Mutations in the B Subunit of Escherichia coli DNA Gyrase That Affect ATP-dependent Reactions*

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We have previously reported specific labeling of Escherichia coli DNA gyrase by the ATP affinity analog pyridoxal 5'-diphospho-5'-adenosine (PLP-AMP), which resulted in inhibition of ATP-dependent reactions. The analog was found to be covalently bound at Lys gaping residues on the gyrase B subunit (Tamura, J. K., and Gellert, M. (1990) J. Biol. Chem. 265, 21342-21349). In this study, the importance of these two lysine residues is examined by site-directed mutagenesis.

Substitutions of Lys result in the loss of ATP-dependent functions. These mutants are unable to supercoil DNA, to hydrolyze ATP, or to bind a nonhydrolysable ATP analog, 5'-adenyl-β,γ-imidodiphosphate (ADPNP). The ATP-independent functions of gyrase, such as relaxation of negatively supercoiled DNA and oxolinic acid-induced cleavage of double-stranded DNA, are unaffected by these mutations, suggesting that the mutant B subunits are assembling correctly with the A subunits. Gyrase with substitutions of Lys retains all activities. However, the affinity of ATP is decreased. The DNA supercoiling activity of gyrase A,B, tetramers reconstituted with varying ratios of inactive mutant and wild-type gyrase B subunits is consistent with a mechanism of DNA supercoiling that requires the interdependent activity of both B subunits in ATP binding and hydrolysis.

DNA gyrase is a bacterial type II topoisomerase that couples the free energy of ATP hydrolysis to the introduction of negative supercoils into closed-circular DNA. (For recent reviews, see Refs. 1-3.) In the absence of ATP, DNA gyrase relaxes negatively, but not positively, supercoiled DNA. In contrast, eukaryotic and T-even phage type II topoisomerases, which are structurally related to DNA gyrase (4), catalyze ATP-dependent relaxation of both positively and negatively supercoiled DNA, but do not supercoil DNA (5). The topoisomerase reactions of all type II enzymes involve the passage of a double-stranded DNA segment through a transient double-strand break, which is then resealed to give changes in the DNA linking number in steps of two.

DNA gyrase from Escherichia coli contains two subunits, A (GyrA) and B (GyrB), which are assembled in an A,B complex (6, 7). GyrA has a molecular weight of 97,000 and is essential for DNA breakage and reunion. An intermediate step in this reaction involves the covalent attachment of Tyr of GyrA at the broken 5'-end of each DNA strand (8). GyrB has a molecular weight of 90,000 and carries a site for ATP binding and hydrolysis (9-12). Affinity labeling studies using the ATP analog, PLP-AMP, have identified Lys and Lys as possible active site residues (13). The presence of Lys at the nucleotide binding site was later confirmed by crystallographic studies on an N-terminal fragment of GyrB with bound ADPNP (14), an ATP analog which cannot be hydrolyzed by gyrase.

The mechanism of energy coupling between ATP hydrolysis and DNA supercoiling is largely unknown. Limited supercoiling of relaxed closed circular DNA occurs upon the binding of ADPNP, suggesting that conformational changes associated with nucleotide binding can induce one supercoiling event while catalytic supercoiling requires hydrolysis of ATP and dissociation of products (15, 16). Once formed, the gyrase-DNA-ADPNP complex does not readily dissociate. Binding of ADPNP to a gyrase-DNA complex is slow, and involves cooperative interactions between the two nucleotide binding sites in the gyrase tetramer (16). Modification of only one of these sites appears to inhibit both ATP hydrolysis and DNA supercoiling (13).

In this work, we introduce mutations into GyrB at Lys and Lys and study the effects of these changes on the ATP-dependent and ATP-independent reactions of DNA gyrase. Evidence is presented indicating that Lys, which is not conserved in the equivalent region of the eukaryotic type II topoisomerases, is essential for the ATP-dependent activities of gyrase. Results of supercoiling experiments combining wild-type and Lys mutant GyrB subunits in the reconstituted enzyme are discussed in terms of a mechanism requiring participation by both nucleotide binding sites of the gyrase A,B tetramer.

EXPERIMENTAL PROCEDURES

Materials—MAX Efficiency DH5αF’IQ™ competent E. coli cells were from Life Technologies, Inc. E. coli strain TG1 was supplied by Amersham Corp. Phage M13mp9gurB was made by inserting a 3.7-kilobase XmaI/HpaI DNA fragment containing most of the coding region of gyrB into M13mp9 as described by Adachi et al. (17). E. coli strain J MtacB (18) was a gift from A. Maxwell. Plasmid pAG111 (19), which carries the E. coli gyrB gene under control of the tac promoter, was prepared by alkaline lysis and CsCl centrifugation from J MtacB. Restriction endonucleases were obtained from New England Biolabs. GyrA protein was purified from E. coli strain N4186 by the method of Mizuuchi et al. (19). Oligonucleotides for mutagenesis and sequencing primers were synthesized using an Applied Biosystems model 380B DNA synthesizer. DNA sequencing was performed using Sequenase Version 2.0 from U. S. Biochemical Corp.

Site-directed Mutagenesis—Specific amino acid substitutions in E. coli GyrB were made by oligonucleotide-directed mutagenesis using M13mp9gurB single strand template DNA and a kit supplied by Amersham Corp. Oligonucleotides were designed to introduce only the desired amino acid substitution while in most cases also creating a new restriction site for rapid screening of clones (Table I). The presence of the required base changes was confirmed by nucleotide sequencing of
the purified single-stranded phage DNAs grown in TG1 cells. The double-stranded replicative forms of the phage DNAs were also prepared and the 428-base pair XmaI/NcoI restriction fragments containing the mutations were used to replace the wild-type XmaI/NcoI fragment in pAG111. After transformation into DH5αF′10⌃, the mutant plasmids were screened by restriction endonuclease digestion for the presence of intact insert ends, and for the newly created restriction sites where applicable. Plasmids meeting these criteria were then sequenced over the entire 428-base pair insert region to ensure that no other mutations had taken place.

Purification of GyrB Proteins—DH5αF′10⌃ cells carrying wild-type or altered pAG111 were grown, induced with isopropyl β-D-thiogalactopyranoside, and harvested as described by Hallett et al. (18). Cell lysis, streptomycin/ammonium sulfate fractionation, heparin-agarose chromatography, and DEAE-Sepharose chromatography were performed as described by Mizuuchi et al. (19), with the following exception. The heparin-agarose columns were developed with 40-column volumes linear gradients of 0.05–0.5 M NaCl in 20 mM Tris-HCl (pH 7.5), 0.2 mM EDTA, 5 mM dithiothreitol, 10% (w/v) glycerol. GyrB protein eluted as two peaks identified by SDS-polyacrylamide gel electrophoresis, and by DNA supercoiling assay of the wild-type protein preparation. The first peak, which contained less GyrB protein but had much greater specific DNA supercoiling activity, the specific activity of the wild-type GyrB and the specific activity of the wild-type protein are also shown.

Information about the specific activity of wild-type GyrB and the specific activity of the wild-type protein are also shown. The method, for kinetic studies of DNA-dependent ATPase activity, was an ATP-regenerating spectrophotometric assay used as described previously (13). The apparent values for $K_m$ and $k_{cat}$ were determined from double reciprocal plots of the turnover rate for ATP hydrolysis against the concentration of ATP (0.1–1.0 mM). Prior to the assay, 1.2 μM GyrA, 1 μM GyrB, 50 μM linear pUC9 DNA, and 0.2 mg/ml bovine serum albumin were preincubated for 1 h at room temperature in buffer 1. ATPase reactions were initiated by the addition of 10 μl of enzyme to 90 μl of the ATPase assay mixture. Binding studies using ADPNP (Sigma) and (α-32P)ADPNP (ICN) were carried out as described elsewhere (16).

### RESULTS

Mutations in E. coli GyrB Protein

**TABLE I**

| Mutation | Oligonucleotide | Gyrb protein specific activity |
|----------|-----------------|-------------------------------|
| K103T    | CAC GCA GGC GGT ACC TTT GAC GAT AAC TCC | $10^8$|
| K103E    | CAC GCA GGC GGT GAA TTC GAC GAT AAC TCC | 0 |
| K103G    | CAC GCC GGC GGT ACC TTT GAC GAT AAC TCC | 0 |
| K110V    | GAT AAC TCC TAC GTA GTG TCC GGC GGT | $4.7 \times 10^5$|
| K110E    | GAT AAC TCC TAT GAA GTG TCC GGC GGT | $4.5 \times 10^5$|
| WT       | CAC GCA GGC GGT AAA TTT GAC GAT | $5 \times 10^6$|

Wild-type and mutant GyrB proteins were expressed in E. coli as described under "Experimental Procedures." Cells producing the lys110 GyrB mutants grew at the same slow rate as those producing the wild-type protein. On the other hand, cells producing the lys103 mutants grew more rapidly, indicating that excess production of the lys103 mutants may be less toxic to the cells than overproduction of the wild-type GyrB protein. Expression of all of the GyrB proteins was very high, averaging about 30% of the total cell protein. However, based on the supercoiling assay, the specific activity of the wild-type GyrB protein in the cell lysate was much lower than expected. Further investigation led to the finding that wild-type GyrB protein could be resolved into two peaks, which eluted at 0.2 and 0.3 M NaCl on a heparin-agarose column. The first peak had a much higher specific supercoiling activity than the second, which may largely consist of improperly folded protein. Wild-type GyrB prepared from a strain carrying the same plasmid as used here was previously found to contain a large amount of low-activity protein, which showed increased activity after renaturation from guanidine hydrochloride solution (12). All of the mutant GyrB proteins were similarly partitioned into two peaks on heparin-agarose columns. DEAE-Sepharose chromatography of the more active wild-type peak fractions produced a nearly homogenous GyrB preparation with a specific activity close to that previously reported (19). This wild-type GyrB and
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**ATP-independent Activities**—To ensure that the mutated forms of GyrB were still able to form a complex with GyrA, we tested the ATP-independent activities of the enzyme. Quinolone antibiotic drugs, such as oxolinic acid, interfere with the DNA breakage-reunion activity of DNA gyrase by trapping the covalent DNA-protein intermediate. Subsequent treatment with SDS and proteinase K results in double-strand breaks in the DNA (20, 21). This cleavage activity occurs in the absence of ATP, as does the relaxation of negatively supercoiled DNA. Both activities can only be supported by the enzyme A<sub>2</sub>B<sub>2</sub> tetramer and not by GyrA or GyrB alone. The ability of the enzymes containing mutant GyrB proteins to catalyze these ATP-independent reactions would indicate proper formation of the enzyme tetramer. The results in Fig. 1 show that in the absence of ATP, the wild type and all of the mutant enzymes were equally capable of promoting oxolinic acid-induced DNA cleavage. Similarly, all of the mutants completely retained the capacity to relax negatively supercoiled DNA.

Both ATP-dependent activities show interesting differences between amino acid substitutions at positions 103 and 110 of GyrB. The K103E and K103I enzymes showed no detectable levels of supercoiling activity; the activity of K103T was reduced 500-fold. On the other hand, both K110V and K110E gyrases had specific activities for supercoiling that were only slightly less than that of the wild-type enzyme (Table I). However, with high enzyme/DNA ratios and longer incubations, the limit of negative supercoiling reached by these mutants was less than that of wild-type GyrB, as visualized by chloroquine gel electrophoresis (Fig. 2). Increasing the ATP concentration to 5.4 mM (added as Mg<sup>2+</sup>) did not increase the supercoiling limit for the Lys<sup>110</sup> mutant enzymes (data not shown).

**ATP Hydrolysis**—The ATPase reaction of gyrase reconstituted with K110V and K110E GyrB subunits was investigated using a spectrophotometric assay. A double reciprocal plot of the results is presented in Fig. 3. The k<sub>app</sub> for ATP of the wild-type enzyme control is 0.24 nM<sup>-1</sup>s<sup>-1</sup>, in good agreement with previously reported values (13, 15). However, the k<sub>app</sub> values for K110V and K110E (1.4 nM<sup>-1</sup>s<sup>-1</sup> and 1.1 nM<sup>-1</sup>s<sup>-1</sup>, respectively), are several-fold higher than for wild-type, suggesting that these mutations decrease the affinity for ATP. The k<sub>app</sub> values for wild-type, K110V, and K110E enzymes are 2.7, 2.3, and 1.3 s<sup>-1</sup>, respectively. Similar analyses of the Lys<sup>110</sup> mutants were not performed because the ATPase activities were below the detection limits of the assay.

Using a more sensitive radioactive assay (see "Experimental Procedures"), the effects of the various mutations on the hydrolysis of [γ-<sup>32</sup>P]ATP were studied. The K103E, K103I, or K103T GyrB proteins had very low levels of ATPase activity, either in the presence or absence of GyrA and DNA. This residual ATP hydrolysis was largely insensitive to novobiocin suggesting that most, if not all, of the activity can be attributed to contaminating ATPases in the preparations. In assays using the K110V or K110E enzymes, DNA-dependent novobiocin-sensitive ATPase activity was observed at levels 2-3-fold lower.
Fig. 4. Binding of ADPNP to wild-type and mutant GyrB proteins and to their complexes with GyrA and DNA. GyrB proteins (0.5 μM) were preincubated alone or with 0.5 μM GyrA protein and 50 μg/ml linear pBR322 DNA in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 10 mM potassium phosphate (pH 7.5), 5 mM dithiothreitol, 1.8 mM spermidine, 6 mM MgCl₂, 0.5 mg/ml bovine serum albumin, and 6.5% glycerol at 37°C for 30 min at 25°C. 50 μM [α-32P]ADPNP was added and the incubation at 25°C was continued for 5 h or 25 h. Unbound nucleotide was removed by rapid gel filtration and stoichiometry of binding was determined as described by Tamura et al. (16).

Fig. 5. Correlation between DNA supercoiling activity and percentage of inactive GyrB protein used in reconstitution of the gyrase tetramer. Wild-type and K103I mutant GyrB proteins were combined in 1.6 μM solutions at molar ratios of 10:0, 9:1, 4:1, 2:1, 1:1, 1:2, 1:4, 1:9, and 0:10. Mixed gyrase tetramers were formed by adding an equimolar amount of GyrA protein to each GyrB solution and incubating for 30 min at 25°C. A series of dilutions of each tetramer preparation was assayed for supercoiling activity. Experimentally determined supercoiling activities of the mixed tetramers (Δ) are expressed as percentages of the activity of tetramers containing 100% wild-type GyrB protein. Theoretical curves 1, 2, and 3 are described under "Results".

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than with the wild-type enzyme (data not shown).

Cleavage Site Preference—In the presence of oxolinic acid, there is a slight enhancement by ATP of DNA cleavage efficiency, and an accompanying change in cleavage site preference is seen with the wild-type, K110V, or K110E enzymes. In contrast, ATP had no effect on cleavage by the mutants K103T, K103E, and K103I (Fig. 1). A change in the DNA cleavage site preference in the presence of ATP is thought to be the result of a conformational change in the enzyme due to ATP binding (22).

ADPNP Binding—In the presence of ADPNP, DNA gyrase can introduce negative supercoiling into a relaxed closed-circular DNA substrate (15, 16). Both of the LysK110 mutants were able to carry out this limited ADPNP-dependent supercoiling while no such activity was detected in all three LysK103 mutants (data not shown). Is the loss of ATP-dependent functions for the LysK103 mutants due to their inability to bind nucleotides? We addressed this question by utilizing spin columns to test the enzymes for their ability to bind ADPNP (Fig. 4). After 5 h at 25°C, both LysK110 mutant GyrB proteins bound ADPNP at about 50% of the wild-type level. We have previously reported a small increase in the rate of ADPNP binding to GyrB in the presence of GyrA and DNA (16). The 80% increase in ADPNP bound to the GyrB in the wild-type A₂B₂-DNA complex after 5 h is consistent with this enhanced binding rate. However, with the LysK110 mutants, there is little or no increase in ADPNP binding in the presence of GyrA and DNA; after 5 h, ADPNP bound to the mutant A₂B₂-DNA complexes is only about 28% of the wild-type level. This value increased to approximately 55–60% after 25 h. In contrast, negligible amounts of bound ADPNP were detected on the LysK103 mutants even after 25 h in the presence of GyrA and DNA. Therefore, LysK103 appears to be essential for nucleotide binding.

Reconstitution of Mixed Gyrase Tetramers—Since we have demonstrated that the LysK103 mutants of GyrB can assemble with GyrA to form a complex capable of catalyzing ATP-independent reactions, we were able to examine whether catalytic DNA supercoiling requires one or two active GyrB subunits in the A₂B₂ tetramer. The DNA supercoiling activity was measured using gyrase tetramers reconstituted with varying ratios of wild-type and mutant (K103I) GyrB proteins. This experiment requires the following assumptions: 1) GyrB proteins exist as monomers in solution and 2) association of the mutant and wild-type GyrB proteins with GyrA proteins is a random process, with no selective mechanism favoring formation of enzyme A₂B₂ tetramers with two wild-type or two mutant GyrB subunits. Cross-linking studies of purified GyrB protein from Micrococcus luteus have shown no higher order complexes, suggesting that the GyrB protein is indeed monomeric in solution (6). Recent work using a 43-kDa N-terminal fragment of GyrB from E. coli showed that this protein is also a monomer, although it forms a dimer in the presence of ADPNP (11). Reconstitution of mixed gyrase tetramers in the absence of nucleotide should yield predictable proportions of enzyme containing two active GyrB subunits, two inactive GyrB subunits, one of each, and one.

Fig. 5 shows the result of the reconstitution experiment. Included in the figure are three theoretical curves, each predicting a different outcome depending on the mechanism. The straight line (curve 1) is subject to two interpretations. First, curve 1 represents the anticipated results if the GyrB protein in solution is already a dimer or if assembly is not random but strongly favors two GyrB subunits of the same type per tetramer. Tetramers containing one active and one inactive GyrB monomer would be unlikely to form; the specific supercoiling activity of the wild-type enzyme would be simply diluted. Alternately, curve 1 would also be obtained if tetramers containing one inactive and one active B monomer are formed, but the activity of the mixed tetramer is proportional to the active GyrB content; that is, a mixed tetramer would have half the activity of a tetramer containing two active B subunits. The upper curve (curve 2) predicts the results if assembly of the gyrase tetramers is random, and catalytic supercoiling can occur with enzyme having one active and one inactive GyrB subunit; it is assumed that an enzyme tetramer containing only one active GyrB subunit has full catalytic activity. This result could also be achieved if a conformational change following
Initially proposed from the results of affinity labeling studies, ing for the region of GyrB comprising amino acids 103–119 was nucleotide binding (23–25). A possible role in nucleotide binding loop structure involved in conformational changes following binding proteins, has been postulated to form part of a flexible II). This region, which is also found on a variety of other ATP bacterial, phage, and eukaryotic type II topoisomerases (Table II). These sequences of residues 96–104 are quite different in both ATP binding sites in the enzyme A2B2 complex.

**DISCUSSION**

ATP-dependent type II topoisomerases possess an amino acid sequence motif predictive of ATP binding. This motif, a highly conserved glycine-rich region (residues 114–119 of GyrB from E. coli) of the sequence GXXGXX, is found in all known bacterial, phage, and eukaryotic type II topoisomerases (Table II). This region, which is also found on a variety of other ATP binding proteins, has been postulated to form part of a flexible loop structure involved in conformational changes following nucleotide binding (23–25). A possible role in nucleotide binding for the region of GyrB comprising amino acids 103–119 was initially proposed from the results of affinity labeling studies, in which Lys103 and Lys110 were specifically modified (13). The importance of this region was shown in more detail in the crystal structure of a 43-kDa N-terminal domain of GyrB containing bound ADPNP (14). The structure shows that residues 96–117 form a loop followed by a short helix composed of residues 118–126. The phosphates of the bound nucleotide are contained in the loop, as well as the base of the helix. Furthermore, the ε-amino group of Lys103 forms a salt bridge with the β-phosphate of the bound ADPNP molecule, and Tyr109 forms a hydrogen bond with N3 of the adenine ring. When the amino acid sequence of E. coli GyrB residues 96–127 is compared with the corresponding region of all other bacterial GyrB proteins for which sequences have been determined, a very high degree of conservation is found. A separate comparison of these regions from the eukaryotic type II topoisomerases also shows very high sequence homology. However, comparing the gyrases to the eukaryotic enzymes reveals variations in this region which may be significant (Table II). The sequences of residues 96–104 are quite different in the two classes of enzyme; in particular, the Gly101 and Gly102 residues of gyrase, which should contribute to the flexibility of the loop, are replaced by serine, and Lys103, which is conserved in all GyrB species, is replaced by asparagine in all of the eukaryotic type II topoisomerases. The Tyr109 residue found in all GyrB proteins is replaced by lysine, glutamine or serine in the eukaryotic type II topoisomerases. Lys110 is commonly found in enzymes from both groups; however, substitutions at this position have been identified in both gyrases and eukaryotic type II topoisomerases. Mutation of the corresponding lysine to alanine in yeast topoisomerase II had little effect on the activity (26). Thus, in eukaryotic type II topoisomerases, neither the equivalent of Lys103 nor of Lys110 is required. It is interesting that this segment of E. coli ParE, a component of bacterial topoisomerase IV, very closely resembles GyrB in overall sequence, while the phage T2 and T4 gene 39 proteins include characteristics of both gyrase and eukaryotic type II topoisomerase. Like gyrase, topoisomerase IV and the T-even phage type II topoisomerases are multimers of two or more different subunits, but they resemble the homodimeric eukaryotic type II topoisomerases in activity.

Our present results suggest that Lys103 is essential for the ATP-dependent activities of DNA gyrase. Mutations at this site resulted in loss of these functions but did not affect ATP-independent activities. The ADPNP binding results, along with the implication of Lys103 in nucleotide binding from the crystal structure, support the hypothesis that it is the ability to bind ATP which is lost. However, we have previously proposed that ATP and its analog ADPNP initially form a rapidly reversible complex with gyrase, followed by a conformational change to a tightly bound state (16). It is possible that mutations at Lys103 do not altogether prevent nucleotide binding, but prevent or alter the conformational changes that follow; our spin column assay for ADPNP binding may not detect bound ADPNP if dissociation of the initial complex is very fast. We can never-
directly involved in ATP binding. Enzyme reconstituted with GyrB containing valine or glutamic acid substituted for Lys110 retains all activities of gyrase. However, the increase in the $K_{m}^{ADP}$ for ATP of these mutants, together with the reduced level of ADPNP binding, suggests that ATP binds with lower affinity. The $K_{m}^{ADP}$ and $k_{cat}$ values must be interpreted with caution; cooperative binding of two nucleotide molecules to the two binding sites in each gyrase tetramer, leading to conformational changes in the protein prior to hydrolysis (11, 16, 27) casts doubt upon the validity of a steady-state approach to gyrase kinetics. However, by using gyrase as the preformed $A_{2}B_{2}$-DNA complex and holding the concentration of this complex constant, we obtain apparent parameters for the wild-type and mutant GyrB proteins which provide a useful means for comparing their relative ATPase activities.

Ali et al. (11) have proposed that the rate-limiting step for the ATPase activity of gyrase is not nucleotide binding, but hydrolysis and release of products. However, if the rate of nucleotide binding is considerably slower for the Lys110 mutants, as suggested by Fig. 4, then ATP binding may become rate-limiting. The binding rate of ADPNP to the wild-type gyrase-DNA complex has recently been found to be dependent on the topology of the substrate DNA, with binding being more rapid if the DNA is negatively supercoiled (28). This increase in nucleotide binding rate at higher levels of supercoiling may be less pronounced with the Lys110 mutants. It is therefore possible that with these mutants the ATP-independent DNA relaxation activity would make a greater contribution to the equilibrium superhelical density (Fig. 2).

Our results on the Lys110 mutants are consistent with the structural information on the 43 kDa fragment of GyrB which shows that Lys110 does not form contacts with bound ADPNP (14). It is probable that amino acid substitutions for Lys110 cause a slight perturbation of the protein conformation involved with ATP binding or subunit interactions. This is not surprising in view of the close proximity of Lys110 to residues known to interact with ATP such as Tyr109, Gly114, Gly117, and Gly119.

The Lys103 mutants have provided a unique tool for obtaining gyrase $A_{2}B_{2}$ tetramers containing a predictable distribution of active and inactive GyrB subunits. They offer an alternative to partial inactivation using nucleotide analogs or other inhibitors which might themselves cause or prevent conformational changes when bound to the protein, perhaps blocking interactions between the active and inactivated subunits. We have exploited the fact that the GyrB mutants at Lys103, while devoid of ATP-dependent reactions when reconstituted with GyrA and DNA, appear to retain the capacity to assemble into an enzyme complex capable of carrying out ATP-independent reactions. In the analysis, it was assumed that wild-type and mutant GyrB proteins in a mixture can assemble randomly and equivalently into the enzyme $A_{2}B_{2}$ complex. The observed supercoiling activities closely follow the predicted theoretical curve of a mechanism in which both GyrB subunits must be functional in ATP binding and hydrolysis to catalyze DNA supercoiling.

The interdependent action of the two ATP binding sites in DNA supercoiling by gyrase has been previously proposed. Thermodynamic calculations of the free energy change required to decrease the DNA linking number by two at the high-supercoiling limit of gyrase action are consistent with the concerted hydrolysis of two molecules of nucleotide (29–32). Furthermore, measurements of the inhibition of ATPase and DNA supercoiling activities of gyrase following reaction with the ATP affinity reagent, PLP-AMP, indicate that modification of only one of the two ATP binding sites can lead to inactivation (13). A cooperative model for ATP binding was proposed based on the rates of ADPNP binding in the presence of ATP (16). This model predicts the interdependence of the GyrB subunits for catalyzing ATP-driven reactions and agrees well with the present results from the subunit mixing experiments. Lastly, there is structural information which supports the functional interaction of the two ATP binding sites. The crystal structure of the N-terminal fragment of GyrB with ADPNP bound shows that the fragment exists as a dimer with dyad symmetry (14). Each subunit contains an N-terminal extension that goes from close proximity to one nucleotide binding site to direct contact with the bound nucleotide on the other subunit. The physical contact between the two subunits provides a possible way of coupling ATP hydrolysis at one site to the participation of the ATP binding site on the other subunit.

Related studies of the yeast type II topoisomerase have come to different though not necessarily contradictory conclusions. ATP binding to the two ATPase sites of the DNA-bound homodimer of this enzyme appears to be cooperative (26). At low ATP concentrations, at which the coupling of ATP hydrolysis to relaxation activity is most efficient, an average of 1.9 ± 0.5 ATP molecules are hydrolyzed for each DNA transport event (26). However, in a yeast heterodimer consisting of one wild-type subunit and one mutant allele defective in ATP binding, binding of ADPNP resulted in a concerted conformational change in both wild-type and mutant subunits (26, 33, 34). This suggests the possibility that binding only one ATP to an enzyme dimer might suffice to drive a DNA transport event. However, strand-passage activity by the heterodimer was not studied.

Further comparison of the yeast heterodimer with our present work is complicated because the inactivating mutation at Gly114 of yeast topoisomerase II does not correspond to Lys103 of gyrase, but to Gly117 in the gyrase ATP binding motif. It remains possible that in a Gly117 gyrase mutant an induced conformational change similar to that seen in the yeast topoisomerase II mutant/wild type heterodimer would be found.

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