Hydrolysis of ATP at Only One GyrB Subunit Is Sufficient to Promote Supercoiling by DNA Gyrase*

(Received for publication, June 22, 1998, and in revised form, August 12, 1998)

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The three-dimensional structure of DNA plays a key role in many biological processes. Reactions such as replication, transcription, or recombination not only are regulated but also have a profound effect on the topology of the DNA molecule. The enzymes responsible for maintaining the topological state of DNA are DNA topoisomerases. One such enzyme is DNA gyrase, a bacterial topoisomerase that introduces negative supercoils into DNA in a reaction coupled to ATP hydrolysis. The action of gyrase involves the creation of a double-stranded break in one DNA segment and the passage of another segment through this enzyme-stabilized DNA gate.

The ability of gyrase to negatively supercoil DNA is unique among topoisomerases and is based on its mode of DNA binding (1, 2). Gyrase wraps DNA in a right-handed manner (1), resulting in the positioning of two segments of DNA in the right orientation for supercoiling. Binding of ATP closes a protein clamp that traps the DNA segment to be transported. The nucleotide is then hydrolyzed, and the free energy is coupled to the supercoiling reaction. After hydrolysis, the enzyme is reset for another round of supercoiling. The limit of supercoiling is believed to be thermodynamic rather than steric, because gyrase can supercoil very small DNA circles, whereas a nucleotide analog ATPos (3) with higher free energy of hydrolysis than ATP is capable of taking the limit of the supercoiling reaction to higher negative superhelical density (3, 4).

Gyrase is a heterotetramer in which two A (GyrA, 97 kDa) and two B (GyrB, 90 kDa) subunits constitute an A2B2 complex (5). There is one ATP-binding site per GyrB, which is situated in the 43-kDa N-terminal domain of the protein. The structure of this domain complexed with the ATP analog 5′-adenylyl-β,γ-imidodiphosphate (ADPNP) was solved by x-ray crystallography and was found to be a dimer (6). Study of the ATPase reaction of this domain revealed that dimerization is an essential step for ATP hydrolysis (7). It is very likely that dimerization of the 43-kDa domain also occurs in the ATPase reaction of intact gyrase (8). The rate of ATP hydrolysis by gyrase is stimulated by the presence of DNA (9), and the kinetics of hydrolysis show positive cooperativity between the two ATP-binding sites (10–12).

A number of issues concerning the mechanism of ATP hydrolysis are still unclear. These include the mechanism of cooperativity between the two sites and the coupling of the free energy produced by these two reactions to a strand passage event. Directly related to these issues is the question of whether ATP hydrolysis can take place only in one of the two sites and the capacity of such a reaction to support supercoiling. At the molecular level the mechanism of hydrolysis of ATP by gyrase involves nucleophilic attack by water on the γ-phosphate of ATP with a Glu42 of GyrB acting as a general base (13). Mutation of Glu42 to Ala in GyrB abolishes ATP hydrolysis but not nucleotide binding (13). We formed heterogeneous gyrase tetramers containing one wild-type and one mutant GyrBAla42 subunit and used these complexes to address the above questions.

Experimental Procedures

Enzymes, DNA, and Assays—Wild-type GyrA and GyrB proteins were prepared as described previously (14). Mutant GyrBAla42 or GyrBAsp42 proteins were a gift of Dr. A. P. Jackson and were made as described previously (13). Relaxed and supercoiled forms of pBR322 DNA were gifts of A. J. Howells (Leicester University), whereas linear pBR322 was made by digestion of the supercoiled form by EcoRI. Limited proteolysis experiments were performed as described previously (8). ATPase assays were performed as described previously (7, 15), in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM MgCl2, 5 mM dithiothreitol, 6.5% w/v glycerol, 2 mM ATP at 25 °C. The extent of supercoiling was determined by removing samples from the ATPase reactions and analyzing them by agarose gel electrophoresis.

Formation of Heterogeneous Tetramers—Heterogeneous tetramers were formed by mixing the indicated amounts of wild-type and mutant GyrB proteins and allowing the mixture to equilibrate at 25 °C for 1 h. GyrA was then added in excess of the total B protein, and the gyrase complexes were allowed to form for 1 h at 25 °C. After DNA was added (where indicated), reactions were incubated at 25 °C for 30 min. 2 mM ATP was then added to initiate the reactions.

Results

ATP Traps A2B2Ala42 in the Dimerized Form (Complex II)—Limited proteolysis has been used as a sensitive probe for detecting conformational changes in DNA topoisomerases (8, 16). Treatment of gyrase with trypsin produces two major fragments, one of ~62 kDa, derived from the A protein, and another of ~25 kDa from the B protein (Fig. 1); this proteolytic fingerprint has been termed complex I (8). Binding of ADPNP induces dimerization of the B subunits resulting in a conformation that protects the 43-kDa domains of GyrB from trypsin digestion. The characteristic fingerprint of this conformation (termed complex II) consists, apart from the two above men-

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† Supported by a BBSRC-CASE studentship funded by the BBSRC and Glaxo-Wellcome and a grant from the Alexander S. Onassis Public Benefit Foundation.

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1 The abbreviations used are: ATPos, adenosine 5′-O-(1-thiotriphosphate); ADPNP, 5′-adenylyl-β,γ-imidodiphosphate.

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Printed in U.S.A.
proteins to equilibrate and then adding GyrA, a random distribution by allowing a mixture of the wild-type and mutant GyrB proteins (17). Therefore, we expect in the absence of GyrA, suggesting that a significant proportion of the two ATP-binding sites in the case where both sites are occupied by the nucleotide. To achieve this we formed heterotetramers of gyrase that contained, apart from the two GyrA protomers, one wild-type GyrB subunit and one mutant GyrBAla42 subunit. Such complexes can be formed by reconstituting the gyrase heterotetramer in the presence of both wild-type and mutant GyrB proteins (17). Electron microscopy and cross-linking studies have failed to detect any dimeric forms of GyrB and mutant GyrB proteins (17). Electron microscopy and cross-linking studies have failed to detect any dimeric forms of GyrB and mutant GyrB proteins (17). Electron microscopy and cross-linking studies have failed to detect any dimeric forms of GyrB and mutant GyrB proteins (17). Electron microscopy and cross-linking studies have failed to detect any dimeric forms of GyrB and mutant GyrB proteins (17).

**ATP Hydrolysis Can Proceed Only at One Site**—We addressed the possibility of ATP hydrolysis occurring only at one of the two ATP-binding sites in the case where both sites are occupied by the nucleotide. To achieve this we formed heterotetramers of gyrase that contained, apart from the two GyrA protomers, one wild-type GyrB subunit and one mutant GyrBAla42 subunit. Such complexes can be formed by reconstituting the gyrase heterotetramer in the presence of both wild-type and mutant GyrB proteins (17).

Fig. 1. **ATP traps A_B^Ala42 in the dimerized form.** Samples contained 0.3 mg/ml GyrA, 0.3 mg/ml GyrB (wild type or mutant), 0.4 mg/ml linear pBR322, and 2 mM ATP or ADPNP (where indicated) and were incubated for 1 h at 25 °C to allow the nucleotide to bind. 10 μg/ml trypsin was then added, and the reactions were incubated for 1 h at 37 °C. The results were analyzed by SDS-polyacrylamide gel electrophoresis. On the right is a diagrammatic representation of the gyrase fragment corresponding to each band. The letter denotes the subunit (A or B), and the number indicates the approximate size of the fragment in kDa.

![Fig. 1](Image)

**Fig. 2.** ATP hydrolysis can proceed only at one site. The concentration of wild-type GyrB, [Bt], was kept constant at 40 nM monomer, whereas the concentration of GyrB^Ala42, [B^*], was varied from 0 to 480 nM monomer. GyrA was in excess over the total GyrB concentration at 750 nM dimer. The concentration of linear pBR322 DNA, where indicated, was 20 nM. These results are the averages of two determinations. To facilitate comparison between the DNA-free and the DNA-bound complexes, the ATPase rates are expressed in terms of the ratio of the rate exhibited by a certain mixture to the rate exhibited by the sample containing only the wild-type complex. The theoretical line describing the situation of complete inhibition is drawn according to the equation shown in the appendix. (Note that the concentrations of wild-type and mutant GyrBs are expressed on a monomer basis.)
FIG. 3. ATP hydrolysis at one site is sufficient for strand passage. A, mixtures of heterogeneous and homogeneous tetramers were formed by keeping the total GyrB (wild type and mutant) concentration, [B]_tot, constant at 40 nM, whereas the ratio of wild type to mutant was varied from 100% GyrBAla42 to 100% wild-type GyrB. The concentration of GyrA was 60 nM, and 20 nM relaxed pBR322 was present where appropriate. The rates shown here are the averages of three determinations. Results are presented as the ratio of the measured rate to the rate of the sample containing only wild-type gyrase. B, samples were removed from the above reactions at 30-min intervals and were analyzed by agarose gel electrophoresis to measure the limit of supercoiling. The 1% agarose gel contains 1 µg/ml chloroquine. At this concentration of chloroquine, the relaxed topoisomers appear positively supercoiled on the gel, whereas the negatively supercoiled products of the reaction still appear negatively supercoiled but with lower absolute superhelical density.

heterogeneous mixture to that supported by an amount of wild-type enzyme equal to the predicted concentration of wild-type complexes in this mixture. In this experiment, at the time when the heterogeneous mixture had reached the limit of the supercoiling reaction, the wild-type enzyme had been unable to support significant levels of supercoiling (data not shown). This extent of supercoiling is due to a catalytic reaction, because the levels of enzyme used are not sufficient to ascribe this result to a stoichiometric reaction. It appears from these results that heterogeneous tetramers have not lost their ability to supercoil DNA but that the limit of this reaction is lower. The experiments reported here with GyrBAla42 were also performed with the GyrBAla42 mutant, which also binds but does not hydrolyze ATP (13), yielding similar results (data not shown).

DISCUSSION

The A2B2Ala42 complex is unable to hydrolyze ATP (13), and this is manifested by the trapping of this protein in the dimerized conformation (complex II) in the presence of the nucleotide.
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The mechanism of ATP hydrolysis by DNA gyrase. This schematic diagram shows the steps involved in the mechanism of hydrolysis by DNA gyrase. The upper shaded part encloses the pathways undergone by the heterogeneous tetramers. E-E is used here to represent the dyadic symmetry of the gyrase molecule. EE indicates the conformation of gyrase where the B subunits are in the dimerized (clamp-closed) form (complex II). The rate constants are discussed in the text; the equilibrium dissociation constants $K_{d1}$ and $K_{d2}$ are according to Ref. 12.

(Fig. 1). This result also reveals that the complex II-characteristic proteolytic signature is not an attribute of the particular complex formed with ADPNP but reflects the conformation of the catalytic intermediate formed in the presence of ATP. Thus, the structure of complex II formed with ATP or ADPNP is indistinguishable, at least at the level detectable by limited proteolysis.

The mechanism of ATP hydrolysis by DNA gyrase can be summarized in the scheme shown in Fig. 4. The central point in this mechanism is the dimerization of the B subunits (formation of complex II). ATP binds to the monomerized subunits within the $A_B$ complex, and dimerization has to occur before hydrolysis can take place (7). Binding of ATP in only one of the two subunits is sufficient for dimerization (16, 20). Although, once the clamp is in the dimerized form, binding of a second ATP molecule appears not to be possible (6), no direct experimental evidence exists on this issue. It has recently been reported that when ATP binding at one of the two sites was abolished, gyrase could not perform catalytic supercoiling (17). However, it is not clear whether this is because the complex with one ATP has poor or no ATPase activity or whether hydrolysis in this case is uncoupled from strand passage. Nevertheless, with wild-type enzyme, occupation of the two ATP-binding sites is cooperative; therefore the complex with two ATP molecules bound should be the predominant species at high ATP concentrations (10–12). Moreover, experiments with the 43-kDa domain of gyrase showed that dimerization of two ATP-bound domains is favored over dimerization of one bound and one free domain by 2 orders of magnitude (20). ADP does not stabilize the dimerized form (7), resulting in the monomerization of the subunits after hydrolysis (Fig. 4). Hydrolysis in the doubly occupied complex comprises two hydrolysis reactions. In the experiments described in this paper, we set out to address the issue of interdependence between these two hydrolysis events and their coupling to strand passage.

We found that complexes containing only one ATPase-proficient GyrB subunit hydrolyze the nucleotide at a lower rate than the wild-type enzyme. The pathway that describes reactivity at one of the two sites is shown in the upper shaded part of Fig. 4. The spectrophotometric assay used in these experiments for determining the rate of hydrolysis measures changes in the concentration of free ADP; therefore the rate of the actual hydrolysis reaction cannot be measured separately from that of the monomerization and product release steps. However, experiments with the 43-kDa domain of GyrB suggested that the rate-limiting step in the mechanism of ATP hydrolysis by gyrase is either a conformational change associated with monomerization or product release (7). Thus, due to the two ATP molecules hydrolyzed per round, the apparent rate constant of the monomerization/product release step in the wild-type complex can be determined to be $k_{app}^{	ext{BB-DNA}}$ = 0.53 s$^{-1}$. In the case of the heterogeneous tetramer, the mutant site would contain the unhydrolyzed nucleotide after hydrolysis on the other site had occurred. This would likely result in stabilization of the dimerized form, inhibiting the rate of the monomerization/product release step. Therefore, in the heterogeneous tetramer-DNA complex, the rate-limiting step slows down to $k_{app}^{	ext{BB-DNA}}$ = 0.19 s$^{-1}$ (note that only one ATP is hydrolyzed per heterogenous tetramer). In the absence of DNA, gyrase exhibits a much lower turnover number, $k_{app}^{	ext{DNA}}$ = 0.12 s$^{-1}$ in our experiments. This is because binding of gyrase to DNA stimulates the ATPase activity of the enzyme (9). It is not yet clear what is the rate-limiting step in the reaction of the DNA-free complex. If monomerization or product release is again the rate-limiting step, then the results obtained here with the heterogeneous tetramer could be explained in terms of the inhibition of the monomerization/product release step by the bound nucleotide. If this is the case, DNA binding should stimulate the ATPase activity by accelerating the monomerization/product release part of the mechanism. However, it is possible that in the absence of DNA, the rate-limiting step is the actual hydrolysis reaction. DNA binding would stimulate the rate of this step, thus making monomerization/product release rate-limiting in the enzyme-DNA complex.

The limit of the supercoiling reaction in the presence of the heterogeneous tetramers was $\Delta Lk$ 17 in contrast with $\Delta Lk$ 20 observed in the case of wild-type gyrase. The value of $\Delta Lk$ 20 is in good agreement with that determined previously for the limit of supercoiling in the absence of spermidine (21). Assuming that the limit of the supercoiling reaction is directly related to the free energy of ATP hydrolysis (4), the lower absolute superhelical density reached in the case of the heterogeneous complex should reflect the reduction in the free energy released when only one ATP is hydrolyzed. However, the free energy released by the extra ATP molecule does not reflect the difference in the free energy of the products, if this is determined according to the relationship described by Cullis et al. (4). In other work, the dependence of the limit of supercoiling on the free energy of ATP hydrolysis was studied by varying the ratio of [ATP]/[ADP] present in the reaction while keeping the total concentration of nucleotides constant (21). These data suggested that, especially in the absence of spermidine, the relationship between linking number change and phosphate potential is not proportional, due to significant ATPase slip and ATP-independent DNA relaxation (21). We believe that the results obtained here reflect a complicated equilibrium established between the ATP-dependent supercoiling and ATP-independent relaxation reactions of the wild-type and heterogeneous complexes. Therefore, making any correlation between the
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limit of supercoiling and the free energy of ATP hydrolysis is beyond the capacity of these experiments.

In conclusion, we found that gyrase can hydrolyze ATP at one active site and can supercoil DNA even with only one ATP hydrolyzed at a time. Although this reaction is unlikely to occur in vivo, due to the physiological ATP concentration being relatively high, the results obtained here provide us with useful information on the mechanism of energy coupling in DNA gyrase.

Acknowledgments—We thank Mary O'Dea, Janid Ali, and Andy Bates for reading the manuscript and Andy Jackson and Ali Howells for gifts of enzyme and DNA.

APPENDIX

If \([B]\) is the concentration of wild-type and \([B^*]\) is the concentration of the mutant GyrB protein, then the total concentration of B protein in a mixture of the above two will be:

\[
[B]_{tot} = [B] + [B^*]
\]

(Eq. 1)

Considering random mixing of the two GyrB proteins and an excess of GyrA over \([B]_{tot}\), the concentrations of the various species of heterotetramer formed would be:

\[
[A_B]_2 = \frac{[B]^2}{2[B]_{tot}} [A_B][B^*] = \frac{[B][B]_{tot} - [B]}{[B]_{tot}}
\]

(Eq. 2)

\[
[A_B^*] = \frac{([B]_{tot} - [B])^2}{2[B]_{tot}}
\]

The rate of hydrolysis \(v = d[ADP]/dt\) exhibited by one such mixture would be the sum of the rates exhibited by the \(A_B^*\) and \(A_BB^*\) complexes (\(A_B^*\) does not hydrolyze the nucleotide):

\[
v = v_{BB^*} + v_{BB^*}
\]

(Eq. 3)

If \(k_{BB^*} = k_{BB^*}/[A_B^*]\) is the turnover number of the wild-type complex (\(A_B^*\) and \(k_{BB^*} = k_{BB^*}/[A_B^*]\) is the turnover number of the mutant complex (\(A_B^*\)), then by combining the above equations, we can estimate the rate of hydrolysis in terms of the starting concentrations of the wild-type and mutant proteins. Thus, the rate exhibited by the sample containing only wild-type \((v_{wt})\) can be expressed in terms of the ratio of the concentration of mutant to the concentration of wild-type protein present in the reaction (\([B^*]/[B]\)):

\[
v = \frac{1 + 2k_{BB^*}([B^*]/[B])}{k_{BB^*}}
\]

(Eq. 5)

In the experiment described in Fig. 3, the total concentration of B protein, \([B]_{tot}\), is constant, whereas the ratio of wild-type to mutant subunit is varied. In this case, the rate exhibited by the sample containing only the wild-type complex is \(v_{wt} = k_{BB^*}[B]_{tot}/2\), because in this particular sample \([B] = [B]_{tot}\). From this and Equation 4 the ratio \(v/v_{wt}\) can be written in terms of the ratio of the concentration of the wild-type subunit to the total concentration of B protein (\([B]/[B]_{tot}\)):

\[
v = \frac{1 - 2k_{BB^*}([B]/[B]_{tot})^2 + k_{BB^*}([B]/[B]_{tot})}{k_{BB^*}([B]/[B]_{tot})}
\]

(Eq. 6)

The theoretical lines describing the situations of complete inhibition or no inhibition can be derived from the above equations by substituting \(k_{BB^*}\) by 0 or \(k_{BB^*}/2\), respectively.

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