nucleotide, giving an upper limit of ATPβS(S_p) hydrolysis of 3.7×10^{-3} s^{-1}. Using the nucleoside phosphorylase assay, ATPβS(R_p) was also shown to be a reasonably good substrate in terms of the gyrase ATPase activity, with a K_M^{app} similar to that of ATP and a k_{cat}^{app} only ~2-fold lower than that of ATP (Figure 1C); i.e. ATPβS(R_p) is hydrolyzed by gyrase 260 times faster than ATPβS(S_p). However, ATPβS(R_p) was a poor substrate for supercoiling, supporting only a very slow reaction. Intriguingly therefore, the hydrolysis of ATPβS(R_p) by gyrase appears to be substantially uncoupled from DNA supercoiling.

Nucleotide Stereospecificity of the N-Terminal Fragment of GyrB (43 kDa Protein)

The ATPase activity of DNA gyrase lies within the 43 kDa domain of GyrB. The rate of ATP hydrolysis by the

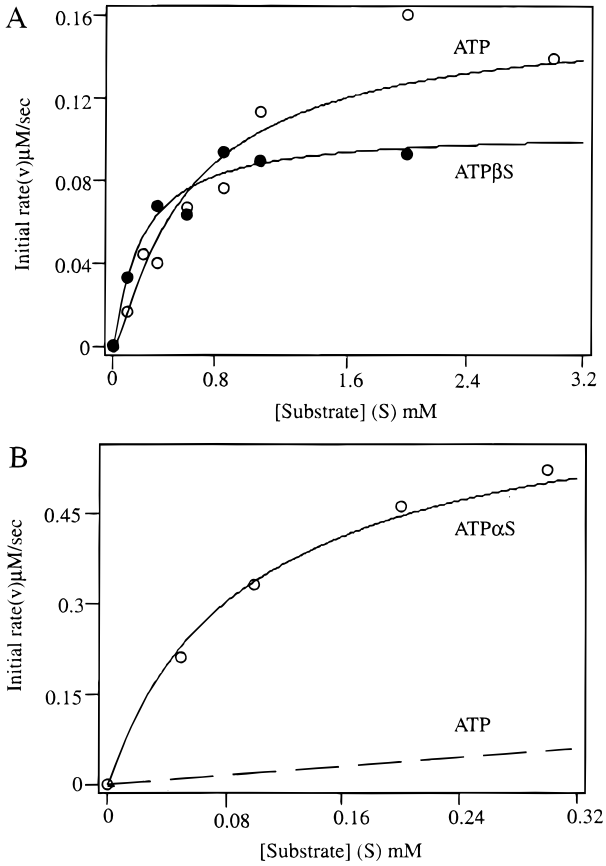
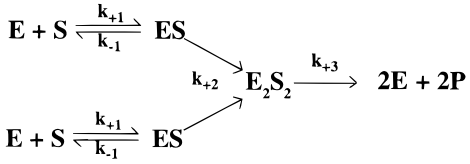


FIGURE 2: Hydrolysis of ATP and thiophosphate analogs by the 43 kDa fragment. ATPase assays were carried out using the fluorescence assay with 15 μM protein. Data were fitted to the steady state rate equation (Ali et al., 1993) derived from Scheme 2. Panel A shows the hydrolysis of ATP (open circles) and ATPβS-(R_p) (filled circles). Panel B shows the hydrolysis of ATPαS(R_p) (open circles), with the fitted curve for ATP hydrolysis from panel A shown for comparison (dashed line).

43 kDa protein exhibits a greater than first-order dependence on protein concentration, consistent with the active form of the protein being a dimer (Ali et al., 1993). Using the fluorescence ATPase assay described above, we observed a similar dependence of the rate on enzyme concentration (data not shown). To simplify the analysis, all ATPase experiments with the 43 kDa fragment were carried out at a protein concentration of 15 μM. At constant enzyme concentrations, the ATPase reaction displays approximately hyperbolic kinetics. Figure 2 shows the steady state kinetics of ATP hydrolysis by the 43 kDa fragment at this protein concentration. It has previously been shown (Ali et al., 1993) that the ATPase reaction of the 43 kDa fragment of GyrB can be accommodated by the following scheme:

Scheme 2



In this scheme, nucleoside triphosphate (S) binds to the 43 kDa protein (E) to form a monomer−NTP complex which can then dimerize. Only this dimer form (E_2S_2) can hydrolyze NTP to release products (P) and the free enzyme.

A steady state rate equation has been derived on the basis of this scheme, and values for the rate constants have been determined (Ali et al., 1993). Under the conditions of these experiments (i.e. enzyme concentrations in the micromolar range), the maximum velocity of the reaction is determined largely by k_{+2} (the dimerization rate constant); *viz.* dimerization is rate-limiting under these conditions. The data shown in Figure 2 for ATP can be accommodated by Scheme 2 with rate constants similar to those previously derived. The hydrolysis of ATPαS(R_p) by the 43 kDa protein can also be accommodated by Scheme 2, but in this case, significant changes to the rate constants must be made. These data can only be fitted with increases in k_{+2} (the dimerization rate constant) of more than a factor of 2. In addition, the equilibrium dissociation constant for nucleoside triphosphate ($K_d = k_{-1}/k_{+1}$) has to decrease by about 10-fold. Hence, ATPαS(R_p) would appear to bind ~10-fold tighter to the enzyme and stimulate an increase in the rate of dimerization of the 43 kDa fragment.

In the case of ATPβS(R_p), the ATPase data shown in Figure 2 can be fitted to Scheme 2 with rate constants very similar to those derived for ATP. In particular, there does not appear to be any significant changes in K_d required; i.e. the affinity of the enzyme for ATPβS(R_p) is similar to that for ATP. It must be pointed out that there is insufficient data in Figure 2 to uniquely define the rate constants in Scheme 2, but it is clear that significant alterations in the rates of one or more of the steps must occur with ATPαS(R_p).

Metal Ion Studies

Mg^{2+} can bind to the phosphate oxygens of ATP, in principle, giving rise to either mono-, bi-, or tridentate chelates. Coordination to the α- and β-phosphates generates new chiral centers, and thus, there are several potential metal chelates in solution from which an enzyme will usually be specific for just one (Huang & Tsai, 1982). Studies using nucleotide thiophosphate analogs can probe the nature and structure of the nucleotide−metal chelation involved in catalytic turnover.

It has been shown by NMR spectroscopy (Jaffe & Cohn, 1978a) and subsequently by measurements of metal−thionucleotide stability constants (Pecoraro & Cleland, 1984) that, in complexes with phosphorothioate nucleotide analogs, Mg^{2+} prefers to chelate *via* oxygen whereas softer metals such as Cd^{2+} prefer sulfur [as would be expected from a consideration of hard and soft Lewis acids and bases (Pearson, 1966; Cotton & Wilkinson, 1972)]. Thus, for example, the S_p epimer of ATPαS (or ATPβS) when complexed to Mg^{2+} has the same relative configuration [as defined by Cornelius and Cleland (1978)] as the Cd^{2+} complex of the R_p epimer (i.e. they have the same relative configuration in terms of the metal chelate).

Through studies on a wide range of phosphoryl transfer enzymes, it has been shown that often where metal chelation occurs to either the α- or β-phosphoryl residues this can be reflected in a marked stereoselectivity for one of the thiophosphoryl diastereoisomers in each case. Unambiguous evidence for metal coordination at a particular site is only available if reversal of the diastereoselectivity is observed on substitution of Mg^{2+} by a softer thiophilic metal such as Cd^{2+}. The classic example of such a reversal of stereoselectivity comes from experiments with hexokinase, where it was found that ATPβS(R_p) was the better substrate with Mg^{2+} while ATPβS(S_p) was favored with Cd^{2+} (Jaffe & Cohn, 1978b, 1979). This reversal of stereoselectivity was taken as evidence of the coordination of the metal to the β-phosphate of ATP in the enzyme reaction. In similar experiments with ATPαS, no reversal of stereoselectivity was observed, and it was concluded that, in the hexokinase−Mg−ATP complex, the metal is probably not coordinated to the α-phosphate.

Table 3: Rates of Hydrolysis of ATP, ATPαS, and ATPβS with Mg^{2+} and Cd^{2+} by the 43 kDa Protein

nucleotide	[nucleotide] (mM)	[Mg^{2+}] (mM)	rate of hydrolysis (μM s^{-1})	[Cd^{2+}] (mM)	rate of hydrolysis (μM s^{-1})	rate of Mg / rate of Cd
ATP	2.0	1.98	0.162	2.19	0.067	2.4
ATPαS(R_p)	0.3	0.57	0.49	0.38	0.014	35
ATPαS(S_p)	0.3	0.57	−	0.38	−	−
ATPβS(R_p)	1.0	1.72	0.10	0.93	0.023	4.4
ATPβS(S_p)	1.0	1.72	−	0.93	−	−

In an attempt to determine whether reversal of stereoselectivity could be demonstrated in the presence of softer metal ions, the ability of other metals to substitute for Mg^{2+} in gyrase-catalyzed reactions was investigated. In supercoiling assays in the presence of ATP, it was found that Mg^{2+}, Ca^{2+}, and Mn^{2+} supported the reaction while Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} did not. ATPαS(R_p) showed behavior similar to that of ATP, but with ATPαS(S_p), only very low levels of supercoiling could be detected with Mg^{2+} and nothing with Cd^{2+}. One difficulty in interpreting these data concerns the multiple roles of metal ions in the supercoiling reaction, where they are involved in the interaction of gyrase with not only ATP but also DNA. The DNA−metal−protein interaction may obscure the nucleotide−metal−gyrase interaction.

In order to focus on the nucleotide−protein interaction, ATPase assays with different metal ions were carried out with the 43 kDa fragment which does not require DNA as an effector. The fluorescence phosphate-coupled assay offered a way of measuring the hydrolysis of ATP and the phosphorothioate analogs with different metal ions, since the coupling reaction, namely the nucleoside phosphorylase-catalyzed phosphorolysis of m^7Guo, does not appear to require a divalent metal ion. This was confirmed by control experiments which showed an identical decrease in fluorescence on addition of a given amount of phosphate in the presence and absence of Mg^{2+} ions. The effect of Cd^{2+} ion concentration on the activity of nucleoside phosphorylase in the coupled assay was also investigated. It was found that, up to 0.5 mM Cd^{2+}, there was no measurable effect on the activity of the phosphorylase enzyme. However, at higher concentrations, Cd^{2+} was found to be inhibitory, reducing the rate of formation of m^7Gua such that this step was partially rate-limiting. The effect of Cd^{2+} ions on the 43 kDa protein was also examined. At high Cd^{2+} concentrations (2 mM and above), the protein (15 μM) was clearly seen to precipitate. In order to avoid these problems, the concentration of free Cd^{2+} in experiments with the 43 kDa protein was kept below 0.2 mM. The rates of hydrolysis of ATP and the thiophosphate analogs by the 43 kDa protein in the presence of Mg^{2+} or Cd^{2+} are shown in Table 3. The concentration of each nucleotide was chosen to be near

saturating, and an excess metal ion concentration was calculated to ensure that 90% of the nucleotide was complexed to the metal. It can be seen from Table 3 that Cd−ATP gives a rate of hydrolysis only 2.4-fold slower than that of Mg−ATP. With the phosphorothioate ATP analogs, the R_p isomers for both ATPαS and ATPβS were better substrates in the presence of Mg^{2+} but were hydrolyzed at measurable rates in the presence of Cd^{2+}. For ATPαS(R_p), there was a 35-fold preference for the Mg complex, while for ATPβS(R_p), the preference for the Mg complex was only 4.4-fold. Since these data were obtained at just one nucleotide concentration, the effects on the rate could result from changes in binding or hydrolysis steps (Scheme 2). The two corresponding S_p diastereoisomers, ATPαS and ATPβS, showed no measurable activity with either Mg^{2+} or Cd^{2+}. Had the diastereoselectivities arisen solely from the sites of metal ion coordination at Pα and/or Pβ, then in each case Mg^{2+} and Cd^{2+} would show opposite stereoselectivities with the epimers of ATPαS and/or ATPβS.

CONCLUSIONS

Catalytic DNA supercoiling catalyzed by DNA gyrase is known to require the hydrolysis of ATP, with a single round of supercoiling requiring the hydrolysis of 2 mol of ATP. It is also clear from studies with the nonhydrolyzable ATP analog ADPNP that a single round of supercoiling can be induced by nucleotide binding and that catalytic turnover requires hydrolysis of the nucleotide. In a recent study using ATPαS(R_p), we reported that the extent of supercoiling supported by this ATP analog was consistently slightly greater than that for ATP itself (Cullis et al., 1992). We were able to show that ATPαS(R_p) had a slightly larger free energy of hydrolysis than ATP ($\sim\Delta\Delta G = 1$ kJ mol^{-1}), and we argued that this was sufficient to account for the additional supercoiling, suggesting that the extent of supercoiling catalyzed by DNA gyrase is limited by the free energy of hydrolysis of the nucleotide. In this present study, we have looked at the full range of thiophosphate analogs of ATP in an attempt to probe further the nature of the coupling between nucleotide hydrolysis and DNA supercoiling.

In the case of ATPγS, substitution of sulfur at the reactive phosphoryl group leads to almost complete abolition of the various gyrase activities. Within the limits of detection, there is no hydrolysis of ATPγS catalyzed by DNA gyrase, although ATPγS does support low levels of DNA supercoiling. The extent of supercoiling observed in these latter experiments could be accounted for by stoichiometric supercoiling, analogous to that seen with ADPNP. However, given that the extent of supercoiling exceeds that which would be found with ADPNP, we are inclined to suggest that ATPγS supports a slow catalytic reaction. We have also shown that ATPγS is a reasonable inhibitor of both the ATPase and the ATP-dependent DNA supercoiling reactions of gyrase, with IC_{50} values in the range of 20−30 μM. The extremely slow rate of hydrolysis of ATPγS by DNA gyrase inferred from these observations has precluded attempts to determine the stereochemical course of the ATPase reaction but is consistent with a number of other phosphatases where the corresponding thiophosphate analogs are not hydrolyzed at any appreciable rates. There are likely to be many possible explanations for this, but in purely mechanistic terms, thiophosphates are considerably less reactive via an associative reaction pathway because of the reduced electrophilicity of the thiophosphoryl center.

The studies with ATPαS indicate a pronounced preference for one of the two diastereoisomers. The R_p diastereoisomer is a very good substrate for DNA supercoiling and nucleotide hydrolysis catalyzed by gyrase, and in terms of the ATPase activity of the 43 kDa GyrB protein. The results are all broadly similar. With DNA supercoiling, the data are consistent with a ~10-fold increased affinity of gyrase for ATPαS(R_p) compared with that for ATP. In the gyrase ATPase reaction, the data suggested a K_M^{app} value reduced by a factor of ~11, while in the case of the 43 kDa protein, the ATPase data were consistent with a K_d value for ATPαS(R_p) ~10-fold lower than that for ATP. Taken together, it seems that the enzyme has an ~10-fold higher affinity for ATPαS(R_p) than for ATP. There are likely to be several possible explanations for this apparent increased affinity. First, the Mg−ATP complex in solution will be an equilibrium between the various coordinated states and diastereoisomeric forms, only one species of which will be recognized as a substrate for gyrase. The substitution of the *pro-R* nonbridging oxygen at Pα for sulfur reduces the number of alternative Mg complexes by favoring coordination to the oxygen at Pα [see also Goody and Hoffman (1980)]. The tighter binding of ATPαS(R_p) may also reflect the expected easier desolvation of thiophosphate esters as compared to that of phosphate esters (Hine & Mookerjee, 1975; Wolfenden et al., 1979) which could result in a more favorable free energy of transfer from bulk water to the gyrase active site. It is also not possible to exclude the possibility of a specific, fortuitous favorable interaction between the protein and the sulfur ligand of ATPαS(R_p).

In stark contrast, ATPαS(S_p) does not support significant levels of DNA supercoiling, although some supercoiling is detectable at very high gyrase concentrations. In keeping with this, ATPαS(S_p) is not appreciably hydrolyzed by either intact DNA gyrase or the 43 kDa GyrB protein. It is not possible to exclude the possibility that the low level of supercoiling seen at high gyrase concentrations could arise from a stoichiometric reaction since ATPαS(S_p) has been shown to bind to the protein, although the binding is relatively weak. The results with the ATPαS diastereoisomers are consistent with a tight coupling between nucleotide hydrolysis and DNA supercoiling. The differences between the two diastereoisomers is likely to be due, at least in part, to metal ion coordination (see below).

For the diastereoisomers of ATPβS, again a marked preference for the R_p isomer was observed. For ATPβS(R_p), a slow supercoiling reaction was observed, whereas, under the same conditions, for ATPβS(S_p), no DNA supercoiling could be detected. In terms of the ATPase reaction of gyrase and of the 43 kDa GyrB protein, ATPβS(R_p) is hydrolyzed almost as efficiently as ATP, whereas ATPβS(S_p) is not hydrolyzed under these conditions. The diastereoselectivity seen for ATPβS can be rationalized in terms of coordination of Mg^{2+} to this center. The intriguing uncoupling of the nucleotide hydrolysis from DNA supercoiling is less easy to account for. Experiments with yeast DNA topoisomerase II (topo II) support a reaction mechanism in which the enzyme contains two protein "gates" (Roca & Wang, 1992, 1994; Berger et al., 1996). One of these gates is an ATP-operated clamp which serves to capture a DNA segment (the "transport" or T-segment) which may

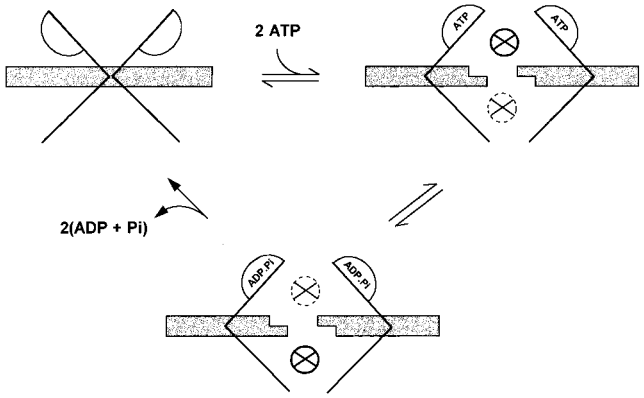


FIGURE 3: Diagrammatic representation of the catalytic cycle of DNA gyrase. The solid bar (gray) represents the segment of DNA that is transiently cleaved (G-segment), and the crossed circle is the translocated DNA segment (T-segment). Gyrase is represented as the "scissor" structure that must undergo a conformational change that operates the entry and exit gates in a cycle that is controlled by the ATPase domain as depicted. The T-segment can be regarded as being in equilibrium between the entry and exit gates in steps 2 and 3.

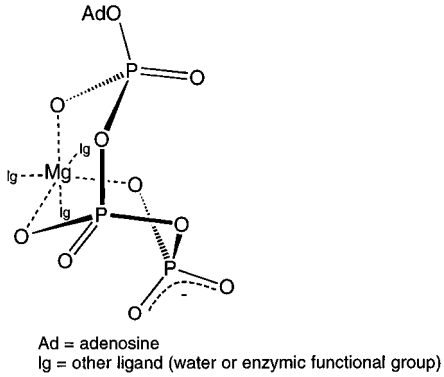


FIGURE 4: Absolute configuration of the catalytically active α,β,γ-tridentate complex between ATP and Mg^{2+}. This Λ-exo configuration (Cornelius & Cleland, 1978) has both the α- and β-phosphoryl residues coordinated through the *pro-S* oxygens in each case and is inferred from the stereoselectivity observed between the two diastereoisomers of ATPαS and ATPβS, respectively. Coordination to the γ-phosphoryl group is invoked on mechanistic grounds and is supported by the X-ray structure of the 43 kDa protein fragment complexed to ADPNP (Wigley et al., 1991).

then pass through a double-stranded break in another piece of DNA (the gate or G-segment) held open by the enzyme. This ATP-operated clamp is equivalent to the 43 kDa N-terminal GyrB fragment of gyrase which is known to undergo cycles of association−dissociation driven by the binding and hydrolysis of ATP (Wigley et al., 1991; Ali et al., 1993). In the case of topo II, the T-segment leaves the enzyme through a second protein gate (Roca & Wang, 1994; Roca et al., 1996). It is very likely that a similar mechanism operates in the case of DNA gyrase. Such a mechanism is depicted diagrammatically in Figure 3. In this figure, no attempt has been made to illustrate the detailed protein structure as much of this is unknown. For simplicity, we show gyrase existing in two conformational states: a nucleotide-free state where the DNA is intact and the "entry" and "exit" gates are both open and an ATP-bound state where the DNA is cleaved and the protein gates are closed. In reality, a number of other states must exist, but we have used a minimal scheme sufficient to account for the data in this study. Here the enzyme is represented as an X-shaped structure which can undergo conformational changes upon ATP binding and hydrolysis. ATP binding closes the entry gate and may trap a DNA segment which can be translocated through the double-standed break in the G-segment. The T-segment can be considered as being in equilibrium between the entry and exit gates. It is not clear what is required to translocate the T-segment from the entry gate to the exit gate, but it is likely that this might depend on the torsional stress of the DNA. The role of ATP hydrolysis in the process is not entirely clear; it may have a role in the translocation process or may merely serve to "reset" the enzyme to the starting conformation. With reference to recent work by Bates et al. (1996) where the coupling probability (the probability of completing a strand-passage event) was found to depend on the specific linking difference (σ) of the substrate DNA, we can suggest that positively supercoiled DNA is more likely to drive the T-segment to the exit gate whereas negatively supercoiled DNA may favor the T-segment remaining in the entry gate. In this latter case, ATP hydrolysis and product release may result in loss of the T-segment from the entry gate and failure to complete a round of DNA supercoiling. This is consistent with the low coupling probability found for negatively supercoiled DNA substrates (Bates et al., 1996). In other work (Tingey & Maxwell, 1996), we have found that mutation of a putative DNA-binding residue in the entry gate (Arg286 of GyrB) abolishes DNA supercoiling and the DNA-dependent ATPase reaction of gyrase. These results are also consistent with the model proposed in Figure 3 and suggest that capture of the T-segment by the clamp (the entry gate) is a prerequisite for DNA-dependent ATP hydrolysis.

In the case of ATPβS(R_p), it is interesting to speculate on the uncoupling between the ATPase and supercoiling reactions in the context of the model in Figure 3. For example, it is feasible that the binding and hydrolysis of the nucleoside triphosphate are very similar to that with ATP but that product release is altered. If ADPβS dissociated rapidly (compared with ADP) and thus disfavored the translocation of the T-segment from the entry gate to the exit gate, then DNA supercoiling would be inefficient; i.e. premature product release would allow the protein gates to open and the loss of the T-segment through the entry gate. Preliminary studies on inhibition of DNA supercoiling by ADP and ADPβS have shown that the latter is indeed less tightly bound by at least a factor of 2 which would be consistent with the above proposal (data not shown).

The marked stereospecificities seen with both ATPαS and ATPβS could be interpreted in terms of the structure of the metal−nucleotide complex dictated by the preference of Mg^{2+} to coordinate with the nonbridging oxygen rather than the sulfur. However, only in the case where it is possible to demonstrate that the diastereoselectivities are reversed when the metal ion is changed from Mg^{2+} to a softer metal such as Cd^{2+} is this conclusion unequivocal. In the case of DNA gyrase, the Mg^{2+} ion is also implicated in interation between DNA and the protein which complicates the interpretation of metal ion changes. But, even in the case of the ATPase reaction of the 43 kDa GyrB protein which does not involve DNA, no metal ion-dependent change in diastereoselectivity for ATPαS or ATPβS could be detected. The S_p isomers in both cases were effectively nonsubstrates. Even in cases where there is a lack of reversal of diastereoselectivity, this could still be compatible with coordination

at this site. In these cases, the enzyme presumably must enforce coordination to one of the oxygens at Pα or Pβ independent of the nature of the metal ion.

Although the absence of metal ion-dependent reversal of diastereoselectivity in this study means that the interpretation is equivocal, the fact that both ATPαS(R_p) and ATPβS(R_p) are such good substrates for gyrase and the 43 kDa GyrB protein we believe is, in part, due to the metal ion coordination. We feel justified in drawing this tentative conclusion because of the nature of the metal coordination seen in the X-ray structure of the 43 kDa GyrB protein complexed with ADPNP-Mg^{2+} in which a tridentate complex is evident (Wigley et al., 1991). If this conclusion is valid then the kinetic data with the thionucleotides are consistent with Mg^{2+} being coordinated to the *pro-S* oxygens of both Pα and Pβ, and on mechanistic grounds probably Pγ, giving rise to the prediction that the Λ-exo configuration of the α,β,γ-tridentate ATP−Mg^{2+} [Figure 4; nomenclature of Cornelius and Cleland (1978)] is involved in catalytic turnover. This is the same absolute configuration as seen with ADPNP−Mg^{2+}, suggesting that this nonhydrolyzable analog in this case is serving as a good surrogate for ATP.

ACKNOWLEDGMENT

We thank Janid Ali, Andy Bates, Andy Jackson and Sotirios Kampranis for helpful discussions and comments on the manuscript.

REFERENCES

Ali, J. A., Jackson, A. P., Howells, A. J., & Maxwell, A. (1993) *Biochemistry 32*, 2717−2724.
Ali, J. A., Orphanides, G., & Maxwell, A. (1995) *Biochemistry 34*, 9801−9808.
Banik, U., & Roy, S. (1990) *Biochem. J. 266*, 611−614.
Bates, A. D., & Maxwell, A. (1989) *EMBO J. 8*, 1861−1866.
Bates, A. D., O'Dea, M. H., & Gellert, M. (1996) *Biochemistry 35*, 1408−1416.
Berger, J. M., Gamblin, S. J., Harrison, S. C., & Wang, J. C. (1996) *Nature 379*, 225−232.
Blandamer, M. J., Briggs, B., Cullis, P. M., Jackson, A. P., Maxwell, A., & Reece, R. J. (1994) *Biochemistry 33*, 7510−7516.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Chan, K., Delfert, D., & Junger, K. D. (1986) *Anal. Biochem. 157*, 375−380.
Cohn, M. (1982) *Acc. Chem. Res. 15*, 326−332.
Cornelius, R. D., & Cleland, W. W. (1978) *Biochemistry 17*, 3279−3286.
Cotton, F. A., & Wilkinson, G. (1972) *Advanced inorganic chemistry. A comprehensive text*, 3rd ed., John Wiley & Sons, New York.
Cullis, P. M., Maxwell, A., & Weiner, D. P. (1992) *Biochemistry 31*, 9642−9646.
Drlica, K., & Coughlin, S. (1989) *Pharmacol. Ther. 44*, 107−121.
Eckstein, F. (1985) *Annu. Rev. Biochem. 54*, 367−402.
Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685−1691.
Frey, P. A. (1989) *Adv. Enzymol. Relat. Areas Mol. Biol. 62*, 119−201.
Goody, R. S., & Eckstein, F. (1971) *J. Am. Chem. Soc. 93*, 6252−6257.
Goody, R. S., & Hoffman, W. (1980) *J. Muscle Res. Cell Motil. 1*, 101−115.
Hallett, P., Grimshaw, A. J., Wigley, D. B., & Maxwell, A. (1990) *Gene 93*, 139−142.
Hine, J., & Mookerjee, P. K. (1975) *J. Org. Chem. 40*, 292−298.
Huang, S. L., & Tsai, M. D. (1982) *Biochemistry 21*, 951−959.
Jackson, A. P., & Maxwell, A. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11232−11236.
Jaffe, E. K., & Cohn, M. (1978a) *Biochemistry 17*, 652−657.
Jaffe, E. K., & Cohn, M. (1978b) *J. Biol. Chem. 253*, 4823−4825.
Jaffe, E. K., & Cohn, M. (1979) *J. Biol. Chem. 254*, 10839−10845.
Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., & Candia, O. A. (1979) *Anal. Biochem. 100*, 95−97.
Lazewska, D., & Granowski, A. (1990) *Nucleic Acids Res. 18*, 6083−6088.
Lewis, R. J., Singh, O. M. P., Smith, C. V., Skarynski, T., Maxwell, A., Wonacott, A. J., & Wigley, D. B. (1996) *EMBO J. 15*, 1412−1420.
Ludwig, J., & Eckstein, F. (1989) *J. Org. Chem. 54*, 631−635.
Marquetant, R., & Goody, R. S. (1983) *J. Chromatogr. 280*, 386−389.
Maxwell, A. (1992) *J. Antimicrob. Chemother. 30*, 409−416.
Maxwell, A. (1993) *Mol. Microbiol. 9*, 681−686.
Maxwell, A., & Gellert, M. (1984) *J. Biol. Chem. 259*, 14472−14480.
Maxwell, A., & Gellert, M. (1986) *Adv. Protein Chem. 38*, 69−107.
Maxwell, A., Rau, D. C., & Gellert, M. (1986) Mechanistic studies of DNA gyrase, in *Biomolecular stereodynamics III. Proceedings of the fourth conversation in the discipline of biomolecular stereodynamics* (Sarma, R. H., & Sarma, M. H., Eds.) pp 137−146, Adenine Press, Albany, NY.
Pai, E. F., Krengel, U., Petsko, G. A., Goody, R. S., Kabsch, W., & Wittinghofer, A. (1990) *EMBO J. 9*, 2351−2359.
Pearson, R. G. (1966) *Science 151*, 172−177.
Pecoraro, V. L., Hermes, J. D., & Cleland, W. W. (1984) *Biochemistry 23*, 5262−5271.
Rádl, S. (1990) *Pharmacol. Ther. 48*, 1−17.
Reece, R. J., & Maxwell, A. (1991) *CRC Crit. Rev. Biochem. Mol. Biol. 26*, 335−375.
Roca, J. (1995) *Trends Biochem. Sci. 20*, 156−160.
Roca, J., & Wang, J. C. (1992) *Cell 71*, 833−840.
Roca, J., & Wang, J. C. (1994) *Cell 77*, 609−616.
Roca, J., Berger, J. M., Harrison, S. C., & Wang, J. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 4057−4062.
Schlichting, I., Almo, S. C., Rapp, G., Wilson, K., Petratos, K., Lentfer, A., Wittinghofer, A., Kabsch, W., Pai, E. F., Petsko, G. A., & Goody, R. (1990) *Nature 345*, 409−415.
Sugino, A., Higgins, N. P., Brown, P. O., Peebles, C. L., & Cozzarelli, N. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4838−4842.
Tamura, J., & Gellert, M. (1990) *J. Biol. Chem. 265*, 21342−21349.
Tamura, J. K., Bates, A. D., & Gellert, M. (1992) *J. Biol. Chem. 267*, 9214−9222.
Tingey, A. P., & Maxwell, A. (1996) *Nucleic Acids Res. 24*, 4868−4873.
Wigley, D. B. (1995) Structure and mechanism of DNA gyrase, in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) pp 165−176, Springer-Verlag, Berlin and Heidelberg.
Wigley, D. B., Davies, G. J., Dodson, E. J., Maxwell, A., & Dodson, G. (1991) *Nature 351*, 624−629.
Wolfenden, R. V., Cullis, P. M., & Southgate, C. C. F. (1979) *Science 206*, 575−577.

BI962725E