The DNA Dependence of the ATPase Activity of DNA Gyrase*

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Anthony Maxwell and Martin Gellert
From the Laboratory of Molecular Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

We have studied the ATPase activity of DNA gyrase both in the absence and presence of DNA. In the absence of DNA we show that the gyrase B protein alone has a very low level of ATPase activity which can be increased many-fold by pretreatment of the B protein with heat or urea. When both the gyrase A protein and linear DNA are also present, the ATPase activity of the untreated B protein is greatly stimulated. We find that the extent of stimulation is dependent upon the length of the DNA but largely independent of DNA sequence. DNA molecules greater than 100 base pairs in length are much more effective in stimulating the gyrase ATPase than those of 70 base pairs or less, although short DNA molecules will stimulate the ATPase at high concentrations. The behavior of long and short DNA molecules with respect to ATPase stimulation is also reflected in their abilities to bind DNA gyrase. To account for these data we propose a model for the interaction of gyrase with ATP and DNA in which ATP hydrolysis requires the binding of DNA to two sites on the enzyme.

This and other evidence shows that the breakage-reunion reaction is carried out principally by the A protein whereas the B protein is responsible for ATP hydrolysis. Inhibition by coumermycin or novobiocin may be used to distinguish the ATPase activity of DNA gyrase from that of other enzymes. Although ATP hydrolysis is an absolute requirement of catalytic supercoiling, the two processes are not necessarily tightly coupled, and highly supercoiled DNA can stimulate the ATPase activity of the enzyme (Mizuuchi et al., 1978).

In this paper we examine the properties of the ATPase of DNA gyrase with particular reference to the stimulation of the ATPase activity by DNA.

EXPERIMENTAL PROCEDURES

Enzymes—Escherichia coli DNA gyrase was prepared as described previously (Mizuuchi et al., 1984). Gyrase A protein was a valine-Sepharose fraction, and gyrase B protein was a hydroxyapatite fraction; each was extensively dialyzed against enzyme buffer (50 mM Tris (pH 7.5), 0.1 mM KCl, 5 mM dithiotreitol, 1 mM EDTA, 10% (w/v) glycerol) prior to use. Both proteins were >98% pure as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and neither contained significant amounts of the other protein as determined by ATPase and supercoiling assays (Mizuuchi et al., 1984). The specific activities of the A and B proteins, as determined by supercoiling assays, were 8 x 10⁶ units/mg and 6 x 10⁵ units/mg, respectively. It was shown that an approximate 1:1 stoichiometry existed between the two proteins for both the ATPase and the binding experiments described in this paper. Protein concentrations were determined by Bio-Rad protein assay using bovine serum albumin as a standard; concentrations of A and B are expressed as molarities of dimer. Restriction enzymes were purchased from New England Biolabs except AatII which was from Toyobo Ltd., Japan. Conditions for restriction enzyme digestes were those recommended by the manufacturer.

DNA—Bacteriophage λ DNA (Bethesda Research Laboratories) was extracted with phenol and chloroform and precipitated with ethanol before use. The DNA of plasmid pXRC24 from the E. coli strain HB101/pXRC24 (gift of Dr. R. T. Simpson, National Institute of Arthritis, Diabctes, and Digestive and Kidney Diseases, National Institutes of Health), was prepared using a chloroform enrichment procedure as described by Tomizawa et al. (1975), except that cells were grown in M9 medium (Miller, 1972). When 3H-labeled deoxyadenosine were added to 800 ml of culture 30 min prior to the addition of chloroform. To prepare DNA fragment 1, pXRC24 DNA (2 mg) was digested with Hhal restriction enzyme, precipitated with ethanol, resuspended in 0.5 ml of 10 mM Tris (pH 7.5), 0.1 mM NaCl, 1 mM EDTA, and passed through a 500-ml Sepharose 3B column (Pharmacia) previously equilibrated in the same buffer. Fragment 1 (4534 bp) eluted with the void volume and was well resolved from the smaller fragments (<400 bp). Peak fractions were pooled and concentrated by butan-1-ol extraction and ethanol precipitation. DNA fragments A-X were prepared by digestion of 100–150 µg of fragment 1 with the appropriate restriction enzyme or enzymes (see Fig. 2), followed by electrophoresis in 12% polyacrylamide gels. DNA bands stained with ethidium were cut from the gel, sliced into small pieces, and soaked for 48 h with agitation in an equal volume of

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1 The abbreviation used is: bp, base pairs.
and was recovered from the filtrate by ethanol precipitation. The DNA was isolated by centrifugation through Bio-Rad Econo-Columns and was recovered from the filtrate by ethanol precipitation. The recovery was routinely 50–85%. Although DNA purified in this way contained some gel material, control experiments showed that this material had no effect on the ATPase experiments described below. Additionally, fragments A and X, when prepared without gel purification, gave identical results to those obtained when these fragments had been eluted from gels.

**ATPase Assay—Crude [γ-32P]-ATP (specific activity >7000 Ci/mmol) was purchased from ICN and repurified by chromatography on a DEAE-Cellulose column (Chaykin et al., 1965) and stored at −20 °C. Typically ATP was recovered >99% pure as judged by thin-layer chromatography on polyethyleneimine-cellulose plates and contained <0.5% inorganic phosphate as determined by the Norit assay described below. ATPase reaction mixtures (30 μl) contained 35 mM Tris (pH 7.5), 24 mM KCl, 6 mM MgCl₂, 1.8 mM spermidine, 0.36 mg/ml bovine serum albumin, 9 μg/ml tRNA, 6.5% (w/v) glycerol, 5 mM dithiothreitol, 10 mM potassium phosphate, and 0.1 mM [3H]glycine (40 Ci, New England Nuclear). gyrase A protein (20–120 μg), and gyrase B protein (10–60 μg), a 2-fold molar excess of DNA over B protein was employed in ATPase experiments. Reaction mixtures (10 μl) were as described for filter binding experiments. ATP hydrolysis was measured by the addition of A protein; addition of both DNA and the A protein led to an increased ATPase activity which, at high concentrations of the B protein, was about a factor of 2 greater than that with the B protein alone. When a more sensitive ATPase assay, we have reinvestigated the phenomenon of the gyrase B ATPase. As shown in Fig. 1a, at a concentration of 12 nM the B protein displayed a level of ATPase which was close to the limits of detection of this assay system. Addition of the A protein led to no increase in ATPase activity. Similarly, addition of DNA alone did not stimulate the ATPase of the B protein (data not shown). However, when both the A protein and DNA were added an appreciable level of ATPase activity was measured. Hence, these data indicate that the level of ATPase due to the B protein alone is insignificant in comparison with the

**RESULTS**

The ATPase of the Gyrase B Protein—It is well established that the ATPase activity of DNA gyrase is strongly stimulated by double-stranded DNA (Mizuzuki et al., 1978; Sugino et al., 1978), but it has not been clear whether gyrase displays any ATPase activity in the absence of DNA. Mizuzuki et al. (1978) showed that in the absence of DNA, gyrase displayed a low level of ATPase, which was sensitive to novobiocin. Sugino and Cozzarelli (1980) reported that while the gyrase B protein alone had no ATPase activity, addition of the A protein gave a low level of ATPase. In contrast, Staudenbauer and Orr (1981) reported that their preparation of the B protein had an appreciable level of ATPase activity which was unaffected by the addition of the A protein; addition of both DNA and the A protein led to an increased ATPase activity which, at high concentrations of the B protein, was about a factor of 2 greater than that with the B protein alone.

Using a more sensitive ATPase assay, we have reinvestigated the phenomenon of the gyrase B ATPase. As shown in Fig. 1a, at a concentration of 12 nM the B protein displayed a level of ATPase which was close to the limits of detection of this assay system. Addition of the A protein led to no increase in ATPase activity. Similarly, addition of DNA alone did not stimulate the ATPase of the B protein (data not shown). However, when both the A protein and DNA were added an appreciable level of ATPase activity was measured. Hence, these data indicate that the level of ATPase due to the B protein alone is insignificant in comparison with the

**FIG. 1. ATPase hydrolysis by the gyrase B protein.** Reaction mixtures containing ATP (0.1 mM), gyrase B protein (12 nM), and, where indicated, gyrase A protein (25 nM) and λ DNA (10 μg/ml) were incubated at 25 °C for 2 h. The amount of ATP hydrolyzed was determined and corrected for that fraction which was insensitive to novobiocin inhibition. Results are expressed relative to the level of ATPase for the untreated B protein in the presence of A and DNA (14.5%). The three panels show different treatments of the B protein prior to inclusion in the reaction mixtures: α, no treatment; β, preincubation of the B protein (0.6 μM) in the presence of 2.5 μM urea in an enzyme buffer at 25 °C for 90 min; and γ, preincubation of the B protein (1.2 μM) in enzyme buffer at 43 °C for 5 min.

0.5 M NH₄(CH₃CO₂), 1 mg/ml sodium dodecyl sulfate, 10 mM Mg(CH₃CO₂),, 1 mM EDTA, at 37 °C (Maxam and Gilbert, 1977). DNA was isolated by centrifugation through Bio-Rad Econo-Columns and was recovered from the filtrate by ethanol precipitation. The recovery was routinely 50–85%. Although DNA purified in this way contained some gel material, control experiments showed that this material had no effect on the ATPase experiments described below. Additionally, fragments A and X, when prepared without gel purification, gave identical results to those obtained when these fragments had been eluted from gels.

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ATPase activity when both A and DNA are also present. When the concentration of the B protein was raised 5-fold, the level of the B ATPase increased at least 10-fold over that shown in Fig. 1a but was still less than 5% of that of the DNA-stimulated ATPase (data not shown). At a similar weight concentration of enzyme, Staudenbauer and Orr (1981) reported that the level of ATPase due to the B subunit was about one-fifth that of the DNA-stimulated ATPase; however, as the specific activities of the gyrase proteins used by these workers were much lower than ours, such a comparison by enzyme concentration may not be appropriate.

Treatment of the B protein with either heat or urea prior to the ATPase reaction led to changes in the properties of the gyrase ATPase. Preincubation of the B protein at temperatures between 42 and 50 °C caused a significant increase in the B ATPase activity (Fig. 1c), even though the DNA-dependent ATPase activity was somewhat reduced by this treatment. For example, preincubation at 43 °C for even very short times (<2 min) led to about a 40-fold increase in the ATPase of the B subunit. The treated B protein could then be stored at 0 or 25 °C for several hours or at −70 °C for several weeks without losing its ATPase activity. Preincubation for longer times at the elevated temperatures led to a time-dependent decrease in both the DNA-dependent and the B ATPase, but the ratio of the B ATPase to the DNA-dependent ATPase remained constant. Under these conditions, we have also observed a diminution of the supercoiling and relaxing activities of the enzyme, which decrease in parallel with the DNA-dependent ATPase (data not shown).

Preincubation of the B protein at less than 42 °C had no apparent effect on the properties of the enzyme, whereas preincubation at 50 °C or higher led to complete inactivation. Incubation of the A protein at high temperatures (>57 °C) also led to a loss in enzyme activity, but no effect on the ATPase activity of B in the presence of A alone was observed.

Similarly, preincubation of the B protein in the presence of urea also led to changes in the properties of the gyrase ATPase. At urea concentrations greater than 0.5 M, a marked increase in the level of gyrase B ATPase was observed, as well as a moderate decrease in the DNA-dependent ATPase activity (Fig. 1b). Preincubation of the B protein at urea concentrations below 0.5 M had no apparent effect on the properties of the enzyme. Treatment with urea or brief incubation at high temperatures did not appear to alter the interactions of the B protein with DNA or the A protein, as appreciable stimulations of the ATPase activity were still recorded when both A and DNA were added (Fig. 1, b and c). We also found that the ATPase activity of the treated B protein showed a greater than first-order dependence on enzyme concentration (data not shown); this had been reported previously by Staudenbauer and Orr (1981).

To exclude the possibility that the observed increase in the ATPase of the B protein shown in Fig. 1b was due to the small amount of urea present in the reaction mixture upon dilution of the treated B protein with the other reaction components, control experiments were performed. Using untreated gyrase proteins, we have shown that urea added to reaction mixtures at 50 mM or less caused no effect on the observed ATPase. So, the changes in the properties of the gyrase ATPase shown in Fig. 1b must be a consequence of preincubation of the B protein in the presence of urea. (The turnover number) for DNA gyrase were found to be 39.5 - 82.4 min⁻¹, and the parameter kcat/Km was 1.87 ±0.02) x 10⁶

The preparations of the gyrase B protein used by Staudenbauer and Orr (1981) had been eluted from a novobiocin-Sepharose column with 5 mM urea. It is, therefore, possible that the high level of B ATPase observed by these workers was a consequence of the exposure of the enzyme to urea. Additionally, we have noted that the levels of ATPase activity in untreated samples of the B protein differ slightly for different enzyme preparations, which might be consistent with variable amounts of the protein in the altered state.

**DNA Dependence**—Although closed circular DNA may be regarded as the usual substrate for the DNA gyrase supercoiling reaction, nicked circular and linear DNA molecules (which cannot be supercoiled) will also interact with the enzyme and stimulate the ATPase activity (Mizuuchi et al., 1978, Sugino and Cozzarelli, 1980). For the purpose of studying the ATPase reaction, DNA can be regarded as a cosubstrate or cofactor of the enzyme. From this viewpoint it is advantageous to use nicked or linear DNA, because their state is not altered by the reaction and they can thus be regarded as true cofactors. We have examined the stimulation of the ATPase reaction of DNA gyrase by linear DNA molecules of different lengths.

In order to prepare linear DNA fragments of defined size and known sequence, we have utilized the DNA of plasmid pXRC24. Plasmid pXRC24 contains part of the sea urchin 5 S rRNA gene (Simpson and Stafford, 1983) cloned, as 25 tandemly repeated copies, into plasmid pARA1 (Hartley and Gregori, 1981). Each of the repeated sequences is 172 base pairs in length. The repeated DNA may be conveniently excised from pXRC24 by digestion with the restriction enzyme HhaI; the repeated sequences lie on one large HhaI fragment which is easily separated from the smaller HhaI fragments derived from pARA1. The 172-bp sequence contains several unique sites for restriction enzymes and thus may be used to prepare a range of DNA fragments of defined sizes (Fig. 2).

Using two of these fragments and phage λ DNA, the kcat and Km values for the gyrase ATPase reaction were determined (Fig. 3). As it has been shown that gyrase is strongly inhibited by the reaction product ADP (Sugino and Cozzarelli, 1980), we determined rates of ATP hydrolysis from several time points taken in the initial part of this reaction. Under these conditions, we found the rates of ATP hydrolysis to be virtually constant over the period of measurement, and these reactions could be analyzed by steady-state kinetics. Previous determinations of the kinetic parameters of DNA gyrase have relied upon the estimation of reaction rates from single time points taken later in the reaction (Sugino and Cozzarelli, 1980; Staudenbauer and Orr, 1981).

In each experiment the DNA concentration was held constant at 10 µg/ml, which was shown to be saturating at these enzyme concentrations for λ DNA and fragment M (Fig. 4) and fragment X (data not shown). Initial rates of ATP hydrolysis showed the expected hyperbolic dependence on substrate concentration and are displayed as Eadie-Hofstee plots (Fig. 3). Different values of the kcat and Km of DNA gyrase were obtained for these three DNA molecules, but the parameter kcat/Km was found to be a constant. The values derived for Km (0.21-0.45 mM) are similar to those previously determined (0.3 mM, Sugino and Cozzarelli, 1980; 0.5 mM, Staudenbauer and Orr, 1981), although these determinations were under slightly different reaction conditions. The values of kcat (the turnover number) for DNA gyrase were found to be 39.5-82.4 min⁻¹, and the parameter kcat/Km was 1.87 (±0.02) x 10⁶

R T. Simpson, unpublished data.
Fig. 2. Map of DNA fragments. The upper part of the figure shows a restriction map of the large HhaI fragment (fragment 1) of pXRC24. The region between the brackets contains two copies of the 172-bp repeated sequence; the dashed line indicates the boundary between the two repeats. Vertical arrows indicate sites of restriction enzyme cleavage: Aa, AatII; Ah, AflIII; AluI, Alu; Au, AulII; Bs, BstNI; Hh, HhaI; Hpi, HpiII; Sn, Sau3AI; Ta, TafI; Xm, XmnI. The bars in the lower part of the figure show the positions of DNA fragments derived from fragment 1; the size of each fragment is given in base pairs excluding any single-stranded termini.

M⁻¹ min⁻¹ for the three DNA cofactors examined.

Using λ DNA and the full range of DNA fragments shown in Fig. 2, we have measured the stimulation of the ATPase reaction of DNA gyrase by fragments of different length (Fig. 5). Although the comparison in Fig. 5 may be regarded as being qualitative these data may also be compared quantitatively if it is assumed that the value of $k_{cat}/K_m$ determined above applies to all the DNA fragments from Fig. 2. Then, provided that the substrate concentration is below the $K_m$, the Michaelis-Menten equation for the velocity of an enzyme reaction simplifies to

$$v = \frac{k_{cat}}{K_m} [E_0][S]$$

where $[E_0]$ is the total enzyme concentration and [S] is the substrate concentration. Thus, at constant $[E_0]$, $k_{cat}/K_m$ approximates to an apparent first-order rate constant, and the amount of product generated in a fixed time period may be compared for different DNA molecules.

The data in Fig. 5 show that there is little variation in the level of ATPase activity for DNA fragments greater than 100 bp in length. However, fragments less than 70 bp produce little or no ATPase stimulation. Comparison of the sequences of these DNA fragments (Fig. 2) indicates that there are no common sequences present in the large DNA fragments which are absent from all of the short ones. Hence, there seems to be a dependence of the stimulation of the ATPase of DNA gyrase on DNA length but no obvious sequence dependence. (These results differ slightly from those of Klevan and Tse (1983) who showed that with Micrococcus luteus DNA gyrase, a 100-bp DNA fragment gave little ATPase stimulation when compared with a 240-bp fragment or phage T7 DNA.) The observation that DNA gyrase demonstrates little or no sequence preference for stimulation of the ATPase is consistent with the observation that DNA homopolymers and alternating copolymers will stimulate the gyrase ATPase activity (Sugino and Cozzarelli, 1980).³ DNA fragments between 70 and 100 bp in size, however, did not show an obvious relationship between ATPase stimulation and DNA length. It is

³ A. Maxwell and M. Gellert, unpublished observations.
possible that in this range DNA sequence becomes a more significant factor.

In experiments where DNA fragments of differing sizes were included in the same reaction mixture, it was found that short DNA fragments (<70 bp) did not significantly inhibit the ATPase stimulation of gyrase by the long fragments (>100 bp). For example, addition of 1 or 10 μg/ml of fragment N (55 bp) to a gyrase reaction mixture containing λ DNA (1 μg/ml) resulted in no change in the ATPase activity when compared with λ DNA alone. These results suggest that the short DNA fragments do not effectively compete with the long DNA fragments for binding to the enzyme and are supported by the binding studies described below. These experiments also exclude the possibility that the short DNA fragments contained a factor which inhibited the gyrase ATPase reaction.

Thus the above data suggest that there is a size limitation on DNA fragments which will stimulate the ATPase reaction of DNA gyrase. One possibility is that gyrase has to make contacts with about 85 contiguous base pairs of DNA in order for it to become a fully active ATPase. A second and more realistic possibility would be that two (or more) DNA-binding sites on the enzyme must be occupied in order to stimulate the ATPase activity. A long DNA fragment (>100 bp) would be able to contact these sites and thus turn on the ATPase activity, whereas a short DNA molecule (<70 bp) would be unable to bridge the sites. One prediction of such a model is that the DNA-binding sites on gyrase could be filled by two (or more) independent DNA molecules, i.e. short DNA molecules at high concentrations should stimulate the gyrase ATPase.

This prediction has been tested by examining the dependence of the ATPase stimulation of DNA gyrase on the DNA concentration of fragment N (55 bp). Fragment N was chosen for these experiments because it does not have single-strand protruding ends, i.e. it cannot form multimers by end-to-end base pairing. As shown in Figs. 5 and 6, fragment N gives very little ATPase stimulation at 10 μg/ml (a concentration of DNA which is above saturation for larger fragments), but at much higher DNA concentrations, significant ATPase stimulation was observed (Fig. 6). Moreover, the ATPase activity showed a sigmoidal dependence upon DNA concentration, suggesting that more than one molecule of DNA interacts with each enzyme molecule. This behavior may be contrasted with that of λ DNA and fragment M (Fig. 4) which stimulate the ATPase activity of gyrase at much lower DNA concentrations and do not show a sigmoidal dependence. We show below that the behavior of fragment N is consistent with a model involving the interaction of two independent DNA molecules with each enzyme molecule to stimulate the ATPase activity.

The variation in the ATPase stimulation of DNA gyrase with DNA concentration was also measured with DNA fragments R (49 bp) and S (66 bp). Neither of these DNA fragments showed significant ATPase activity at 10 μg/ml DNA (Fig. 5), but at higher DNA concentrations (e.g. 100 μg/ml) an appreciable level of ATPase stimulation was observed (data not shown). However, as both fragments R and S have a protruding single-strand end, the possibility remains with these fragments that the observed increase in the ATPase at high DNA concentrations was a consequence of dimerization. This cannot be the case with fragment N.

Binding Studies—We have shown above that the ATPase activity of DNA gyrase is stimulated by DNA fragments greater than 100 bp in length but that shorter fragments (<70 bp) do not stimulate the ATPase unless high DNA concentrations are employed. Although these short fragments are unable to stimulate the gyrase ATPase at low DNA concentrations, it is possible that they may still bind to gyrase under these conditions. We have examined the binding of DNA fragments to gyrase using filter binding and a gel method.

It has already been shown that DNA gyrase forms complexes with DNA which may be detected by their retention on nitrocellulose filters (Higgins and Cozzarelli, 1982). Using a similar method, we have examined the formation of complexes between DNA gyrase and some of the DNA fragments from Fig. 2 (Table I). In this experiment, the DNA concentration was maintained at 10 μg/ml for each fragment, and the enzyme concentration was 200 nM. (This enzyme concentration is higher than that employed in Fig. 5 in order that a significant fraction of the DNA could be retained on the filter.) Table I shows that only the DNA fragments which were effective as cofactors for the ATPase reaction showed...
 TABLE I  
 Filter binding of DNA fragments

| DNA fragment | Length (bp) | Concentration (µM) | Retention (%) |
|--------------|------------|--------------------|---------------|
| X            | 171        | 0.09               | 42            |
| B            | 124        | 0.12               | 38            |
| M            | 117        | 0.13               | 31            |
| N            | 55         | 0.27               | 1             |
| C            | 46         | 0.31               | 0             |

 FIG. 7. Filter binding of DNA gyrase and fragment X (171 bp). Filter binding was performed as described in the legend to Table I except that the concentration of fragment X was varied. The line drawn is the theoretical curve for the formation of a bimolecular complex between gyrase and DNA with an equilibrium dissociation constant of 0.54 nM.

 FIG. 8. Filter binding of DNA gyrase and fragment N (55 bp). Filter binding was performed as described in the legend to Fig. 7.

 FIG. 9. Gel assay of DNA binding to gyrase. Reaction mixtures containing DNA gyrase (210 nM) and varying concentrations of fragments X or N were incubated for 1 h at 25 °C and then applied to a 5% polyacrylamide gel. After electrophoresis the gel was stained with ethidium and photographed. Lane a is fragment X alone (0.09 µM), and lane b is fragment N alone (0.27 µM); figures above other lanes indicate the molar ratio of DNA to DNA gyrase in that lane. X marks the position of the free DNA band of fragment X, N marks the free DNA band of fragment N, and E:DNA shows the position of gyrase-DNA complexes as determined by protein staining.

appreciable binding to the enzyme under these conditions.

We have further examined the formation of DNA gyrase-DNA complexes by varying the DNA concentration in filter-binding experiments, using both long and short DNA fragments. Fig. 7 shows the retention of fragment X by DNA gyrase on nitrocellulose filters. The efficiency of retention of fragment X by gyrase was determined by the method of Yarus and Berg (1970) to be 0.41, and the data were found to be consistent with the formation of a bimolecular complex between gyrase and DNA with an equilibrium dissociation constant of 0.54 nM. (This value may be compared to that determined by Higgins and Cozzarelli (1982) who found, under slightly different conditions, that complexes between DNA gyrase and ColE1 DNA had a dissociation constant of 0.2 nM.) In a similar experiment, we have also examined the binding of fragment N to gyrase at a range of DNA concentrations (Fig. 8). Unlike fragment X, little retention of fragment N was observed at low DNA concentrations; however, the retention was greatly increased at high DNA concentrations. Moreover, like the ATPase data for fragment N (Fig. 6), the binding curve takes the form of a sigmoid. Although the efficiency of retention of fragment N cannot be determined, it is clear that these data cannot be explained by the formation of a simple bimolecular complex between enzyme and DNA.

DNA-protein interactions may also be examined using gel methods (Garner and Revzin, 1981). Reaction mixtures containing DNA gyrase and DNA were applied directly to polyacrylamide gels and analyzed by electrophoresis. In this way the binding of fragments X and N to DNA gyrase has been measured (Fig. 9). At low concentrations of fragment X, most of the DNA is found in a band migrating more slowly than the free DNA band; this band was shown by protein staining to contain DNA gyrase in addition to DNA. At molarities of DNA which exceeded that of the enzyme, free DNA was apparent, indicating that the enzyme was becoming saturated with DNA. In contrast, at low concentrations of fragment N, the DNA was mostly uncomplexed; comparable amounts of enzyme-DNA complex to those achieved with fragment X are only apparent at much higher concentrations of fragment N. When the bands on the photographic negative of Fig. 9 were quantitated by densitometry, it was found that the
amount of enzyme-DNA complex for fragment X was in good agreement with the data shown in Fig. 7, provided that the efficiency of filter retention was taken into account. There was also a qualitative agreement in the binding data for fragment N from Figs. 8 and 9 when they were compared in this way. So, although neither of these binding assays is strictly an equilibrium technique, their validity is strengthened by the agreement found between the two methods.

These binding data suggest that, in the absence of ATP, DNA gyrase binds efficiently to long DNA fragments but binds poorly to short DNA molecules. This discrimination of DNA fragments by size parallels the observations made in the ATPase experiments described above. Thus, it appears that the DNA length dependence of the ATPase reaction (Fig. 5) can be attributed to the different abilities of long and short DNA molecules to bind to DNA gyrase. Additionally, the binding of gyrase to fragment N showed a sigmoidal dependence upon DNA concentration which suggests the interaction of two or more DNA molecules with the enzyme, with positive cooperativity between binding sites. This behavior is similar to that observed in ATPase experiments (Fig. 6); we conclude that two or more molecules of fragment N must bind to DNA gyrase in order to stimulate the ATPase activity.

**DISCUSSION**

DNA gyrase provides a relatively straightforward example of energy coupling in biological systems. Here the free energy derived from the hydrolysis of ATP is used to fuel the introduction of negative supercoils (and thereby torsional stress) into DNA. Important to the understanding of energy transduction in DNA gyrase is the relationship between the interaction of DNA with the enzyme and the triggering of the gyrase reaction. We have shown that gyrase exhibits a DNA-independent ATPase activity which is a property of the B protein alone. Although the level of ATPase activity of the untreated B protein is very low, treatment with urea or heat substantially increases this activity. It is possible that these treatments induce some conformational change in the B protein whereby it becomes a more active ATPase. This conformation may be identical to that adopted in the presence of the A protein and DNA. One consequence of conformational change in the B protein might be dimerization. The data of Klevan and Wang (1980) suggest that the B protein exists as a monomer in free solution but that two copies of the B protein are present in the active gyrase complex. Additionally, the ATPase of the B protein alone has been shown to exhibit a greater than first order dependence on B concentration, suggestive of an active dimer and inactive monomer (Staudenbauer and Orr, 1981). Hence, conditions which promote dimerization (including interactions with the A protein and DNA) may also stimulate the ATPase of the B protein. The possibility of the B protein existing in different states under some conditions may explain the differences in the extent of the ATPase activity of the B protein which have been reported. However, it is not clear whether the low level of ATPase activity of the untreated B protein reported in this paper reflects an intrinsic low level activity of the native protein or represents some fraction of the B preparation which has been converted to another state.

To study the stimulation of the ATPase of DNA gyrase by DNA, we employed part of the sea urchin 5 S rRNA gene. This particular DNA sequence was not chosen for its ability to bind or to be cleaved by DNA gyrase but merely because of the possibilities of preparing DNA fragments of known sequence in large amounts. However, we have found that fragments derived from this DNA will stimulate the ATPase activity of DNA gyrase (Fig. 5), will bind to the enzyme (Table 1), and that in the presence of oxolinic acid, fragment 1 and fragment X are cleaved by gyrase at discrete locations.

Using bacteriophage λ DNA and two fragments from the 5 S sequence, we have shown that the values of \(k_{\text{cat}}\) and \(K_m\) for the gyrase ATPase reaction vary depending upon the DNA cofactor employed (Fig. 3). This implies that the nature of the DNA cofactor determines to some extent the rate of ATP hydrolysis at the catalytic site. Whereas fragments X and M probably bind only 1 gyrase/DNA molecule (Figs. 7 and 9), many gyrase tetramers can bind to each molecule of λ DNA, so the \(k_{\text{cat}}\) and \(K_m\) values for fragments X and M refer \(\alpha\) interactions with particular DNA sequences, but those derived for λ DNA represent mean values averaged over many DNA sequences.

Despite the variation in the values of \(k_{\text{cat}}\) and \(K_m\) for these three DNA cofactors, the value of \(k_{\text{cat}}/K_m\) was a constant. The significance of \(k_{\text{cat}}/K_m\) depends upon the mechanism of the enzyme reaction. For example, if the gyrase-catalyzed hydrolysis of ATP is represented by the standard Michaelis-Menten scheme

\[
E + S \xrightleftharpoons[k_{-1}]{k_1} ES \xrightarrow[k_{\text{cat}}]{k^{-1}} E + P
\]

where \(E\) is DNA gyrase, \(S\) is ATP, \(P\) is ADP and inorganic phosphate, and \(k\) is rate constant, then,

\[
\frac{k_{\text{cat}}}{K_m} = \frac{k_{\text{cat}} \cdot k_1}{k_{-1}}.
\]

If \(k_{\text{cat}} \gg k_{-1}\), then

\[
\frac{k_{\text{cat}}}{K_m} = k_1.
\]

Thus, in this instance, \(k_{\text{cat}}/K_m\) would be equal to the "on" constant for ATP binding, a rate constant which would probably not be expected to vary as a function of the DNA cofactor employed. However, Equation 1 is probably an oversimplification of this reaction, and \(k_{\text{cat}}\) and \(K_m\) are more likely to be the resultants of many rate constants.

Using DNA fragments ranging in size from 46 to 171 bp, we have found, under certain conditions, that while fragments of 100 bp or more will act as cofactors in the gyrase ATPase reaction, those of 70 bp or less will not. No sequence dependence for DNA gyrase based on these data could be derived. However, these data do not exclude the possibility of there being preferred sequences on DNA to which gyrase binds but do support the idea that the enzyme's preference for DNA sequence is not stringent. We have, therefore, concluded that the discriminating factor in the gyrase ATPase experiments described above is the length of the DNA fragments employed.

Previous studies on DNA gyrase have indicated that the enzyme interacts with a long stretch of DNA. Nucleosome protection studies employing staphylococcal nuclelease, exonuclease III, or pancreatic DNase I have suggested that the enzyme protects 120–155 bp of DNA (Fisher et al., 1981; Morrison and Cozzarelli, 1981; Kirkegaard and Wang, 1981), the extent of protection being dependent upon the DNA sequence and the nucleosome employed. Additionally, cleavage of DNA gyrase-DNA complexes with DNase I suggested that part of the protected DNA may be wrapped around the enzyme in a structure resembling the nucleosome (Liu and Wang, 1978). Thus, it appears that gyrase has the capacity to interact with a relatively large region of DNA.

The results in this paper suggest that the minimum length of DNA which will stimulate the ATPase activity of the
enzyme is 85 bp (±15 bp), somewhat smaller than those reported from nuclease protection studies. However, our data will probably underestimate the binding-site size, as these experiments are concerned only with those interactions necessary to turn on the gyrase ATPase; it is likely that there are interactions spanning a greater length of DNA which are important in the topoisomerization reactions but do not influence the ATPase reactions. Nevertheless, the estimates of the length of DNA bound to gyrase derived from the nuclease studies and the ATPase studies are not widely different. It must, however, be noted that neither of these methods measures the amount of DNA to which gyrase binds while actively engaged in DNA supercoiling.

Although the ATPase data in Fig. 5 suggested that short DNA fragments (<70 bp) were unable to stimulate the ATPase reaction of DNA gyrase, the possibility remained that these DNA molecules could still bind to the enzyme under these conditions. This possibility was excluded by showing that these short DNAs bound very poorly to DNA gyrase under conditions where the large fragments (>100 bp) were efficiently bound (Table 3), implying that the discrimination of DNA gyrase with respect to DNA length in the ATPase reactions was due to the different abilities of long and short fragments to bind to the enzyme.

At much higher concentrations of the short DNA fragments, both binding to DNA gyrase and ATPase stimulation was observed (Figs. 6 and 8). Here the dependence of DNA binding and ATPase stimulation on DNA concentration was found to be sigmoidal, suggestive of the binding of two or more DNA molecules to the enzyme with positive cooperativity between the binding sites. It is not clear whether these are identical sites in the two half-molecules or whether one is a breakage-reunion site and the other a site for the binding of a DNA segment to be translocated (Gellert, 1981).

The ATPase data also suggest that the binding of DNA to gyrase mediates the transition between two states of the enzyme, from an inactive to an active ATPase. We have already reported in this paper that under certain conditions the B protein alone can exist in an altered state which is more active as an ATPase. It is possible that DNA binding to gyrase at the appropriate sites promotes the same change in the B protein.

In Fig. 10 we suggest a model for the hydrolysis of ATP by DNA gyrase incorporating the ATPase and binding data reported in this paper. In this scheme the enzyme molecule is represented as a dimer with each half containing a single equivalent binding site for DNA. (In relation to the proposed structure of gyrase (AB2), each half of the dimer could be considered to be composed of one molecule of the A protein and one of the B protein.) We have assumed no preferred order of binding of DNA and ATP, as it has been shown that gyrase can bind to DNA in the absence of ATP (Table 1) (Liu and Wang, 1978) and that ATP can bind to the enzyme in the absence of DNA (Mizuuchi et al., 1978). The important feature of this model is that DNA must be bound at both DNA-binding sites on the enzyme before hydrolysis of ATP can occur. Such a requirement ensures the coupling between ATP hydrolysis and the binding of DNA in the appropriate manner. When both DNA-binding sites are occupied, the enzyme is proposed to undergo a conformational change whereby it becomes an active ATPase. The binding of DNA to the two binding sites is cooperative and the binding of DNA to one site only will not elicit the conformational change. A single large DNA molecule (>100 bp) would thus occupy both sites, the binding to the two sites being essentially a single event, whereas a single short DNA molecule (<70 bp) would be unable to contact both binding sites and the binding of a second DNA molecule would be required to activate the ATPase.

In this scheme the hydrolysis of ATP is assumed to be irreversible and the concentration of product (ADP) is negligible compared with product release. Using computer-assisted modeling, we have shown that the hydrolysis of ATP by DNA gyrase in the presence of varying concentrations of fragment N is consistent with this scheme (Fig. 6). The ATPase data derived for longer DNA molecules (Fig. 4) are also in agreement with such a scheme, but in these cases the scheme may be simplified by having the DNA binding to the enzyme in a single event.

Although the model in Fig. 10 satisfies the data presented in this paper, it takes no account of the translocation of DNA during supercoiling (Brown and Cozzarelli, 1979; Mizuuchi et al., 1980); this translocation step would require further enzyme-DNA complexes to be included in Fig. 10. However, the data in this paper do not address the question as to whether the observed ATP hydrolysis in the presence of DNA fragments is coupled to any DNA movement. Also, in view of the AB2 structure of the gyrase complex, the enzyme will contain two ATP-binding sites. No account of this has been taken in Fig. 10, and only one ATP molecule is shown to be hydrolyzed during each catalytic cycle; it is quite possible that ATP molecules are hydrolyzed pairwise in a coupled process. In conclusion, the data in this paper are consistent with a model...
in which DNA gyrase contains two (or more) DNA-binding sites which mediate the ATPase stimulation and exclude a model involving the interaction of DNA with a single site on the enzyme to stimulate the ATPase.

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