Energy Coupling in *Escherichia coli* DNA Gyrase: the Relationship between Nucleotide Binding, Strand Passage, and DNA Supercoiling†

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ABSTRACT: Binding of the nonhydrolyzable ATP analogue 5′-adenylyl-β,γ-imidodiphosphate (ADPNP) to *Escherichia coli* DNA gyrase can lead to a limited noncatalytic supercoiling of DNA. Here we examine the efficiency of coupling between ADPNP binding and the change in linking number either of positively or negatively supercoiled plasmid DNA or of small DNA circles. The coupling efficiency varies from 100% (ΔLk = −2 per gyrase tetramer, a stoichiometry of 1) with positively supercoiled substrates under certain reaction conditions to an undetectably low value with moderately negatively supercoiled substrates ($\sigma = -0.046$) or small circular substrates. Furthermore, the rate of ADPNP binding to the gyrase−DNA complex is also dependent on the topological state of the DNA; the previously observed slow binding of ADPNP to the complex of gyrase with linear DNA is accelerated 16-fold when the substrate DNA is negatively supercoiled, suggesting a functional interaction between the nucleotide-binding and DNA-binding domains which is independent of the strand-passage process. The implications for the normal ATP-dependent supercoiling reaction of the enzyme are considered and the results discussed in terms of current mechanistic models for DNA gyrase action and the possible *in vivo* roles of the enzyme.

DNA gyrase is unique among the type II DNA topoisomerase enzymes in its ability to utilize the free energy of ATP hydrolysis for the introduction of negative supercoiling into closed-circular DNA. All other type II enzymes are able only to relax supercoiling already present in DNA. Gyrase provides an accessible system exemplifying energy transduction in biological processes (Reece & Maxwell, 1991).

Active *Escherichia coli* DNA gyrase is a tetramer of two A and two B subunits, of 97 and 90 kDa, respectively. The tetramer binds to double-stranded DNA, with approximately 130 base pairs (bp)[1] of DNA being wrapped around the complex in a positive superhelical sense (Orphanides & Maxwell, 1994). The DNA is cleaved in both strands, roughly in the center of the wrapped segment, via phosphodiester exchange with the Tyr-122 residues of the A subunits, leading to covalent attachment of the 5′-ends of the cleaved strands to the protein. Another segment of DNA, which in the supercoiling reaction is probably near or within the wrapped region, is then passed through the double-stranded break, resulting in the reduction of the linking number (Lk) of a closed-circular DNA by two. The strands are then resealed by the reverse exchange reaction. At some point in this process ATP is hydrolyzed by the B subunits, providing energy for the incorporation of torsional stress into the DNA molecule. The exact mechanism of the coupling of ATP hydrolysis to the introduction of supercoiling is not known in detail, although the ability of the nonhydrolyzable ATP analogue 5′-adenylyl-β,γ-imidodiphosphate (ADPNP) to promote limited noncatalytic supercoiling of relaxed DNA by gyrase has led to the suggestion that the initial binding of nucleotide causes the translocation of DNA through the double-stranded break (i.e., the supercoiling event) and that hydrolysis and product release are responsible for the cycling of the complex to allow catalytic supercoiling (Sugino et al., 1978). It has been suggested in the case of the homologous eukaryotic enzyme, topoisomerase II, that the strand-passage process proceeds by the trapping of the translocated strand through the closing of a nucleotide-operated clamp (Roca & Wang, 1992). Gyrase can also relax positive supercoils, in an ATP-dependent reaction essentially equivalent to the introduction of negative supercoils, and relax negative supercoils in a nucleotide-independent fashion.

A variety of evidence suggests that two molecules of ATP are hydrolyzed per supercoiling cycle. The assumption that one nucleotide molecule binds to each B subunit has been confirmed from studies of the binding of nucleotide analogues to the gyrase−DNA complex (Tamura et al., 1992; Tamura & Gellert, 1990). In addition, the X-ray-derived structure of crystals of the N-terminal 43 kDa portion of the B subunit in the presence of ADPNP has revealed a dimer containing one molecule of the ATP analogue per monomer (Wigley et al., 1991). It has been shown that the supercoiling of a plasmid DNA substrate by gyrase eventually reaches a limit corresponding to a specific linking difference (σ) of −0.11 (Bates & Maxwell, 1989; Westerhoff et al., 1988). This corresponds to a reduction in the linking number of pBR322 DNA (length N = 4361 bp) of ∼46. The free

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[1] Abbreviations: bp, base pairs; Lk, linking number; ADPNP, 5′-adenylyl-β,γ-imidodiphosphate; σ, specific linking difference; N, length of DNA in base pairs; EtBr, ethidium bromide; ΔLk, linking difference; GyrA, gyrase A protein; GyrB, gyrase B protein; DTT, dithiothreitol; BSA, bovine serum albumin; oc, open-circular (nicked) DNA.

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energy required for the final supercoiling step may be estimated from previous determinations of the free energy of DNA supercoiling to be close to the free energy available from the hydrolysis of two ATP molecules under similar conditions, thus suggesting that the process may be thermodynamically limited by the involvement of two ATP molecules per supercoiling cycle. This conclusion has been strengthened by the finding that the additional free energy available from the hydrolysis of $ATP_{\alpha}S(S_p)$ by gyrase may be correlated with the extra supercoiling introduced (Cullis et al., 1992). Tamura et al. (1992) studied the rates of binding and dissociation of ADPNP to the gyrase−DNA complex and, with evidence from structural and other work, proposed a cooperative model for the binding of two nucleotide molecules to the complex and its coupling to the translocation of DNA in the supercoiling event. The coupling of ATP hydrolysis to strand-passage is not absolute, however. Simultaneous measurements of rates of ATPase and supercoiling are difficult to correlate, since the supercoiling of relaxed DNA initially leads to a wide distribution of topoisomer products, but it is clear that the ATPase reaction continues after the substrate DNA has reached its limit of supercoiling and that in general it exceeds the rate of the supercoiling reaction (Sugino & Cozzarelli, 1980).

It has previously been shown that gyrase can carry out the supercoiling of very small circular DNA substrates, down to 174 bp in length, and can effect a linking number change in substrates as small as 116 bp in the presence of ethidium bromide (EtBr), which removes the thermodynamic constraint on the reaction. At such substrate sizes, the enzyme can carry out only one round of supercoiling before reaching the thermodynamic limit to supercoiling discussed above (Bates & Maxwell, 1989).

In this study, we have investigated the efficiency of the coupling of nucleotide binding to DNA supercoiling in both a 174 bp DNA circle and plasmid DNA substrates of varying degrees of supercoiling. The results suggest that a nucleotide-binding event leads with high probability to a strand-passage event only in the case of a positively supercoiled substrate and that the coupling efficiency depends on the torsional stress present during the strand passage step.

EXPERIMENTAL PROCEDURES

Enzymes. *E. coli* DNA gyrase was prepared as described previously (Mizuuchi et al., 1984). Preparations of the gyrase A (GyrA) and gyrase B (GyrB) proteins used in all studies were fractions from valine-Sepharose and leucine-agarose columns, respectively. Each was extensively dialyzed against 50 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM DTT, 1 mM EDTA, and 10% (w/v) glycerol prior to use in DNA supercoiling and nucleotide binding assays. The protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin (BSA) as standard. The derived concentrations were adjusted from previous calibration by amino acid analysis of GyrA and GyrB ([GyrB] = 0.7 × BSA-calibrated measurement; [GyrA] = 1.4 × BSA-calibrated measurement). The A protein was in 1.6-fold molar excess over the B protein in the supercoiling and binding assays. The concentrations of DNA gyrase are quoted as molarities of A_2B_2 complex (defined by half the limiting concentration of B protein). The specific supercoiling activity of the stock A_2B_2 complex was 1.5×10^5 units mg^{-1} GyrB determined as described previously (Reece & Maxwell, 1989); the specific activity of the enzyme used in ADPNP binding studies was 1×10^5 units mg^{-1} GyrB. Pyruvate kinase and lactate dehydrogenase were purchased as a mixture in 50% glycerol solution from Sigma.

DNA. 174 bp circular DNA was prepared by Cre-*loxP* recombination as described previously (Bates & Maxwell, 1989; Bednar et al., 1994). Plasmid pBR322 DNA was prepared from *E. coli* strain MG1182 by the alkaline lysis method (Birnboim & Doly, 1979) or the lysozyme/Triton method (Miller, 1987). Supercoiled DNA was isolated after two rounds of centrifugation on cesium chloride gradients containing EtBr. Relaxed closed-circular pBR322 DNA and samples of increasing negative supercoiling were prepared by relaxation with eukaryotic topoisomerase I in the presence of varying concentrations of EtBr as described previously (Bates & Maxwell, 1989). Positively supercoiled pBR322 DNA was prepared by the ligation of nicked plasmid with *E. coli* DNA ligase in the presence of DNA gyrase, as described (Liu & Wang, 1978) and modified (Clark & Felsenfeld, 1991). Nicked-circular DNA was prepared according to Wang (1974).

DNA Supercoiling Assays: 174 bp Circle. Reactions (15 μL) were carried out for 1 h at 25 °C in 35 mM Tris-HCl (pH 7.5), 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 9 μg mL^{-1} tRNA, 5 mM dithiothreitol (DTT), 6.5% (w/v) glycerol, and 100 μg mL^{-1} bovine serum albumin (BSA) ("standard" conditions) containing relaxed 174 bp circle (12 nM), gyrase (12 nM), ATP or ADPNP (1 mM), and EtBr as indicated and were terminated by the addition of 1 μL of EDTA (500 mM), followed by incubation at 80 °C for 2 min, and extraction with phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitation. Samples were subjected to electrophoresis in 5% polyacrylamide gels [acrylamide/bis (19:1)] in 90 mM Tris-borate (pH 8.0) and 1 mM EDTA containing 0.6 μg mL^{-1} EtBr and silver stained.

pBR322 DNA. Reactions (30 μL) were carried out for the indicated times at 25 °C under three different reaction conditions: 35 mM Tris-HCl (pH 7.5), 9 μg mL^{-1} tRNA, and 5 mM DTT containing 24 mM KCl, 4 mM $MgCl_2$, 1.8 mM spermidine, 6.5% (w/v) glycerol, and 100 μg mL^{-1} BSA (standard conditions); 24 mM KCl, 1.6 mM $MgCl_2$, 5 mM spermidine, 6.5% (w/v) glycerol, and 100 μg mL^{-1} BSA ("nonrelaxation" conditions); and 18 mM KPO_4 (pH 7.5), 6 mM $MgCl_2$, 1.8 mM spermidine, and 360 μg mL^{-1} BSA ("relaxation" conditions) (Gellert et al., 1977). All reactions contained pBR322 DNA at 10 μg mL^{-1} (3.5 nM) and gyrase as indicated. The samples were preincubated for 15 min at 25 °C before the addition of ATP or ADPNP as indicated. In the case of time courses, 30 μL samples were removed from a larger reaction volume at the indicated times. The samples were quenched and treated as described above and electrophoresed in 0.8% agarose gels in 40 mM Tris base, 30 mM NaH_2PO_4, and 1 mM EDTA containing chloroquine at the indicated concentrations. Gels run in the presence of varying concentrations of chloroquine suggest that in all cases (Figures 2−4) the resolved topoisomers are either all positively or all negatively (Figure 4) writhed, and the approximate changes in Lk can be determined simply by counting. Reactions using positively supercoiled DNA were performed as described above, with the DNA concentration reduced to 4 μg mL^{-1} (1.4 nM). Electrophoresis was carried out in 2.5% agarose gels in 40 mM Tris-acetate and 1 mM

EDTA. Gels were stained using EtBr and visualized under UV light.

ATPase Assays. ATPase measurements were performed under nonrelaxation conditions with 20 nM gyrase and 51 nM relaxed or open-circular (nicked) pBR322 in a total volume of 200 μL using a continuous spectrophotometric assay that couples the hydrolysis of ATP to the oxidation of NADH (Tamura & Gellert, 1990). Samples (10 μL) removed at various times from the assays containing relaxed DNA were treated as described above and analyzed by electrophoresis.

ADPNP Binding Assay. ADPNP binding to gyrase in the presence of nicked-circular and negatively supercoiled DNA was measured using a spin-column method, as described previously (Tamura et al., 1992). [α-^{32}P]ADPNP was purchased from ICN. Assays were carried out using 100 μM ADPNP under nonrelaxation conditions.

Quantitation of Gels. The differences in linking number resulting from the gyrase supercoiling assays were determined by scanning and quantitation of gel negatives using an LKB Ultroscan XL densitometer. The distributions of topoisomers were assumed to be Gaussian, and the centers of the distributions were determined from the relative intensities of the resolvable bands. The integral linking difference (ΔLk) of topoisomer bands was determined by band counting on agarose gels (Keller, 1975).

RESULTS

Gyrase Reaction of Small DNA Circles. The binding of the nonhydrolyzable ATP analogue, ADPNP, to DNA gyrase complexed with a relaxed plasmid substrate is sufficient to drive a single supercoiling event in a proportion of the gyrase tetramers (Sugino et al., 1978; Tamura et al., 1992). The fact that the ATP-dependent gyrase supercoiling reaction of a small circular substrate, for example, a 174 bp circle, exhibits only a single cycle of reaction leading to a reduction in Lk of two (Bates & Maxwell, 1989) suggests that a single nucleotide binding event should suffice to carry out the appropriate strand-passage process, i.e., the reaction should proceed in the presence of ADPNP. However, the data presented in Figure 1 suggest that ADPNP will not support the supercoiling of a 174 bp circle. Under the standard DNA supercoiling conditions, ATP supports the supercoiling of the topoisomer of Lk = 17 as seen previously (Bates & Maxwell, 1989). The substitution of ADPNP for ATP abolishes this supercoiling reaction, although the enzyme is present in stoichiometric quantities. In view of a previous report that ADPNP could support catalytic relaxation of positively supercoiled plasmid DNA (Gellert et al., 1980), the supercoiling of the 174 bp circle was carried out in the presence of increasing concentrations of EtBr, which introduces positive writhe into the DNA molecule. This converts the reaction into a relaxation of positive supercoils (technically positive writhes) rather than an introduction of negative supercoils, thus abolishing the requirement for a thermodynamic input into the reaction. It has been shown that the limiting size of DNA that can be supercoiled by gyrase is well below 174 bp in the presence of EtBr (Bates & Maxwell, 1989). Figure 1 further demonstrates that the inclusion of EtBr in the reaction increases the rate of the ATP-dependent reaction, and allows the Lk = 16 isomer to become a substrate, but does not apparently promote the

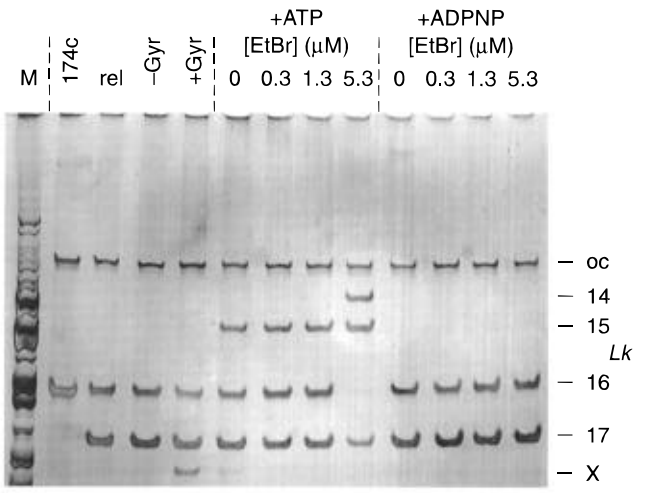

FIGURE 1: Supercoiling of 174 bp DNA circle. 174 bp DNA circle (12 nM) prepared by Cre-*loxP* recombination (174c) was relaxed with eukaryotic topoisomerase I (rel) and incubated without (−Gyr) and with (+Gyr) gyrase (12 nM) as described under Experimental Procedures. Incubations were carried out with gyrase in the presence of ATP or ADPNP (1 mM) and 0, 0.3, 1.3, and 5.3 μM EtBr. The linking numbers of the closed-circular species are indicated. oc, nicked (open-circular) DNA. Track M contains linear DNA markers (pBR322 digested with *Hha*I). The band indicated as X is probably the Lk = 18 topoisomer formed by gyrase-mediated nucleotide-independent relaxation of the Lk = 16 isomer. The 5% polyacrylamide gel contained 0.6 μg mL^{-1} EtBr.

ADPNP-dependent reaction. In fact, careful study of the original of Figure 1 reveals very faint bands in the final two tracks corresponding to the Lk = 15 topoisomer. This implies a very low level of ADPNP-dependent reaction in the presence of the highest concentrations of EtBr.

ADPNP-Dependent Supercoiling of Plasmid Substrates. With this indication that ATP, but not ADPNP, could be used to supercoil some DNA species, we tested the ADPNP-dependent supercoiling of plasmid substrates as a function of their negative supercoiling, to investigate the coupling of nucleotide binding to the strand-translocation reaction of DNA gyrase.

A series of negatively supercoiled samples of pBR322 was prepared by relaxation using eukaryotic topoisomerase I in the presence of increasing concentrations of EtBr, and the DNA was purified by cesium chloride/EtBr density gradient centrifugation. It was quickly realized that the nucleotide-independent relaxation of negative supercoils by DNA gyrase (Gellert et al., 1977; Sugino et al., 1977) would mask the detection of low levels of supercoiling of the negatively supercoiled substrates under normal supercoiling conditions ("standard" conditions; see Experimental Procedures). However, it has been demonstrated that the relaxation reaction of gyrase is inhibited in the presence of high levels of spermidine and lower levels of magnesium than normal (Gellert et al., 1977).

ADPNP-dependent supercoiling reactions were therefore carried out in the presence of 5 mM spermidine and 1.6 mM $MgCl_2$ ("nonrelaxation" conditions). Figure 2 shows the effect of incubation of relaxed DNA (average $\sigma = 0$) and negatively supercoiled DNA (average $\sigma = -0.012$) for 60 min with two concentrations of DNA gyrase in the presence and absence of ADPNP. The DNA topoisomers in this and subsequent experiments are optimally resolved in agarose gels containing 5−15 μg mL^{-1} chloroquine. Under these conditions relaxed closed-circular topoisomers run with

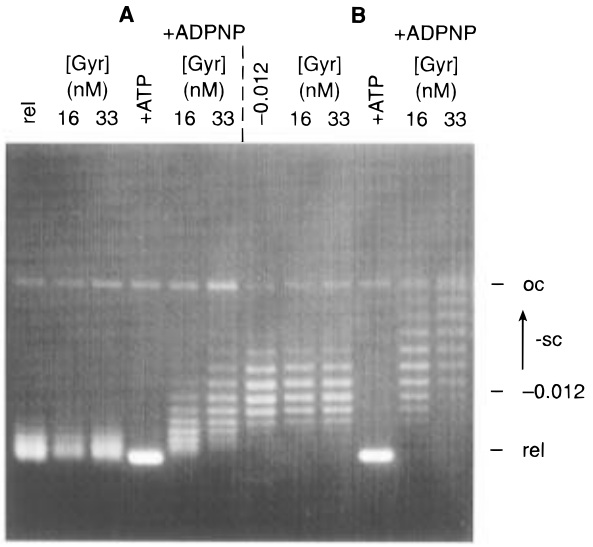

FIGURE 2: ADPNP-dependent supercoiling of pBR322 by gyrase. Relaxed (rel) and negatively supercoiled pBR322 ($\sigma = -0.012$) (**A** and **B**, respectively) were treated under nonrelaxation conditions with the indicated concentrations of gyrase in the presence or absence of ADPNP (500 μM) for 60 min as described under Experimental Procedures. +ATP indicates control incubations containing ATP in place of ADPNP ([gyrase] = 16 nM). The arrow indicates the direction of resolution of more negatively supercoiled topoisomers. oc, nicked (open-circular) DNA. The 0.8% agarose gel contained 9 μg mL^{-1} chloroquine. Under these conditions, relaxed DNA (rel, left-hand lane) has almost the same mobility as highly negatively supercoiled DNA (+ATP lanes), although they are running as positively and negatively writhed species, respectively.

positive writhing on the gel and have a higher mobility than open-circular DNA molecules (oc). The introduction of negative supercoiling results initially in a decrease in mobility, but more highly negatively supercoiled isomers run with increasing mobility, eventually, at the highest levels of supercoiling, appearing as an unresolved band of high mobility. With relaxed DNA as substrate, gyrase in the presence of ADPNP promotes the expected limited supercoiling reaction (Sugino et al., 1978; Tamura et al., 1992) (Figure 2). The concentration of ADPNP used (500 μM) is such that the reaction will have gone to completion in the time scale of the experiment (Tamura et al., 1992). The level of ADPNP-promoted supercoiling achieved using relaxed DNA as a substrate (approximately 30% of the theoretical maximum of two superhelical turns per gyrase tetramer) is consistent with that obtained in the original studies (Sugino et al., 1978). This value is consistently higher than that achieved under standard supercoiling conditions [25% of theoretical maximum (Tamura et al., 1992)]. Supercoiling is also apparent with the $\sigma = -0.012$ substrate, although it can be seen that the extent of the supercoiling reaction is reduced in comparison with that of relaxed DNA (ΔLk is approximately -3 compared with around -5 using relaxed DNA at the higher gyrase concentration, 33 nM). Control experiments (Figure 2) demonstrate that gyrase alone has no effect on the DNA substrates and that either DNA becomes fully negatively supercoiled in the presence of ATP.

One possible explanation of the lower ADPNP-dependent supercoiling is that the rate of the reaction may be reduced on a negatively supercoiled substrate. This possibility was tested in the experiment illustrated in Figure 3. The rate of ADPNP-dependent supercoiling of relaxed DNA is compared

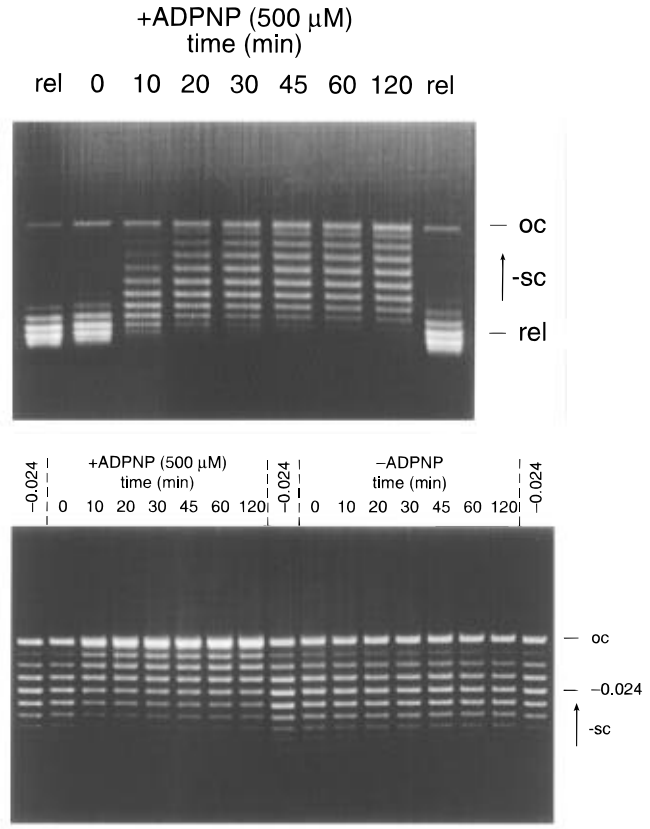

FIGURE 3: Time courses of ADPNP-dependent supercoiling of pBR322. (A, top) Relaxed pBR322 (rel) was incubated with gyrase (33 nM) in the presence of ADPNP (500 μM) under nonrelaxation conditions for the indicated times. The arrow indicates the direction of resolution of more negatively supercoiled topoisomers. The 0.8% gel contained 5 μg mL^{-1} chloroquine. (B, bottom) pBR322 DNA with $\sigma = -0.024$ was incubated with gyrase (33 nM) in the presence or absence of ADPNP (500 μM) under nonrelaxation conditions for the indicated times. oc, nicked (open-circular) DNA. The 0.8% agarose gel contained 15 μg mL^{-1} chloroquine.

with that of a substrate with higher negative supercoiling (average $\sigma = -0.024$). Figure 3A illustrates a time course of supercoiling of relaxed pBR322 DNA by 33 nM gyrase A_2B_2 complex in the presence of 500 μM ADPNP. It can be seen that the reaction is essentially complete after 20 min; little further change in the DNA pattern is apparent. This is consistent with previous determinations of the rate of the ADPNP-dependent supercoiling reaction and the rate of binding of ADPNP to the complex (Tamura et al., 1992). The maximal extent of supercoiling, from measurement of the topoisomer distributions (see Experimental Procedures) is ΔLk = -5.8. Figure 3B shows an analogous time course using substrate DNA with $\sigma = -0.024$, in the presence and absence of ADPNP. Several points should be noted. The extent of limited supercoiling achieved using this substrate is further reduced; a ΔLk of -1.8 is measured. Furthermore, the reaction follows the same time course as previously, being complete by 10−20 min; no change is seen thereafter. The control experiment in the absence of nucleotide confirms that no relaxation of this substrate takes place in 2 h under these conditions.

The situation is more complicated with a still more supercoiled substrate (average $\sigma = -0.046$), since even the altered reaction conditions (5 mM spermidine, 1.6 mM $MgCl_2$) are unable to completely halt the nucleotide-independent relaxation reaction. In a single time point experiment (Figure 4A; 30 min time point), no reaction

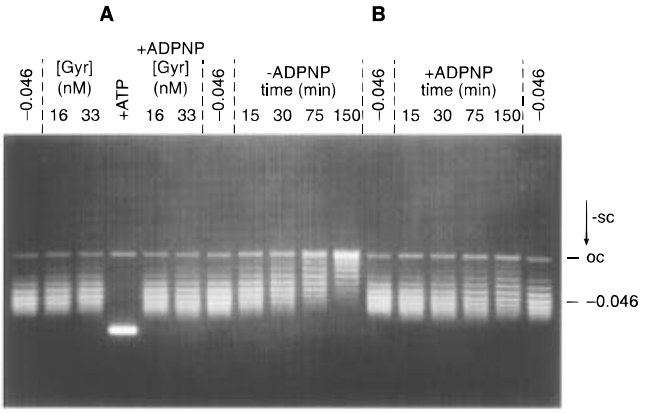

FIGURE 4: ADPNP-dependent gyrase reaction of pBR322 ($\sigma = -0.046$). (A) pBR322 DNA ($\sigma = -0.046$) was incubated with gyrase and ADPNP or ATP for 30 min as described in the legend to Figure 2. (B) The DNA substrate was incubated with gyrase (33 nM) under nonrelaxation conditions for the indicated times in the presence and absence of ADPNP (500 μM). The arrow indicates the direction of resolution of more negatively supercoiled topoisomers. oc, nicked (open-circular) DNA. The 0.8% agarose gel contained 5 μg mL^{-1} chloroquine.

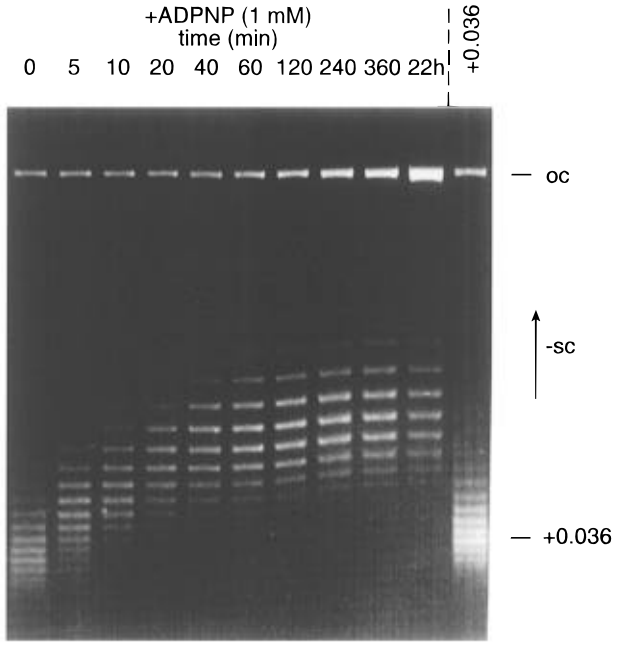

FIGURE 5: ADPNP-dependent gyrase reaction of pBR322 ($\sigma = +0.036$). Positively supercoiled pBR322 (1.4 nM) was incubated with gyrase (5.6 nM) in the presence of ADPNP (1 mM) under relaxation conditions for the indicated times. oc, nicked (open-circular) DNA. The increase in the open-circular band with time is due to adventitious nicking of the substrate DNA with prolonged incubation at the very high gyrase concentration used here. The arrow indicates the direction of resolution of more negatively supercoiled (less positive) topoisomers. The 2.5% agarose gel in TAE buffer contained no chloroquine.

occurs in the presence of ADPNP, and a small relaxation of the substrate is apparent without nucleotide. In the time course experiment (Figure 4B), an inhibition of relaxation by ADPNP is particularly apparent. Without nucleotide, slow but significant relaxation takes place (ΔLk = +5.2 after 150 min), but this relaxation is substantially inhibited in the presence of ADPNP; a very small amount of relaxation can be seen at the 150 min time point (ΔLk = +0.6). At no time is any net negative supercoiling of the substrate apparent. Hence, the ADPNP-dependent supercoiling reaction is essentially abolished with this substrate.

The ADPNP-dependent supercoiling reaction was also investigated using positively supercoiled DNA as a substrate. Gyrase will relax positively supercoiled DNA in the presence of ATP, in a reaction which is thought to be equivalent to the introduction of negative supercoils. Although it was previously reported that in the presence of ADPNP gyrase performs catalytic relaxation of positive supercoils (Gellert et al., 1980), no evidence for such a catalytic activity was demonstrated in these experiments. However, under the standard and nonrelaxing conditions, the coupling of ADPNP binding to strand passage is more efficient with positively supercoiled DNA than with relaxed DNA. The efficiency of coupling is −1.3 to −1.4 ΔLk per gyrase tetramer, or around 70% of the theoretical maximum coupling (see below in Figure 7 and Discussion). Furthermore, the use of conditions which are known to favor the relaxation of negative supercoils by gyrase, including 18 mM potassium phosphate in place of KCl ("relaxation" conditions; see Experimental Procedures), increased the efficiency of the reaction to approach the theoretical maximum of −2.0 ΔLk per gyrase tetramer (Figure 5). The precise value determined from quantitation of the gel was −1.93 ΔLk/tetramer. Even under these conditions, there was no relaxation of positively supercoiled DNA in the absence of ADPNP (not shown).

ATPase vs Supercoiling. While these results suggest that the ADPNP-dependent supercoiling reaction of gyrase is abolished at moderate levels of negative supercoiling, gyrase can supercoil DNA to a much higher level ($\sigma = -0.11$) in the presence of ATP, although the rate of the supercoiling reaction becomes very low (Cullis et al., 1992; Sugino & Cozzarelli, 1980). Gyrase bound to negatively supercoiled DNA must hence be capable of binding and hydrolyzing ATP. It has been suggested that negatively supercoiled DNA is a poorer cofactor for ATP hydrolysis than relaxed or topologically unconstrained DNA (Sugino & Cozzarelli, 1980). This effect is not very marked, however, and depends on how the experiment is carried out. When relaxed DNA in large excess over gyrase was allowed to supercoil, and the gyrase ATPase was simultaneously followed (Sugino & Cozzarelli, 1980), the rate of the ATPase reaction was found to be largely independent of the supercoiling reaction. Under conditions in which DNA was limiting, the V_{max} for the ATPase was slightly reduced, by a factor of around 1.5.

We have performed an analogous experiment. The ATPase activity of gyrase was followed using a continuous coupled assay (Tamura et al., 1992), with relaxed and open-circular DNA as cofactors. In the case of the relaxed DNA, samples were taken from the reaction at various time-points, and the level of supercoiling of the DNA was analyzed on an agarose gel (not shown). The DNA became highly supercoiled, and the reaction began to plateau after around 5 min, but the apparent k_{cat} of the ATPase reaction was steady throughout the experiment (>1 h) at 0.89 s^{-1}. Using open-circular DNA as cofactor, the apparent k_{cat} was determined as 0.87 s^{-1}. Thus, the rate at which gyrase binds and hydrolyzes ATP is independent of the (negative) σ of the DNA cofactor, although the rate of the supercoiling reaction is decreased as the DNA becomes more highly negatively supercoiled.

ADPNP Binding. The results of Tamura et al. (1992) suggest that increasing levels of negative supercoiling of the DNA used as the cofactor of gyrase can cause up to 10-fold

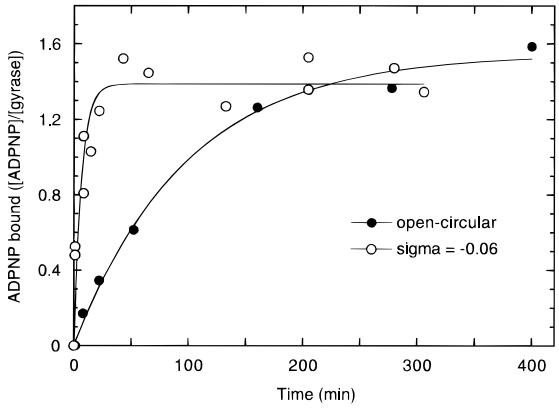

FIGURE 6: ADPNP binding to DNA gyrase, using open-circular and negatively supercoiled pBR322 DNA as cofactors. Gyrase (49 nM) was preincubated with open-circular (filled symbols) or negatively supercoiled ($\sigma = -0.06$) pBR322 DNA (open symbols) (26 nM) under nonrelaxation conditions, and then [α-^{32}P]ADPNP (100 μM) was added at time 0. Aliquots were removed at various times and the free and bound ADPNP separated using a "spin column" (see Experimental Procedures). The stoichiometry of ADPNP binding, determined as mol of ADPNP per mol of gyrase tetramer, was plotted against time (min).

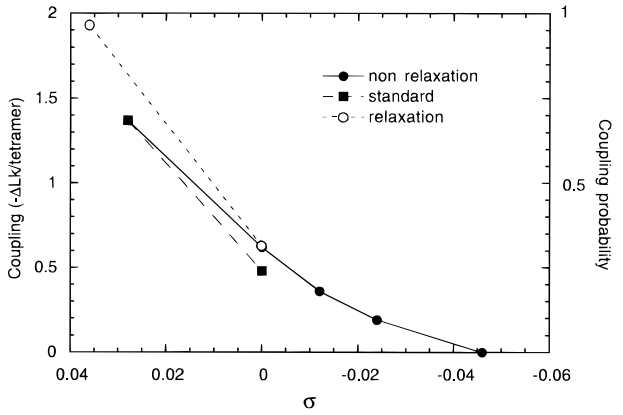

FIGURE 7: Dependence of the coupling of ADPNP binding to strand passage on the specific linking difference of pBR322 DNA substrate. The efficiency of coupling of ADPNP binding to strand-passage by DNA gyrase (see Discussion for details) was plotted against the specific linking difference (σ) of the substrate DNA for three different solution conditions as indicated. The data are also plotted in terms of coupling probability on the right-hand axis (see Discussion for details).

increase in the very low rate of dissociation of ADPNP from the gyrase-DNA complex, from approximately 10^{-6} to 10^{-5} s^{-1}. Thus, it may be that gyrase does not become saturated with ADPNP at high levels of negative DNA supercoiling. This possibility was investigated using an assay of the binding of radiolabeled ADPNP to gyrase complexed with nicked DNA and with negatively supercoiled DNA with $\sigma = -0.06$, a value more negative than that which exhibited no ADPNP-dependent supercoiling (see Figure 4). Binding assays were performed under the nonrelaxing conditions used above. The binding of ADPNP (100 μM) was followed with time, the amount of bound nucleotide being determined by rapid separation of complex and unbound nucleotide on a gel filtration column (spin column) (Tamura et al., 1992). The results are illustrated in Figure 6; the data for negatively supercoiled cofactor are taken from two independent binding experiments. Two conclusions can be drawn. First, the binding of ADPNP saturated at the same stoichiometry with both nicked and negatively supercoiled DNA (1.4−1.5 mol/mol A_2B_2). Second, the rate of binding of ADPNP to the gyrase−DNA complex is significantly increased in the presence of negatively supercoiled DNA, compared with nicked DNA. The apparent second-order rate constants were determined by a pseudo-first-order method as described by Tamura et al. (1992) to be $k_1 = 100$ M^{-1} min^{-1} (nicked) and $k_1 = 1600$ M^{-1} min^{-1} (supercoiled). The former value is in good agreement with the rate determined for linear DNA cofactor (120 M^{-1} min^{-1}) under rather different conditions (Tamura et al., 1992). In fact, the rates of binding and dissociation of ADPNP to gyrase are both increased by approximately the same factor (around 10-fold) in the presence of negatively supercoiled DNA (Figure 6 and Tamura et al., 1992). This implies that the affinity of binding is essentially unchanged.

DISCUSSION

We have shown that the limited negative supercoiling produced by DNA gyrase in the presence of ADPNP is highly sensitive to the initial state of the DNA. The reaction is moderately efficient with relaxed plasmid substrates, but with increasingly negatively supercoiled pBR322 plasmids as substrates, the extent, though not the rate, of the ADPNP-dependent supercoiling reaction decreases markedly. The ADPNP-dependent reaction is not apparent at all using a substrate of specific linking difference of −0.046. In contrast, the use of positively supercoiled DNA substrates ($\sigma = +0.029$ to $+0.036$) leads to an increase in the efficiency of the ADPNP-dependent supercoiling reaction over that of relaxed DNA. In this case, the efficiency is dependent on the conditions of the reaction and, in the case of one set of conditions, close to the maximum possible efficiency of −2.0 ΔLk per gyrase tetramer is observed. The previously reported catalytic ADPNP-dependent relaxation of positive supercoils by gyrase (Gellert et al., 1980) is not apparent. The results of measurements of the efficiency of the ADPNP-dependent supercoiling reaction of DNA gyrase are collected together and illustrated graphically in Figure 7. The values are quoted in terms of a coupling efficiency, that is, the negative linking difference generated in a substrate pBR322 molecule of varying σ per gyrase tetramer present.

It is clear in general terms that the coupling between nucleotide binding and strand-passage is far from perfect, since the ATPase reaction of gyrase continues at a constant rate while the negative supercoiling reaction decreases in rate as the DNA approaches the limit of DNA supercoiling (Sugino & Cozzarelli, 1980; this work). The present experiments using the nonhydrolyzable ATP analogue ADPNP allow a finer dissection of this process. Since the product of the cooperative binding of ADPNP to the gyrase−DNA complex is a very long-lived, essentially dead-end complex (Tamura et al., 1992), each gyrase tetramer has a single opportunity to perform a supercoiling DNA strand-translocation reaction, and the efficiency of coupling in Figure 7 may be thought of as a measure of the probability of a supercoiling event occurring during a given nucleotide-binding event. A coupling efficiency of −2 ΔLk per gyrase tetramer is equivalent to a translocation probability of 1 (Figure 7).

The primary result of this paper is that with pBR322 the coupling probability is dependent on the level of supercoiling of the substrate DNA. It is highest (approaching 1) when

the substrate is positively supercoiled and too small to measure when the DNA is moderately negatively supercoiled. In other words, when gyrase is bound to positively supercoiled DNA, an ADPNP-binding event has a high probability of resulting in the occurrence of a supercoiling event, whereas with moderately negatively supercoiled DNA ($\sigma = -0.046$) that probability is very low; perhaps less then 1 in 100 gyrase molecules will give rise to a supercoiling event. The probability of strand-passage with relaxed DNA (around 0.3) is in between these two extremes.

The fact that the coupling probability can achieve a value of 1 under appropriate conditions means that the imperfect coupling with relaxed substrate is not a consequence of inactive enzyme in the preparation, as has previously been suggested (Reece & Maxwell, 1991; Sugino et al., 1978), but rather of an actual uncoupling of the enzyme.

There could be several mechanistic reasons for this range in probabilities. The conformation of the initial gyrase−DNA complex could depend on the specific linking difference of the substrate DNA in such a way as to influence the overall activity of the enzyme. However, the fact that the ATPase reaction of the gyrase−DNA complex is independent of the supercoiling in the DNA cofactor suggests that this may not be the case.

Alternatively, the coupling probability could be dependent on the free energy required for the gyrase-dependent supercoiling step. This will vary from a negative value for a positively supercoiled substrate to a more and more positive value as the substrate becomes increasingly negatively supercoiled.

The gyrase nucleotide-dependent supercoiling reaction can be considered, by analogy with the mechanistic model proposed for eukaryotic topoisomerase II by Roca and Wang (1992), as being initiated by the capture of the DNA "T-segment" to be translocated through the double-strand DNA break in the wrapped DNA (the "G-segment") by a nucleotide-operated clamp closed by the dimerization of the N-terminal domains of the GyrB protein (Wigley et al., 1991).

The explanation most consistent with our results is that the coupling probability is a function of the ease with which the T-segment can achieve the required conformation to be trapped by the nucleotide-operated clamp. If we assume that the approach of the T-segment to the clamp involves a movement in the same direction as the overall translocation and strand-passage process, then the energetics of this process will be determined by the torsional stress in the DNA. In the case of positively supercoiled DNA, the gyrase reaction is thermodynamically favorable, and the T-segment may be thought of as being primed to translocate in the correct direction. Capture of the T-segment would then have a high probability. As the DNA becomes more negatively supercoiled, any movement of the T-segment towards translocation is opposed by the torsional stress in the DNA, and the ease with which the T-segment is presented for translocation is reduced, until eventually the probability of it being in the correct conformation as the "trap springs shut" is very low. In these circumstances, the gross level of supercoiling seen with ADPNP will be essentially zero.

This interpretation is also consistent with the small circle results (Figure 1). In all circumstances, the small size of the substrate circle means that the process of strand-passage will have a high activation energy; the circular DNA must be forced transiently through a figure-eight conformation. If the required conformation for T-segment trapping approaches this situation, then this will be an improbable event, even if the overall free energy of the gyrase reaction is negative, as it would be in the presence of sufficient concentrations of EtBr. Hence the coupling of nucleotide binding to strand-passage in a small circle will always be low, as observed. From inspection of the original photograph of Figure 1, the coupling probability in even the most favorable case, i.e., with the highest EtBr concentration, is perhaps less than 0.01.

The above argument is consistent with the idea that the translocated segment of DNA is generally close to or within the region wrapped in a positive superhelical sense around the gyrase tetramer, a conclusion which is itself suggested by the reaction of gyrase with small DNA circles (Bates & Maxwell, 1989). One possibility is that there is a particular conformation of the wrapped DNA which leads to efficient T-segment capture and that the probability of achieving that conformation is controlled by the level of supercoiling of the substrate. Positively supercoiled DNA would be most favorably wrapped on the enzyme tetramer and hence have the highest probability of achieving the correct conformation for translocation, whereas negatively supercoiled substrate would be wrapped in a less favorable conformation and hence have only a low probability of capture. In the case of small circles, the probability of the DNA achieving the required wrapped conformation would be low in all circumstances, since a large deformation would be required whatever the topological state of the DNA. This argument is speculative, however, as there is to date no evidence for actual conformational differences between the complexes of gyrase with different DNA species and indeed some evidence that complexes with small circles and their linear equivalents have similar conformations (Bates & Maxwell, 1989). Nevertheless, it is likely that the wrapping of DNA around the gyrase tetramer determines the directionality of the reaction (decrease in Lk) and the preference of gyrase for supercoiling over other reactions such as decatenation (see below).

The Effect of Solution Conditions. The coupling probability in our experiments varies with the solution conditions. With negatively supercoiled substrates, experiments were carried out only under conditions where relaxation of negative supercoils is suppressed (nonrelaxation conditions), involving an increase in spermidine concentration to 5 mM and reduction in $MgCl_2$ to 1.6 mM compared with standard conditions. These conditions were taken from the literature and were shown to be substantially effective (Figures 2−4). With relaxed and positively supercoiled substrates, in addition to the standard and nonrelaxation conditions, conditions known to favor the relaxation of negative supercoils by gyrase (including 18 mM potassium phosphate) were also investigated; these conditions led to the highest coupling probability with positively supercoiled substrates. The effect of changing one component alone has not been systematically examined; however, the variation in coupling probability may be consistent with changes in the free energy of supercoiling caused by changes in the counterions available to balance the charge on the DNA phosphates (Bednar et al., 1994).

Rate of Nucleotide Binding. It has previously been shown that the rate of ADPNP binding to gyrase complexed with linear DNA is very low. The apparent second order rate constant k_1 is equal to 120 M^{-1} min^{-1}, which probably

reflects a slow conformational change of the enzyme (E to E*) occurring after binding of ADPNP to one of the B subunits, as proposed by Tamura et al. (1992):

$$E + \text{ADPNP} \underset{K_d}{\overset{\text{fast}}{\rightleftharpoons}} E{\cdot}\text{ADPNP} \overset{\text{slow}}{\rightleftharpoons} E^*{\cdot}\text{ADPNP} \overset{+\ \text{ADPNP fast}}{\rightleftharpoons} E^*{\cdot}(\text{ADPNP})_2$$

The rate of the ADPNP-dependent supercoiling reaction was observed to be the same as that of nucleotide binding, implying that this conformational change probably represents the step leading to strand passage. We have shown that the apparent second-order binding constant for ADPNP binding is increased 16-fold from 100 to 1600 M^{-1} min^{-1} in the presence of negatively supercoiled DNA under nonrelaxation conditions, even though this DNA ($\sigma = -0.06$) is too negatively supercoiled to show significant coupling to strand passage with ADPNP. Negative supercoiling of the DNA cofactor must increase the rate of ADPNP binding, either by increasing the rate of the slow conformational change or by increasing the initial affinity of the complex for ADPNP (decreasing K_d above). From the data presented (Figures 2−4), it is difficult to judge whether the rate of the ADPNP-dependent supercoiling reaction is influenced by the topological state of the substrate, since the magnitude of the supercoiling reaction becomes very small at higher levels of negative supercoiling. The most that can be said is that the rate remains at least as fast as with relaxed DNA. Conversely, there is no evidence of a significantly lower rate of ADPNP-dependent supercoiling with positively supercoiled DNA as a substrate. The data presented in Figure 5 can be interpreted in terms of an apparent second-order rate constant for ADPNP binding of 90 M^{-1} min^{-1}, a value not significantly different from that determined using open-circular DNA and labeled ADPNP, particularly as the solution conditions are different. The conclusion which can be drawn is that there is a direct influence of the topological state of the DNA substrate on the rate of binding of nucleotide, independent of whether strand-passage is taking place, i.e., presumably an effect on the site of nucleotide binding in the N-terminal region of GyrB. It cannot be argued that there is a different rate of nucleotide binding depending on whether strand passage does or does not take place, since this would imply a biphasic binding curve in the case of nonsupercoiled DNA substrate, which is not observed (Tamura et al., 1992; this work). The rate of ATP binding might also be dependent on the topology of the substrate DNA, but this effect would not be reflected in the overall rate of ATP hydrolysis, since it is thought that product release is the rate-determining step of the ATPase reaction of gyrase (Ali et al., 1993). Indeed, we and others (Sugino & Cozzarelli, 1980) have shown that the rate of ATP hydrolysis is independent of the level of negative supercoiling of the substrate. The relationship between DNA topology and nucleotide binding rates is under further investigation.

The ATP-Dependent Supercoiling Reaction of Gyrase. It is reasonable to assume that the ATP-dependent DNA gyrase reaction is analogous to the ADPNP-dependent reaction, i.e., that cooperative binding of ATP to the two B subunits in the gyrase−DNA complex causes the conformational change leading to the strand-passage event. The primary difference is of course that ATP can be hydrolyzed and the products dissociated from the enzyme, leading to a catalytic reaction. It has been shown previously (Sugino & Cozzarelli, 1980), and we have confirmed, that ATP hydrolysis by gyrase becomes uncoupled from supercoiling when high levels of negative supercoiling have been generated in the substrate.

Unless there are major differences in the conformational changes caused by ATP and ADPNP binding, it is likely that the coupling probabilities we have measured for ADPNP binding and strand passage by gyrase will also be applicable to the ATP reaction. The measurement of coupling probability is experimentally difficult in the case of the ATP-dependent reaction.

Hence, although the coupling probability is so low as to lead to no measurable reaction in the case of ADPNP with a negatively supercoiled substrate, where ADPNP has one chance to promote a strand-passage reaction, the enzyme can continue to turn over ATP until this low probability is converted into a measurable reaction. This explains why the limit of supercoiling achievable by gyrase with ATP is much higher ($\sigma = -0.11$, under standard conditions) than the apparent limit of the ADPNP reaction ($\sigma \approx -0.04$; Figure 7). Likewise, gyrase is able to supercoil small circular substrates in the presence of ATP, although this reaction is very slow (Bates & Maxwell, 1989; Figure 1) due to the low coupling probability, even when the substrate is relaxed. This also explains why gyrase is able to catalyze other reactions, such as catenation and decatenation, in the presence of ATP (Kreuzer & Cozzarelli, 1980), although there is presently no evidence for gyrase-mediated decatenation in the presence of ADPNP. The T-segment in the case of a catenation/decatenation or knotting/unknotting reaction cannot be part of the wrapped DNA region and hence will always have a low (although not zero) probability of capture and translocation. In recent experiments using 1000-fold more enzyme than that required to see decatenation in the presence of ATP, no ADPNP-stimulated decatenation was detected (A. P. Tingey and A. Maxwell, personal communication).

The Role of DNA Gyrase in vivo. Our measurements of coupling efficiencies in DNA gyrase perhaps allow some deductions to be made about the role of gyrase compared with other type II topoisomerases *in vivo*. As pointed out above, gyrase is inefficient in carrying out decatenation, although eukaryotic topoisomerase II can efficiently perform this reaction even in the presence of ADPNP (Roca & Wang, 1994). It is likely that topoisomerase II is involved in the decatenation of daughter chromosomes at the termination of replication (DiNardo et al., 1984). By the argument above, this would be explained by the fact that topoisomerase II, which has no wrapped region (Roca et al., 1993), cannot readily distinguish between close and distant T-segments or even T-segments on separate molecules. Topoisomerase II can therefore be thought of as translocating indiscriminately any DNA segment which can approach the T-segment site. Whether this results in a relaxation or a decatenation reaction, for example, will perhaps depend only on the topology and conformation of the substrate DNA. Recent results suggest that the homologous type II enzyme from *E. coli*, topoisomerase IV, is a much more efficient decatenase than is gyrase (Peng & Marians, 1993b), and it seems likely that topoisomerase IV is the replication decatenase in *E. coli* (Adams et al., 1992; Peng & Marians, 1993a), i.e., the analogue of eukaryotic topoisomerase II.

Gyrase, on the other hand, by virtue of its wrapping of the substrate DNA, is configured to perform efficient intramolecular negative supercoiling (technically, reduction in Lk) and not decatenation. This activity is of course appropriate for the maintenance of negative supercoiling in the *E. coli* cell in concert with topoisomerase I and for the removal of any torsional stress generated ahead of transcription or replication complexes (Liu & Wang, 1987). Our results suggest, however, that the efficiency of the negative supercoiling process is dependent on the topology of the substrate DNA. At the steady-state level of unconstrained negative supercoiling thought to be present in the bacterial cell, $\sigma \approx -0.03$ (Bliska & Cozzarelli, 1987; Pettijohn & Pfenninger, 1980; Sinden et al., 1980), gyrase is only between 5 and 10% efficient at converting nucleotide turnover into supercoiling (Figure 7). The increasing efficiency of the process as DNA substrates become more relaxed would contribute to an effective buffering of the steady-state level of supercoiling by gyrase, although whether the high efficiency of gyrase in the relaxation of positive supercoils has any *in vivo* relevance depends on whether positive supercoils are transiently generated in wild-type *E. coli* cells by the processes of transcription or replication.

The results presented here suggest considerable complexity of interaction between gyrase, nucleotide, and the DNA substrate but do not address the structural basis of these interactions. Clarification of the detailed processes involved will require further investigation of the structure and conformation of the gyrase−DNA complex.

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