Investigating the Role of the Latch in the Positive Supercoiling Mechanism of Reverse Gyrase†

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ABSTRACT: Reverse gyrase is the only topoisomerase known to positively supercoil DNA and the only protein unique to hyperthermophiles. The enzyme comprises an N-terminal ATPase domain and a C-terminal topoisomerase I domain, which interact to couple the hydrolysis of ATP to the overwinding of DNA. The part of the ATPase domain termed the "latch" represses topoisomerase activity in the absence of nucleotide. Here I provide evidence that the latch, in addition to its regulatory role, participates in the supercoiling mechanism during the DNA cleavage and religation steps. The latch also contributes to the coordination of ATP hydrolysis and positive supercoiling by inhibiting ATPase activity in the absence of supercoiling. The latch therefore plays an important role in the communication between the two domains of reverse gyrase.

Topoisomerases change the topological state of DNA by altering its linking number, or the number of times that one strand of the double helix wraps around the other. They participate in practically every DNA transaction in the cell, including transcription, replication, recombination, and chromosomal segregation (*1*). Reverse gyrase is the only topoisomerase capable of introducing positive supercoiling (overwinding) into DNA. While its physiological role remains unclear (*2*), its activity appears to be essential for life at extremely high temperatures. The enzyme is found only in hyperthermophiles, which live at or above ∼70 °C, but not in mesophiles or even moderate thermophiles (*3*, *4*). In fact, a systematic study of microbial genomes has identified reverse gyrase as the only protein specific to hyperthermophiles (*5*). Studies of this enzyme may help to resolve the ongoing controversy concerning thermophily and the divergence of prokaryotes and eukaryotes (*6*, *7*).

Reverse gyrase uses the energy of ATP hydrolysis to generate positive supercoils in DNA. Prokaryotic gyrase, which is the only other topoisomerase capable of generating supercoils, can use ATP to introduce negative supercoils (underwinding) in DNA. Both enzymes are therefore important for understanding energy transduction in biological systems. Reverse gyrase has a distinct advantage over gyrase as a model system because the crystal structure of the intact enzyme is known (*8*). This should allow detailed structure−function analyses aimed at understanding how nature can convert the energy of ATP into superhelical strain in DNA.

The crystal structure of reverse gyrase showed it to have the same ATPase domain seen in helicases, fused with a domain homologous to type IA topoisomerases (*8*). The mechanism of the topoisomerase domain can be extrapolated from studies of other type IA enzymes. These proteins use a three-step mechanism of cleavage, strand passage, and religation (*1*). In the first step, a nucleophilic tyrosine attacks the phosphodiester backbone of one of the strands, cleaving the DNA and creating a covalent adduct of protein−DNA, termed the "cleavage complex". The ends of the cleaved strand are separated, and the intact strand is passed through the gap. The protein then reseals the backbone of the cleaved DNA and releases the product.

In contrast to the topoisomerase domain, how the ATPase domain functions in catalysis is unclear. Despite its homology to the ATPase module of helicases (*8*, *9*), it does not appear to couple hydrolysis to processive strand displacement (*10*). Instead, it may use hydrolysis to drive conformational changes necessary for positive supercoiling (*8*, *10*). The key to understanding the mechanism of reverse gyrase therefore lies in studying how the two domains interact during catalysis.

One of the ways in which they interact is through a region of the ATPase domain dubbed the "latch". The latch plays a regulatory role in the enzyme, repressing topoisomerase activity in the absence of nucleotide (*11*). Here I present the results of further studies into the role of the latch that suggest that it participates in the catalytic mechanism during cleavage and religation, and that it helps to coordinate nucleotide hydrolysis by the ATPase domain with supercoiling activity by the topoisomerase domain.

EXPERIMENTAL PROCEDURES

All proteins were overexpressed and purified as described previously (*11*). "Full-length" *Archaeoglobus fulgidus* reverse gyrase and its deletion mutants all contain the mutations Pro719Leu and Leu1046Met, and all bear an N-terminal FLAG epitope and a C-terminal hexahistidine tag. "Wild-type" enzyme retains both tags but lacks the two point mutations. Protein concentrations were determined using the BCA assay (Pierce). Standard reaction buffer consists of 50

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mM Tris-Cl (pH 8.0), 30 mM NaCl, 10 mM $MgCl_2$, 0.02% NaN_3. The salt concentration includes the contribution from the protein solution. Protein dilutions were prepared in the same buffer as described previously (*11*). Tubes incubated at 80 °C were overlaid with mineral oil (Sigma) to prevent evaporation.

Concentrations for DNA substrates are given in terms of molecules, not bases. Values plotted in the figures or cited in the text are averages of *n* independent experiments, where *n* is stated together with the measurement or in the relevant figure legend. Experimental error is given as the standard error of the mean (SEM).[1]

DNA Cleavage Assay. Reactions (30 μL) containing 20 nM ϕX174 single-stranded virion DNA (New England Biolabs) and the indicated amount of protein were set up in standard reaction buffer in the presence or in the absence of 1 mM ATP. Tubes were incubated for 30 min at 80 °C, and then SDS was added to a final concentration of 1% (w/v), followed by 3 μL of 35 mg/mL proteinase K (Sigma). The reactions were incubated for 60 min at 55 °C, supplemented with 10 μL of loading buffer [5× concentrations: 48% glycerol (v/v), 40 mM EDTA, 0.05% NaN_3], and loaded onto a 1.4% agarose gel (20 × 17 × 0.6 cm) prepared in 1× TBE (90 mM Tris-borate, 2 mM EDTA). The gel was run in 1× TBE at 200 V at 4 °C for 6−7 h, stained in 4 μg/mL ethidium bromide for 35 min, destained in water, and photographed with a digital camera.

Quantitation of DNA bands was carried out using GeneTools software (Syngene, Cambridge, UK). Integrated peak volumes were confirmed to vary linearly with the amount of DNA used in the experiments. For each lane, the integrated volumes of the circular and linear ϕX174 bands were added together to give the sum *C*, and this was compared to the sum C_0 in control reactions lacking enzyme:

$$\text{percent of substrate cleaved} = [1 - (C/C_0)] \times 100\%$$

Occasionally, a cleavage fragment was observed migrating just below the circular species, and this was also included in the determination of *C*.

DNA Religation Assay. Reactions were set up and incubated as described for the cleavage assay. Enzymes with deletions were used at protein-to-DNA (P/D) molar ratios of 8−10, causing disappearance of 80−100% of the substrate; full-length enzyme was used at P/D = 60 and caused the disappearance of 50−90% of the substrate. After the cleavage incubation, control tubes received 7.5 μL of 10% SDS (w/v). Sample tubes received 7.5 μL of water or of an aqueous solution of NaCl to final molar concentrations of 0.25, 0.5, 0.75, or 1. To allow religation, sample and control tubes were returned to 80 °C for 3 min, a time period chosen because pilot experiments showed maximal religation to occur by this point (data not shown). After this second incubation, the sample tubes received 7.6 μL of 10% SDS, and the NaCl concentration of all tubes was adjusted to 0.8 M. Proteinase K treatment was carried out as described for the cleavage assay, as were the subsequent electrophoresis and digital photography.

For each lane, the sum *C* was quantitated as for the cleavage assay, and C_0 was determined from control tubes that were set up without any enzyme and that received SDS after the initial 80 °C incubation. The resulting ratio C/C_0 reflects not only DNA religated by the enzyme during the second 80 °C incubation but also DNA substrate that remained uncleaved after the first incubation. To eliminate the contribution of the latter, the sum C_{SDS} was determined from control tubes that were incubated with enzyme and that received SDS after the initial incubation. The fraction of original substrate that was religated by the enzyme was estimated from the equation

$$\text{percent of substrate religated} = [(C/C_0) - (C_{SDS}/C_0)] \times 100\%$$

When observed, the cleavage fragment running just below the circular species was included in the determination of *C*.

DNA Binding Assay. The DNA substrate contained a 22-bp duplex region with a 5′-tail of 30 deoxythymidine residues. The "upper" strand has the sequence 5′-T_{30}CGAGCACCGCTGCGGCTGCACC. The "lower" strand (complementary to the underlined region) was labeled with polynucleotide kinase as previously described (*11*) and supplemented with a 10-fold molar excess of unlabeled oligonucleotide. The labeled strand was mixed with a 6-fold molar excess of the unlabeled partner and annealed as previously described (*11*). Binding reactions (30 μL) were assembled at room temperature in bandshift buffer (*11*) lacking nucleotide and containing annealed substrate at a final concentration of 10 nM for the Δlatch titration or 20 nM for the other titrations. Reactions were left for 30−45 min at room temperature and then electrophoresed on 10% native polyacrylamide gels as previously described (*11*).

ATP Stoichiometry Assay. Negatively supercoiled pBR322 was obtained from New England Biolabs or purified from *Escherichia coli* DH5α using the QIAfilter Plasmid Mega Kit (Qiagen). The fraction of supercoiled dimeric plasmid in these preparations did not exceed 15% based on quantitation in agarose gels and was not considered when calculating supercoiling rates. The DNA was positively supercoiled by incubation for 30 min at 80°C with a 1- to 2-fold molar excess of reverse gyrase in standard reaction buffer containing ATP. The DNA was extracted once with phenol/chloroform/isoamyl alcohol and once with chloroform/isoamyl alcohol, precipitated with ethanol, resuspended in TE [10 mM Tris-Cl (pH 7.4), 1 mM EDTA], and stored as aliquots at −20 °C. DNA concentration was determined from its absorbance at 260 nm. The number of supercoils in the DNA at ∼39 °C was estimated from one-dimensional electrophoresis in Tris-acetate buffer containing 10 mM Mg^{2+}. This value was increased by 5 to estimate the number of positive supercoils at 80 °C (*12*, *13*).

Reactions (30 μL) were prepared in duplicate, with radiolabeled ATP in one set of tubes and unlabeled ATP in the other. "Cold" supercoiling reactions were set up from a "master mix" containing standard reaction buffer supplemented with 0.4−1 pmol (1.1−2.8 μg) of DNA substrate and ATP (Sigma) to the concentration indicated in the figure

[1] Abbreviations: ADPNP, adenylylimidodiphosphate (AMP−PNP, App(NH)p); P/D, protein-to-DNA molar ratio; FL, full-length reverse gyrase; Δlatch, reverse gyrase lacking residues 360−418; Δ370, reverse gyrase lacking residues 370−381; Δ857, reverse gyrase lacking residues 857−866; σ, superhelical density; SEM, standard error of the mean; SC, supercoiling or supercoiled; Lk, linking number; ΔLk, linking number difference (from an arbitrary reference Lk).

and table legends. "Hot" reactions were set up from an aliquot of the same master mix spiked with [γ-^{32}P]ATP (~3000 Ci/mmol; Amersham), such that each tube contained ~2.4 μCi. To measure background ATP hydrolysis, control reactions were set up with hot master mix in the absence of any enzyme. The three sets of reactions—hot, cold, and control—were incubated in parallel at 80 °C for different lengths of time. At each time point, reactions were halted with 5× "stop" buffer [identical to the 5× loading buffer described above, supplemented with 5% (w/v) SDS] and placed on ice.

The cold tubes were subjected to gel electrophoresis and quantitation as described for the cleavage assay, except that the electrophoresis time was extended to 9.5−11 h. The first resolvable topoisomer band was assigned an arbitrary number of positive supercoils, which was taken as the reference value for the linking number (Lk). Using the band-counting method (*14*), the center of the topoisomer distribution at each time point was calculated relative to this reference value to give the linking number difference (ΔLk). The ΔLk values were plotted as a function of time, and the linear part of this plot was used to estimate the rate of positive supercoiling.

The hot and control tubes were processed in the charcoal adsorption assay (*11*) to determine the extent of ATP hydrolysis. In each experiment, remaining hot master mix was added directly to scintillation vials to build a calibration line for converting counts per minute into nanomoles of ATP. The amount of ATP hydrolyzed was plotted as a function of time, and the ATPase rate was estimated from the linear portion of the graph. The rate of spontaneous ATP hydrolysis was then subtracted from the measured rates to yield the "background-corrected" values reported in the text and figures.

Standard Positive Supercoiling Assay. Reactions were carried out and analyzed by two-dimensional electrophoresis using chloroquine in the second dimension, as described previously (*11*).

RESULTS

A previous study demonstrated that the latch represses topoisomerase activity in the absence of nucleotide (*11*), but it did not address whether the latch participates in the topoisomerase mechanism. Therefore, the same deletion mutants from the previous study were analyzed in DNA cleavage and religation assays (Figure 1A). The mutants lack essentially the entire latch (Δlatch, lacking residues 360−418); the tip of the latch that interacts with the topoisomerase domain in the crystal structure (Δ370, residues 370−381); or, conversely, the part of the topoisomerase domain that interacts with the tip of the latch (Δ857, residues 857−866). *E. coli* topoisomerase I and reverse gyrase are structurally homologous immediately upstream and downstream of residues 856−870, but this region itself is absent from topoisomerase I (Figure 1B). Hence, this "topoisomerase insertion" is likely to have a function unique to reverse gyrase.

The Latch and the Topoisomerase Insertion Are Involved in the DNA Cleavage Step. Like many topoisomerases, reverse gyrase cleaves DNA in a fairly sequence-independent manner, although it prefers to cleave at sites having a

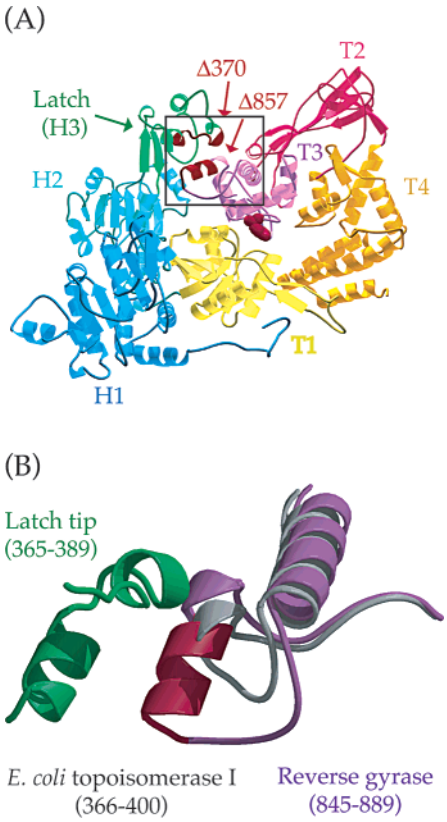

FIGURE 1: Reverse gyrase mutants examined in this study. (A) The crystal structure of reverse gyrase is colored by subdomains as in ref *8*. The nucleophilic Tyr809 in the topoisomerase active site is indicated in red space-filling representation. The Δlatch mutant lacks essentially the entire latch subdomain, the Δ370 mutant lacks the tip of the latch, and the Δ857 mutant lacks the part of the topoisomerase domain interacting with the latch. The Δ370 and Δ857 deletions are colored dark red. A black box indicates the region depicted in (B). (B) Close-up view of the interaction of the latch and the topoisomerase domain in the reverse gyrase crystal structure. Shown are the tip of the latch (green) and part of the topoisomerase domain (purple) of reverse gyrase; in gray are the corresponding regions of *E. coli* topoisomerase I positioned based on a superposition of the structures of the two topoisomerase domains (*8*, *41*). Residue ranges are given in parentheses, and the region deleted in the Δ857 mutant is colored dark red. These images were generated with BOBSCRIPT (*42*) and rendered with POV-Ray (www.povray.org) (A) or Raster3D (*43*) (B).

cytosine at the fourth position upstream of the cleavage site (*15*−*17*). For sequence-nonspecific topoisomerases, the cleavage and religation reactions can be studied using long molecules of single-stranded viral DNA, which should contain multiple cleavage sites (*18*−*21*).

Full-length and mutant reverse gyrase enzymes were assayed for their ability to cleave the single-stranded DNA of bacteriophage ϕX174. The substrate consists of single-stranded DNA in primarily circular form, with some linear form also present (Figure 2A). Titrating reverse gyrase into the reaction causes the disappearance of both substrate forms into fragments that have been cleaved multiple times (Figure 2A), as observed previously with *Drosophila* topoisomerase II (*19*). Titrations were performed for all of the reverse gyrase proteins (Figure 2B), and cleavage activity was estimated from the total amount of circular and linear ϕX174 remaining at the end of the incubation. All three mutant enzymes show significantly higher specific cleavage activity than the full-length protein (Figure 2C). With the mutants, no circular or

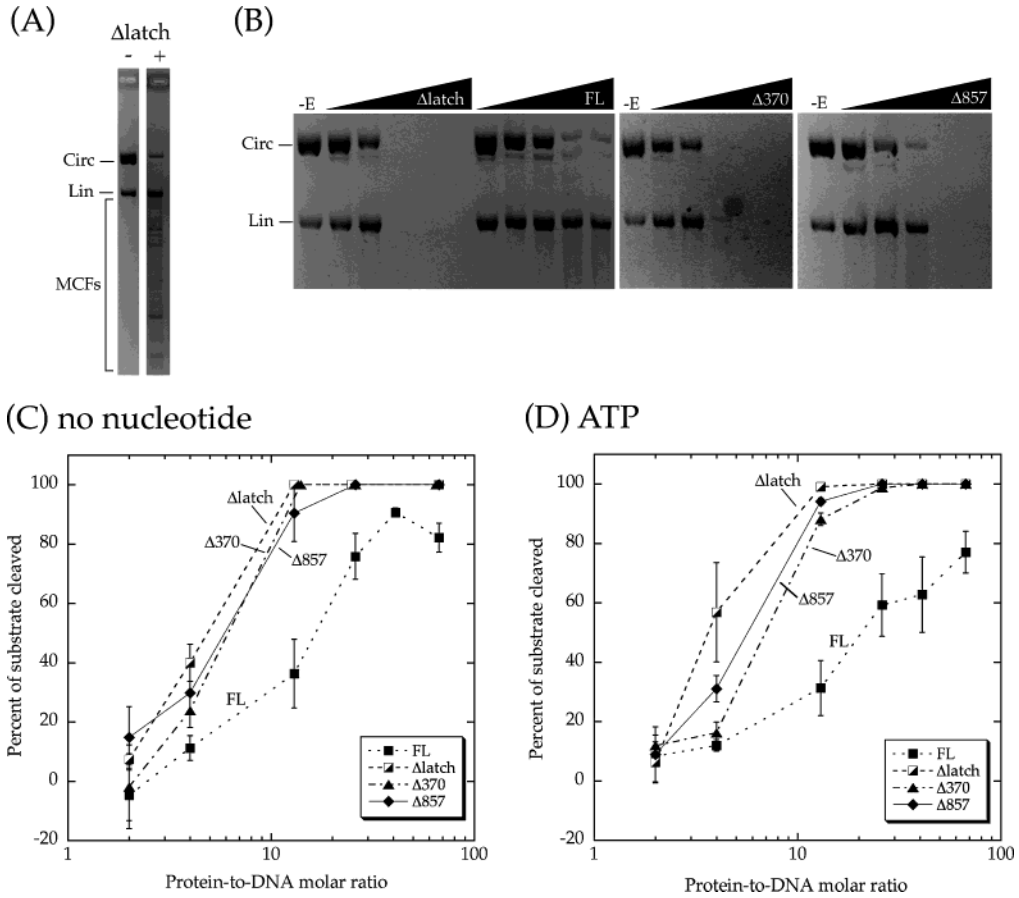

FIGURE 2: Mutations in the latch and the topoisomerase insertion affect the specific cleavage activity of reverse gyrase. Cleavage assays were performed as described in the Experimental Procedures in the presence of full-length (FL) or mutant enzymes at increasing P/D ratios. (A) The ϕX174 substrate after 30 min of incubation at 80 °C in the absence (−) or in the presence (+) of the Δlatch mutant. The circular (Circ) and linear (Lin) species are cleaved a number of times to yield multiply cleaved fragments (MCFs). Because of the difficulty of measuring the appearance of MCFs, cleavage activity was estimated from the disappearance of the Circ and Lin bands. (B) Cleavage assays carried out in the absence of nucleotide. Control reactions were performed in parallel in the absence of enzyme ("−E"). In the experiment shown, P/D values were as follows: 2, 4, 13, 26, 67 (Δlatch, FL, Δ857); 2, 4, 14, 26, 65 (Δ370). (C) Percent cleavage was estimated as described in the Experimental Procedures ($n = 2-3$). (D) Cleavage reactions performed in the presence of 1 mM ATP ($n = 2-4$).

linear substrate is detectable by a P/D ratio of ∼13−26, whereas at least 20% of the two substrate forms persists for the full-length enzyme even up to P/D = 67. In fact, this assay probably underestimates the difference in specific cleavage activity, given that it was impossible to compare the extent of cleavage once all the linear and circular molecules had disappeared. Thus, deletions in the latch, as well as in the topoisomerase insertion, stimulate cleavage activity, which implicates both regions in the cleavage step of the topoisomerase mechanism.

To test whether nucleotide binding or hydrolysis is important for cleavage activity, the cleavage titrations were repeated in the presence of 1 mM ATP (Figure 2D) and in the presence of 1 mM adenylylimidodiphosphate (ADPNP), a nonhydrolyzable ATP analogue (data not shown). In fact, three different preparations of full-length enzyme were tested in the ADPNP reactions. The results do not differ significantly from those in the absence of nucleotide, suggesting that neither ATP binding nor hydrolysis significantly affects the specific cleavage activity of reverse gyrase.

In scoring cleavage as the disappearance of both circular and linear DNA, the analysis in Figure 2 requires that the DNA be cleaved at least twice. When the data were reanalyzed and cleavage was scored as the disappearance of circular DNA only, results similar to those in Figure 2 were obtained (data not shown). Under all three nucleotide conditions (none, ATP, ADPNP), the deletion mutants cleave 100% of the circular DNA at P/D ≈ 13−26, whereas full-length enzyme cleaves 84−94% at P/D = 26. Even when full-length protein is used at P/D = 67, 4−10% of the circular DNA remains uncleaved.

The Latch and the Topoisomerase Insertion Are Involved in the Religation Step. To examine whether the deleted regions play a role in DNA religation, the enzymes were tested in a religation assay in which the protein was incubated with substrate DNA to allow cleavage complex to form, and then large amounts of salt were added to induce the enzyme to reseal the DNA and dissociate from it (*17*). The substrate was the same ϕX174 DNA used in the cleavage experiments (Figure 3A). The amount of DNA religated by the enzyme under these conditions was estimated from the amount of circular and linear ϕX174 bands remaining before and after high salt was added (Figure 3B). At 1 M NaCl, full-length reverse gyrase and the Δ857 mutant religate less than 60% of the cleaved substrate, whereas the Δlatch and Δ370 mutants religate 80% of the cleaved substrate by 0.75 M NaCl. The religation activity of the Δ857 mutant is substantially lower than that of the full-length enzyme at NaCl concentrations below 1 M. Similar results were obtained when the assay was repeated in the presence of 1 mM ATP

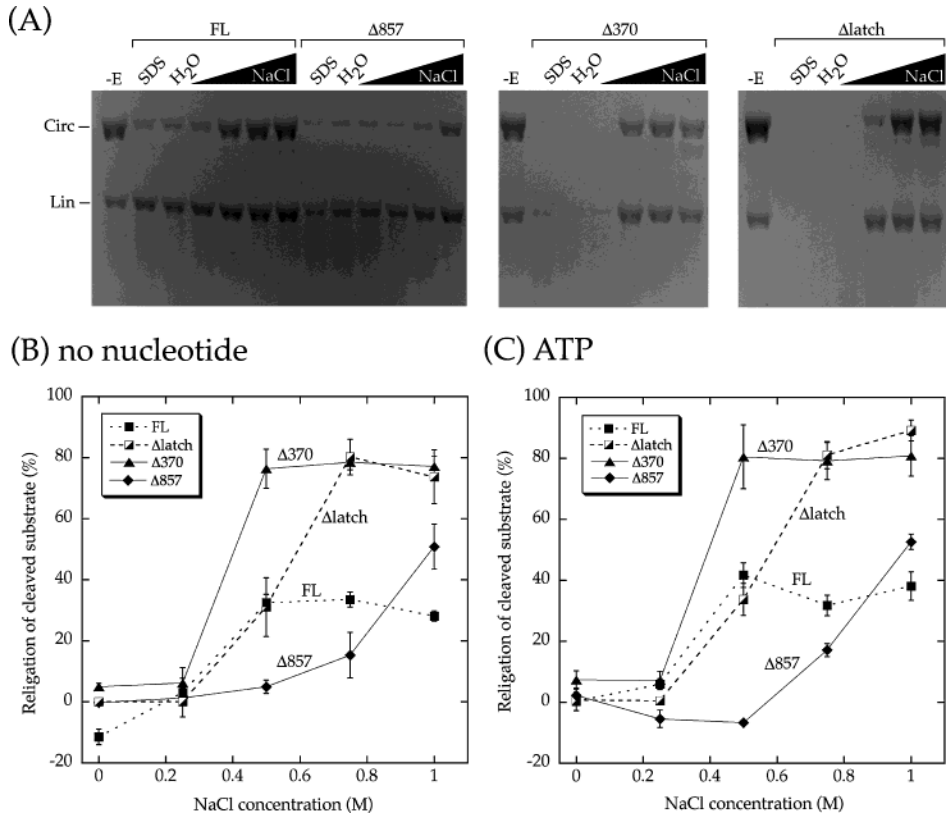

FIGURE 3: Removing the latch and the topoisomerase insertion affect the religation activity of reverse gyrase. (A) Five identical reactions with full-length (FL) reverse gyrase or a deletion mutant were set up with ϕX174 DNA and incubated at 80 °C for 30 min to allow the cleavage complex to form. A sixth control reaction was set up in parallel in the absence of enzyme ("−E"). At the end of 30 min, one tube received SDS to denature the enzyme, and another received water. The remaining tubes received NaCl to final concentrations of 0.25, 0.5, 0.75, and 1 M to induce the religation of DNA trapped in cleavage complexes. The tubes were incubated at 80 °C for 3 min and processed as described in the Experimental Procedures. The assays shown were performed in the presence of 1 mM ADPNP. (B) Religation experiments performed in the absence of nucleotide ($n = 3$). The percent of religated substrate was estimated as described in the Experimental Procedures. (C) Religation experiments performed in the presence of 1 mM ATP ($n = 2-4$).

(Figure 3C) or 1 mM ADPNP (Figure 3A; data not shown).

Removing either the latch or the topoisomerase insertion alters the extent of salt-induced religation, suggesting that both regions of the enzyme are involved in the religation step. In fact, both types of deletion cause a shift in the minimum salt concentration needed for efficient religation, but they do so in opposite directions: the latch deletions render the cleavage complex more sensitive to salt, whereas removing the topoisomerase insertion makes it more resistant to salt.

The Latch Helps To Determine the Cleavage−Religation Equilibrium. Formation of the cleavage complex is reversible (*22*), and normally topoisomerases balance their rates of cleavage and religation to prevent the accumulation of free DNA ends. The much higher specific cleavage activity of the mutants in Figure 2 may reflect a shift toward cleavage in the cleavage−religation equilibrium, but this assumes that the reactions in Figure 2 had reached a steady state. To determine whether this was true, cleavage kinetics for the enzymes were examined under the conditions used in Figure 2. Both full-length and Δlatch enzymes reach a steady state within the first minute of the assay (Figure 4), which means that the reactions in Figure 2 can be taken as evidence that the latch influences the cleavage−religation equilibrium.

The Δ370 mutant shows a similarly rapid burst of cleavage and appears to reach a steady state in the case of no nucleotide (Figure 4A), but in the presence of ATP, the amount of cleavage steadily decreases over the 30-min incubation and may increase at the end (Figure 4B). A similar decrease followed by an increase is observed in the presence of 1 mM ADPNP (data not shown). On the basis of these results, it is unclear whether the Δ370 reactions in Figure 2 had reached a steady state. The same holds for the Δ857 reactions, since the time course in Figure 4 shows a burst of cleavage within the first ∼10 s, after which the amount of cleavage increases at a slow but steady rate. From these assays, it is not possible to conclude whether deleting the tip of latch or the topoisomerase insertion affects the cleavage−religation equilibrium. Nevertheless, the markedly different behavior of the Δ857 mutant from the other enzymes may mean that the topoisomerase insertion influences the rate at which equilibrium is reached, even if it does not affect the amount of cleavage complex at equilibrium.

Deleting the Latch or the Topoisomerase Insertion Does Not Significantly Affect DNA Binding Affinity. A simple explanation for the results of the cleavage and religation assays is that the latch and the topoisomerase insertion are involved in binding DNA. To test this idea, bandshift experiments were performed using an oligonucleotide substrate that reverse gyrase binds with high affinity (*11*). None of the mutants shows a significantly reduced DNA binding affinity relative to the full-length enzyme (Figure 5); in fact, all of the proteins bind the substrate with nanomolar affinity

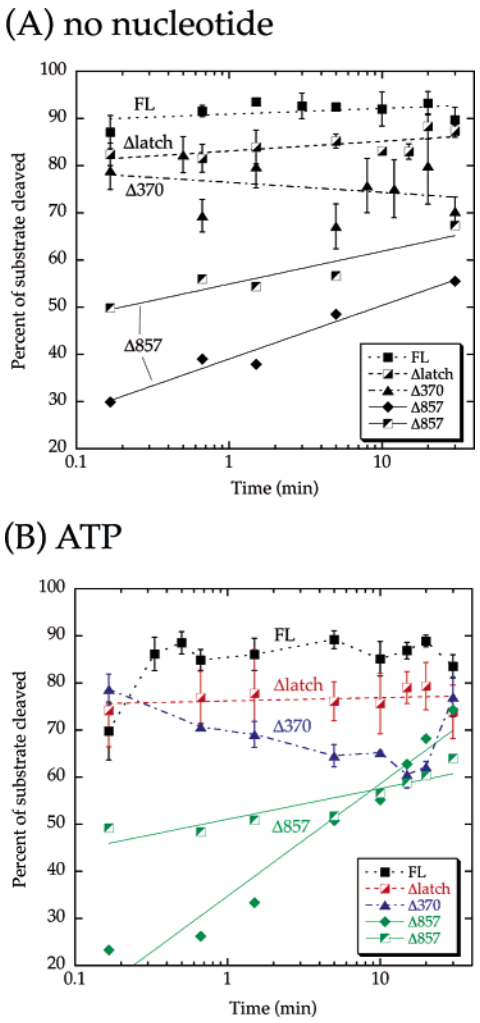

FIGURE 4: The reactions in Figure 2 reflect the amount of cleavage at equilibrium for full-length reverse gyrase and the Δlatch mutant. Cleavage assays were performed as in Figure 2, except that protein concentration was held constant at P/D = 30 for full-length enzyme and P/D = 5 for the mutants, and reactions were sampled at different time points. Results of two independent experiments with the Δ857 mutant are shown separately. For the other enzymes, $n = 2-6$. (A) Time course of cleavage in the absence of nucleotide. (B) Time course of cleavage in the presence of 1 mM ATP.

under these conditions. This suggests that, at least for steady-state binding, the latch and the topoisomerase insertion are not responsible for making most of the contacts with the DNA substrate. If these regions do contact DNA, they probably do so only transiently during catalysis.

Reverse Gyrase Does Not Tightly Coordinate ATP Hydrolysis and Positive Supercoiling. The ATPase and topoisomerase domains of reverse gyrase must communicate to couple the hydrolysis of ATP to the positive supercoiling of DNA. A role for the latch in coordinating the two activities was suggested indirectly by the fact that, in the presence of single-stranded DNA, the ATPase rate of the Δlatch and Δ370 mutants is significantly higher than that of the full-length enzyme (*11*).

To assess whether deletions in the latch affect the coordination of ATP hydrolysis and positive supercoiling, it was first necessary to examine the coordination in the full-length enzyme. The fact that reverse gyrase hydrolyzes ATP in the presence of single-stranded DNA (*11*, *23*) already suggests that the two processes are not tightly coordinated,

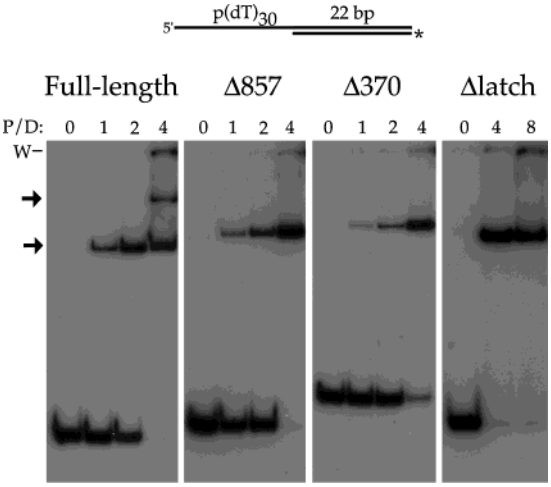

FIGURE 5: Comparable DNA binding affinity by full-length reverse gyrase and deletion mutants. Proteins were mixed in the indicated P/D ratios with the substrate depicted at the top of the figure. On the upper strand, a poly(dT)$_{30}$ tail extends off the 5′-end of a 22-bp duplex. The asterisk indicates the position of the radiolabel on the 5′-end of the lower strand. The "W" marks the position of the wells, and the arrows indicate protein−DNA complexes. Differences in the migration of the free DNA between panels are due to slight variations in electrophoresis time.

but conclusive evidence requires that ATPase activity be measured during the supercoiling reaction. Therefore, reactions with pBR322 plasmid were carried out in parallel with unlabeled ATP or radiolabeled ATP and analyzed separately: reactions with unlabeled ATP were analyzed by gel electrophoresis to determine the rate of positive supercoiling, and reactions with labeled ATP were analyzed by charcoal adsorption assay to determine the rate of ATP hydrolysis. The plasmid substrate already contained a few positive supercoils at the start of the reaction, to ensure that the supercoiling rate reflected the introduction of positive supercoils rather than the removal (relaxation) of negative ones. Dividing the rate of ATP hydrolysis by the rate of positive supercoiling provides an estimate of the ATP stoichiometry, which is the number of ATP molecules hydrolyzed per supercoil introduced.

An example of an ATP stoichiometry experiment performed with 1 mM ATP is shown in Figure 6A. The supercoiling rate remains linear for 8 min in the assay and then falls to zero when no more positive supercoils can be added to the plasmid (Figure 6A, middle panel). The ATPase rate, on the other hand, remains linear over a much longer period (Figure 6A, right panel), implying that the two processes are not tightly coordinated. Further evidence for this comes from the fact that the ATP stoichiometry calculated from this experiment is ∼2400, indicating that the number of ATP molecules hydrolyzed exceeds the number of positive supercoils by 3 orders of magnitude. These results lead one to question whether reverse gyrase coordinates the two processes at all. The ATP stoichiometry experiments were therefore repeated over a broad range of ATP concentrations (Figure 6B). The turnover numbers for both hydrolysis and positive supercoiling increase 36- and 9-fold, respectively, between 1 and 100 μM ATP. Above 100 μM ATP, supercoiling turnover remains constant, and ATPase turnover increases less than 2-fold. Thus, reverse gyrase coordinates the two processes to some degree, but the coordination is loose: even at 1 μM ATP, the enzyme

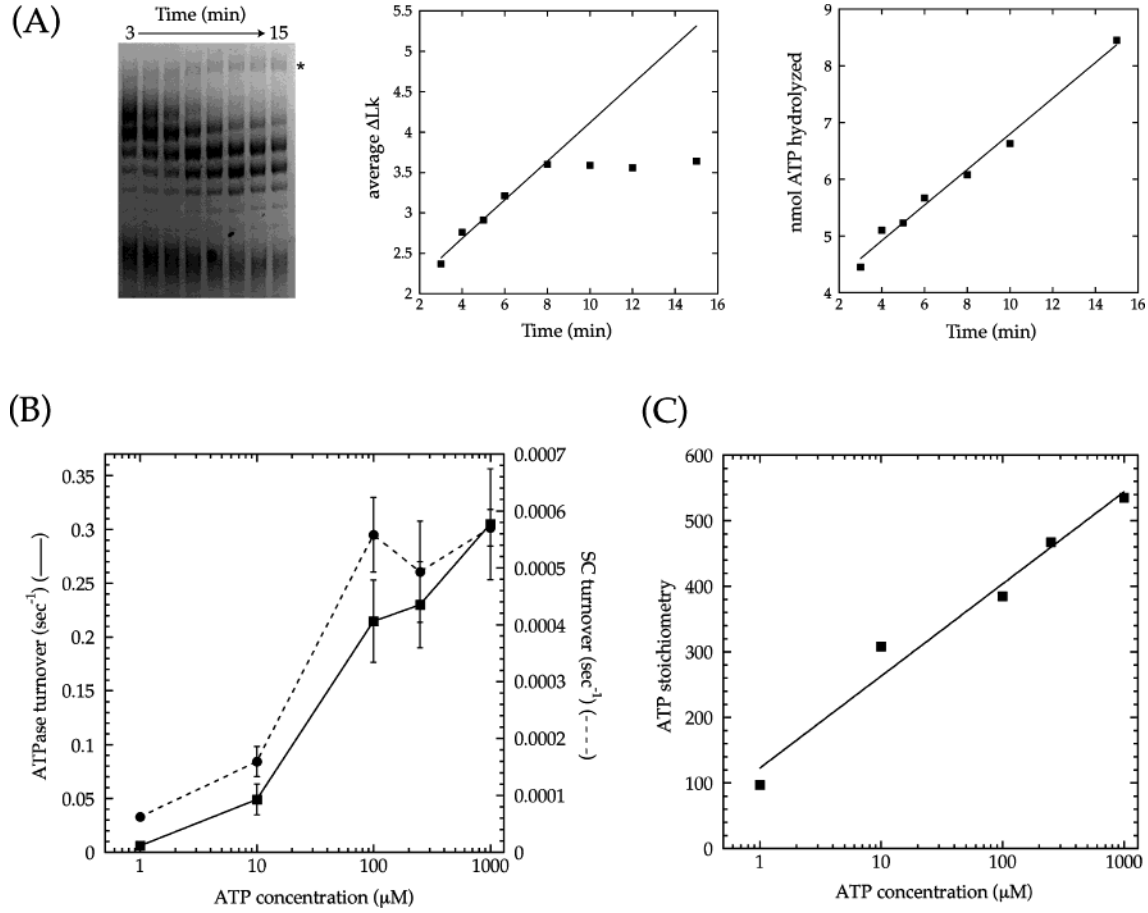

FIGURE 6: Loose coordination of ATPase and supercoiling activities in full-length reverse gyrase. Positive supercoiling reactions were set up with full-length reverse gyrase and positively supercoiled pBR322 as described in the Experimental Procedures. (A) Results from a typical experiment conducted with enzyme at P/D = 12 and ATP at 1 mM. Reactions were analyzed at various time points with agarose gel electrophoresis (left panel). (Examples of control reactions incubated in the absence of enzyme are shown in Figure 9.) The asterisk marks the migration position of nicked DNA. The centers of the topoisomer distributions were used to determine the average ΔLk, which reflects the average number of positive superhelical turns in the product DNA (middle panel). The reactions were also analyzed in a charcoal adsorption assay to determine the rate of ATP hydrolysis (right panel). The plasmid used in this experiment initially contained ~9 positive supercoils under the reaction conditions, corresponding to a superhelical density (σ) of +0.022. (B) ATP stoichiometry experiments were performed using full-length enzyme at P/D = 12 over a broad range of ATP concentrations ($n = 2-4$), and the ATPase and supercoiling (SC) rates were determined as in (A). The plasmid used in these studies initially contained ~5 positive supercoils ($\sigma \approx +0.012$) under the reaction conditions. (C) Plot of the ATP stoichiometry as a function of ATP concentration, derived from the data in (B). For each ATP concentration, the ATPase turnover was divided by the corresponding supercoiling turnover. The lower initial superhelicity of the substrate in (B) compared with that used in (A) may explain the difference in ATP stoichiometry measured with 1 mM ATP (535 vs 2400, respectively).

hydrolyzes ~100 ATP molecules per positive superhelical turn (Figure 6C).

The full-length enzyme used in these and previous studies (*8*, *11*) contains two mutations, Pro719Leu and Leu1046Met. The former mutation reduces the toxicity of the expression construct significantly, making it possible to express recombinant enzyme in *E. coli* in quantities sufficient for structural studies. To eliminate the possibility that the high ATP stoichiometry measured here was an artifact of these mutations, wild-type reverse gyrase lacking any mutations was purified from *E. coli* (Figure 7A) and tested in the stoichiometry assay with 1 mM ATP (Figure 7B). As with the full-length enzyme, the wild-type version maintains a linear ATPase rate even as supercoiling ceases. The turnover numbers for ATPase activity and for positive supercoiling were found, respectively, to be 0.019 ± 0.001 and (1.8 ± 0.4) × 10^{-3} s^{-1} at 1 μM ATP ($n = 2$), and 1.1 ± 0.2 and (2.10 ± 0.06) × 10^{-3} s^{-1} at 1 mM ATP ($n = 2$). In these experiments, the starting substrate contained approximately five positive supercoils ($\sigma \sim +0.012$), just as in the experiments in Figure 6B. The ATPase turnover at each ATP concentration is 3-fold higher than the corresponding turnover for the full-length enzyme, and the supercoiling turnover is 29-fold higher at 1 μM ATP and 4-fold higher at 1 mM ATP. In fact, the difference in ATPase and supercoiling rates for the two proteins is probably greater, because the presence of an appreciable amount of ~27-kDa contaminant in the wild-type preparation (Figure 7A) means that the protein concentration overestimates the amount of reverse gyrase. Therefore, the presence of one or both mutations in the full-length construct causes a decrease in both the ATPase and supercoiling rates. It may be significant that, for the wild-type enzyme, the supercoiling rate is the same at 1 μM and 1 mM ATP, whereas it correlates with ATP concentration in the full-length protein (Figure 6B), but further studies are needed to address this question.

Calculating ATP stoichiometry for wild-type reverse gyrase from experiments such as those in Figure 7B gives values of ~11 at 1 μM ATP and ~511 at 1 mM ATP. The

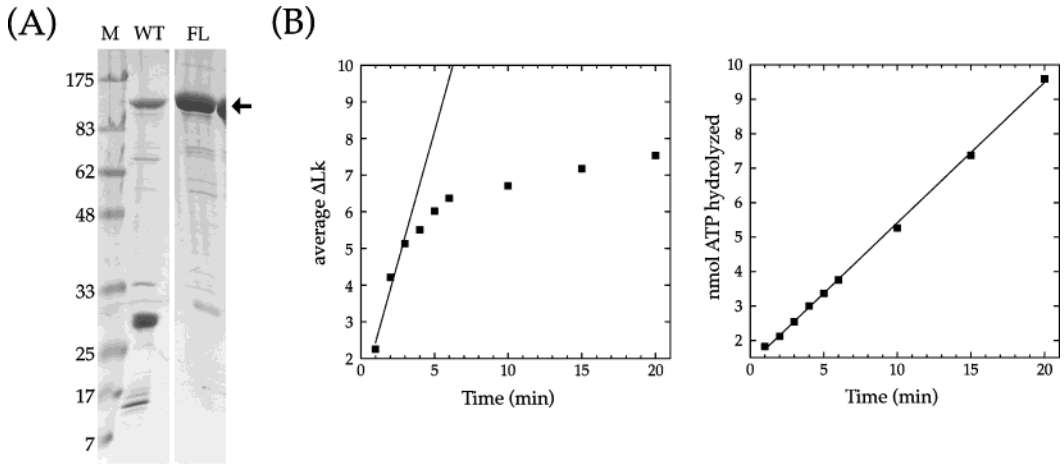

FIGURE 7: Loose coordination of ATPase and supercoiling activities by reverse gyrase lacking the Pro719Leu and Leu1046Met mutations. (A) Wild-type reverse gyrase (WT) was expressed in *E. coli* and purified following the same procedure as for the full-length enzyme (FL). An equal amount (1.25 μg) of each purified preparation was analyzed by SDS−PAGE and Coomassie Blue staining. The "M" lane contains molecular mass standards, with their masses (in kilodaltons) indicated to the left. (B) Representative results of an ATP stoichiometry experiment with wild-type reverse gyrase at P/D = 12 and 1 mM ATP. The plasmid substrate initially contained ~5 positive supercoils ($\sigma \approx +0.012$) under the reaction conditions.

stoichiometry at 1 mM ATP is similarly high for both the wild-type and full-length enzymes. The stoichiometry at 1 μM ATP is ~10-fold lower for the wild-type protein but still too high to be tightly coordinated with strand passage. Moreover, for both proteins, ATPase activity can continue at a linear rate even when supercoiling stops. Therefore, reverse gyrase with or without the two point mutations shows loose coordination of ATP hydrolysis and positive supercoiling.

It is possible that some or all of the "excess" ATP hydrolysis observed in the stoichiometry experiments is due to a contaminating ATPase activity. To test this possibility, stoichiometry reactions at 1 mM ATP were set up in the absence of any DNA but in the presence of 160 nM enzyme (equivalent to P/D = 12). Time courses were performed over 30 min, and the rate of ATP hydrolysis without any added protein was found to be 0.072 ± 0.002 nmol of ATP per minute (n = 4), whereas the rate in the presence of full-length reverse gyrase was found to be 0.070 ± 0.002 nmol of ATP per minute (n = 2). The same control experiments in the presence of wild-type enzyme showed a hydrolysis rate of 0.086 ± 0.009 nmol of ATP per minute (n = 2), compared with 0.09 ± 0.03 nmol of ATP per minute (n = 2) in the absence of added protein. Therefore, ATP hydrolysis measured in the stoichiometry assay is likely to be due solely to reverse gyrase activity.

Instead of a loose coordination between ATPase and supercoiling activities, the high ATP stoichiometry could mean that reverse gyrase tightly coordinates its ATPase and supercoiling activities, and the excess hydrolysis is due to "treadmilling" or "stuttering". In other words, the enzyme is performing strand passage in both the forward and reverse directions, with one or two ATP molecules hydrolyzed each time. Because the stoichiometry experiments measure net supercoiling, only a fraction of the actual strand passage events would be counted, and therefore the ATP stoichiometry would appear artificially high. This scenario seems unlikely on the basis of a previous study showing that reverse gyrase can perform strand passage bidirectionally in the presence of ADPNP but only unidirectionally with ATP (*11*). Unfortunately, the P/D ratios used in the ATP experiments in that study were higher than those at which bidirectional strand passage was observed with ADPNP, so the ATP experiments might have missed bidirectional strand passage if it was occurring. Therefore, positive supercoiling assays with full-length enzyme were performed in the presence of ATP over a wide range of P/D ratios, and control reactions were performed in parallel in the presence of ADPNP (Figure 8). As reported previously, a reversal in the direction of strand passage is observed with ADPNP; the transition occurs here at a higher P/D than in the previous study. With ATP, however, no reversal is observed, suggesting that nucleotide hydrolysis forces the enzyme to perform strand passage exclusively in the direction of positive supercoiling. Therefore, the most likely explanation for the high ATP stoichiometry is that reverse gyrase does not tightly coordinate its ATPase and positive supercoiling activities, and that the excess ATP consumption is not linked to strand passage.

The Latch Helps To Coordinate ATP Hydrolysis and Positive Supercoiling. To test whether deletions in the latch affect the coordination between the two activities, the Δ370 and Δlatch mutants were examined in the ATP stoichiometry assay (Figure 9 and Table 1). The full-length enzyme was tested in parallel, and the stoichiometry was found to be ~2100, which is significantly higher than in the experiments in Figure 6B. This is likely due to the fact that the plasmid substrate in the Figure 9 studies initially contained more positive supercoils, which may indicate that the enzyme consumes more ATP per strand passage as it approaches its supercoiling limit. The Δ370 mutant shows an ATPase rate more than 3-fold higher than that for the full-length enzyme, yet its supercoiling rate is the same (Table 1). Moreover, like the full-length enzyme, the Δ370 mutant maintains a linear ATPase rate after supercoiling has stopped (Figure 9A). The Δlatch mutant shows an even greater "uncoupling" of ATP hydrolysis and positive supercoiling: its ATPase rate is 14-fold faster than that of the full-length enzyme, despite its having impaired supercoiling activity (Figure 9B). Within the first few minutes of the assay, the Δlatch mutant produces a broad distribution of positive topoisomers containing up

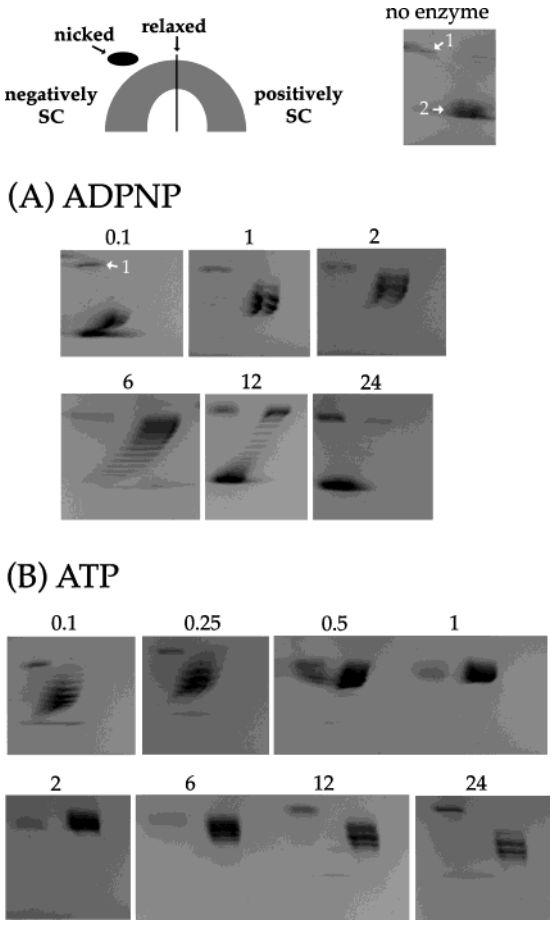

FIGURE 8: Reverse gyrase does not perform strand passage bidirectionally in the presence of nucleotide hydrolysis. Standard positive supercoiling reactions were carried out with full-length reverse gyrase in the presence of 1 mM ADPNP (A) or 1 mM ATP (B). The P/D ratio is indicated above each reaction. The diagram at the top provides a key to interpreting the results of the two-dimensional electrophoresis. The panel at the top shows a control supercoiling reaction lacking any reverse gyrase. The band marked "1" corresponds to nicked plasmid; the band marked "2" corresponds to the negatively supercoiled input plasmid. SC, supercoiled.

Table 1: Coordination of ATPase and Supercoiling Activities in Deletion Mutants of Reverse Gyrase[a]

protein	(a) ATPase turnover (s^{-1})	(b) SC turnover (s^{-1})	(a)/(b) ATP stoichiometry
full-length	0.7 ± 0.1 (7)	$(3.3 \pm 0.4) \times 10^{-4}$ (6)	2100
Δ370	2.3 ± 0.5 (7)	$(3 \pm 1) \times 10^{-4}$ (4)	7700
Δlatch	10 ± 1 (5)		

[a] Assays were performed as described in Figure 9, using a range of P/D ratios for each protein: 5−12 (full-length); 5−25 (Δ370); 2.5, 5, 8 (Δlatch). Lower ratios were used for the Δlatch mutant to minimize the nicking activity observed previously (*11*). Values are the average (±SEM) of the number of independent experiments given in parentheses. SC, supercoiling.

to ~14 supercoils ($\sigma \approx +0.034$). This distribution remains essentially constant for the rest of the incubation, even as ATP hydrolysis continues at a linear rate (Figure 9B). The results with the Δ370 and Δlatch mutants suggest that the latch plays a role in coordinating ATP consumption and positive supercoiling by reducing the amount of hydrolysis occurring in the absence of strand passage.

Using the same procedure as before, control experiments were performed with the mutant enzymes to detect contaminating ATPase activity. While the background rate of hydrolysis in the absence of added protein was 0.072 ± 0.002 nmol of ATP per minute ($n = 4$), the rates in the presence of the Δ370 or Δlatch mutant were found to be respectively 0.076 ± 0.009 and 0.08 ± 0.01 nmol of ATP per minute ($n = 2$ for each).

DISCUSSION

Reverse gyrase comprises an N-terminal ATPase domain and a C-terminal toposiomerase domain, which cooperate to positively supercoil DNA using the energy of ATP hydrolysis. The latch subdomain in the ATPase module represses topoisomerase activity in the absence of nucleotide (*11*), preventing reverse gyrase from acting as an ATP-independent relaxing enzyme like other type I topoisomerases. The studies described here suggest that the latch plays more than just a regulatory role in catalysis by participating in both the cleavage and religation steps of the supercoiling reaction, and by helping to coordinate ATP hydrolysis and strand passage.

Deletion of the latch shifts the cleavage−religation equilibrium toward cleavage, and deletion of all or part of the latch renders the cleavage complex more salt-sensitive. The simplest explanation for these results is that the latch helps to position the DNA substrate in the topoisomerase active site. A recent electron microscopy study of reverse gyrase bound to DNA (*24*) is consistent with a DNA-binding function for the latch. Furthermore, similar binding affinity for the mutant and full-length enzymes suggests that the protein−DNA contacts lost in the mutants serve only a supporting role during catalysis, instead of functioning continuously to ensure strong steady-state binding.

To determine the effects of nucleotide binding and hydrolysis on individual steps of the supercoiling mechanism, the cleavage and religation experiments were performed under different conditions: without nucleotide, with ATP, or with the nonhydrolyzable analogue ADPNP. Previous studies have shown that reverse gyrase can cleave DNA in the absence of nucleotide (*15*−*17*, *25*, *26*). The present work extends these findings by showing that neither nucleotide binding nor hydrolysis significantly affects the specific cleavage or religation activities. Hydrolysis may therefore occur independently of cleavage and religation. Conclusive studies to address this question will probably require a rapid-quench kinetics approach like that applied to yeast topoisomerase II (*27*).

Experiments correlating the rate of ATP hydrolysis and positive supercoiling under identical conditions indicate that reverse gyrase does not tightly coordinate the two processes. ATP turnover exceeds supercoiling turnover by 1−3 orders of magnitude, even at low ATP concentrations. To achieve maximal positive superhelical density in the substrate, the stoichiometry experiments were performed with a molar excess of enzyme. Positive supercoiling assays examining reverse gyrase from four different organisms suggest that maximal superhelical density is achieved only when the enzyme is present in significant molar excess (*11*, *25*, *28*−*30*), unless a macromolecular crowding agent such as poly(ethylene glycol) is used (*11*, *28*). Maximal positive supercoiling of a plasmid in vitro may depend on the binding of multiple reverse gyrase molecules to each DNA molecule.

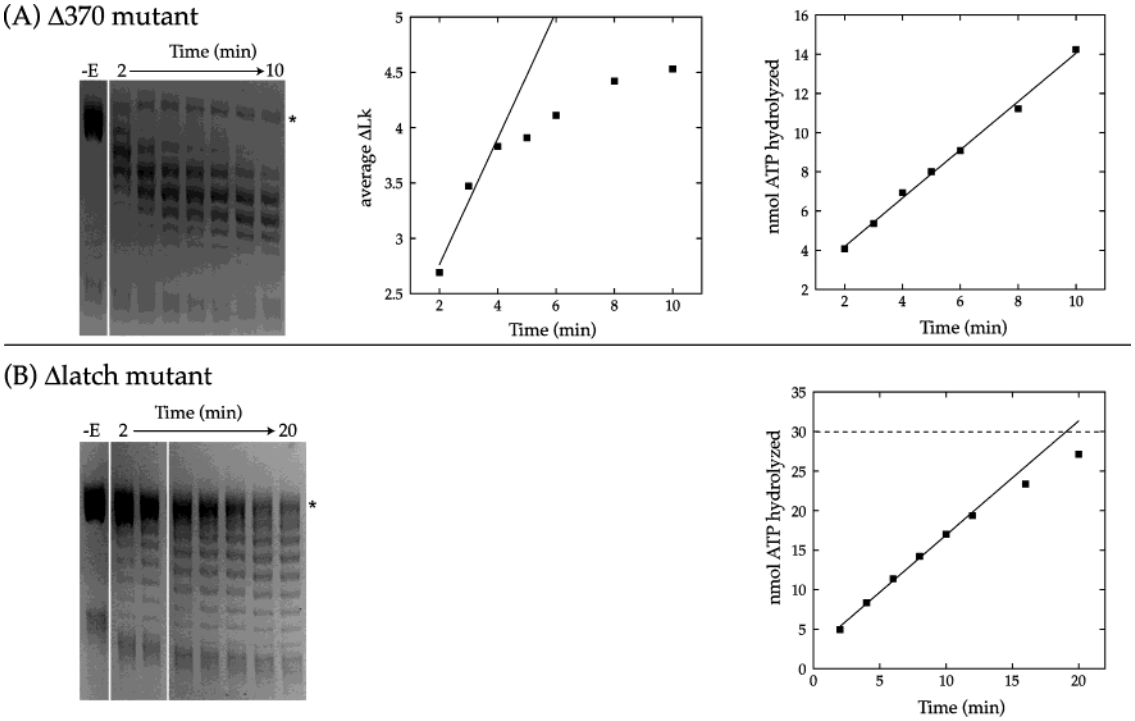

FIGURE 9: The latch helps to coordinate ATP hydrolysis and positive supercoiling. Stoichiometry experiments with 1 mM ATP were performed as described in the Experimental Procedures using the same substrate as described in Figure 6A. (A) An experiment performed with the Δ370 mutant at P/D = 25. A control reaction ("−E") was incubated for 10 min in the absence of enzyme. (B) An experiment performed with the Δlatch mutant at P/D = 5. The "−E" reaction was incubated for 20 min. The supercoiling rate for the Δlatch mutant was not determined (see text for discussion). The dashed line indicates the amount of ATP present at the start of the reaction. The asterisk marks the migration position of nicked DNA.

Electron microscopy studies suggest that the decrease in supercoiling activity with increasing salt concentration is due to a decrease in the number of enzyme molecules bound per plasmid (*24*). If we assume that multiple reverse gyrase molecules are bound to each plasmid during the supercoiling reaction, it is possible that only a few of the molecules are performing strand passage with tight coordination between the ATPase and supercoiling reactions, while the rest of the molecules on the same plasmid are engaging in hydrolysis not linked to strand passage. This would imply a functional difference between otherwise identical protein molecules, and the little evidence available argues against this. Because reverse gyrase unwinds DNA upon binding, the extent of DNA unwinding can be measured as a function of increasing enzyme concentration. Such a titration experiment shows that unwinding increases linearly on going from P/D ≈ 6 to P/D ≈ 24 (*26*). This suggests that, even when multiple enzyme molecules are bound to the same plasmid, they are functionally identical, at least at the start of the supercoiling reaction. Therefore, it seems more likely that all of the enzymes bound to a given plasmid are supercoiling the DNA with loose coordination of ATP hydrolysis and strand passage.

Is the excess hydrolysis that results from this loose coordination truly nonproductive, or could it be driving other steps in the supercoiling mechanism? Reverse gyrase unwinds DNA upon binding to it (*10*, *26*), and this is thought to constitute an early step in the reaction pathway (*2*, *11*), so perhaps the enzyme uses hydrolysis to drive this step. This possibility seems unlikely in light of studies showing that the unwinding occurs in the absence of ATP (*10*, *26*) and the extent of unwinding is not increased by nucleotide binding or hydrolysis (*10*, *31*). Another possibility is that reverse gyrase uses ATP hydrolysis to translocate along the DNA through its N-terminal domain, which shows homology to the ATPase module of helicases (*8*). ATP-dependent translocation could help the enzyme to separate the DNA strands by processive strand displacement, or to unwind the DNA through a mechanism like that recently proposed for some chromatin remodeling complexes (*32*, *33*). But reverse gyrase lacks processive strand displacement activity (*10*), and its unwinding activity does not require ATP hydrolysis. Although translocation along the DNA remains a possibility, the simplest interpretation of the excess hydrolysis measured here is that it does not contribute to supercoiling.

The use of excess enzyme in the stoichiometry experiments makes it difficult to compare the stoichiometry values for reverse gyrase with those for type II topoisomerases, which are the only other topoisomerases that hydrolyze ATP. Type II enzymes that only relax DNA show stoichiometries ranging from 1 to 7 ATP molecules per strand passage at ATP concentrations from 100 μM to 1 mM (*34*−*36*). *E. coli* gyrase shows a stoichiometry of ∼2 ATP molecules per negative supercoil introduced at 0.5 mM ATP (*37*, *38*). It can hydrolyze ATP at a linear rate, even after it has reached a supercoiling limit (*37*, *39*), just as observed here for reverse gyrase. And like reverse gyrase, other type II enzymes show robust ATPase activity in the presence of DNA that cannot be supercoiled (*34*−*37*).

Stoichiometry experiments with the Δ370 and Δlatch mutants suggest that the latch helps to coordinate ATP hydrolysis and positive supercoiling by inhibiting nonproductive hydrolysis. The coordination defect in these mutants may be due to the fact that they undergo ATP-dependent conformational changes less efficiently, that their interaction

with DNA during catalysis is compromised, or both. Because the cleavage and religation assays also suggest a defect in DNA interaction, loss of important protein−DNA contacts may explain all the differences observed so far between the latch deletion mutants and full-length reverse gyrase.

The studies described in this report and elsewhere (*11*) support a mechanistic model for reverse gyrase in which ATP-dependent conformational changes in the latch help to drive strand passage during positive supercoiling. In fact, ATP hydrolysis may normally be limited by the need to drive conformational changes in the latch, which would explain why the Δ370 and Δlatch mutants hydrolyze ATP faster than the full-length enzyme in the presence of single-stranded (*11*) and plasmid DNA (Table 1). According to this model, the excess hydrolysis behind the high ATP stoichiometry of reverse gyrase does not lead to strand passage because it fails to trigger the required conformational changes in the latch, or because the conformational changes do not always result in strand passage.

The present studies have focused on the role of the latch in mediating cross-talk between the ATPase and topoisomerase domains. However, the latch is unlikely to be the only structural feature required for this communication. Previous studies have speculated that the disulfide bridges and noncovalent interactions connecting the domains near the N-terminus are involved in coupling the energy of ATP hydrolysis to positive supercoiling (*11*). Further work is needed to address whether these interactions share in the work of the latch to coordinate hydrolysis and strand passage, and to position the DNA substrate for cleavage and religation.

The importance of the latch to the communication between the ATPase and topoisomerase domains of reverse gyrase makes it functionally analogous to residues 350−407 of human topoisomerase IIα, which lie at the interface between the ATPase and cleavage−religation domains. A mutant topoisomerase IIα lacking this region, like the Δlatch and Δ370 mutants of reverse gyrase, shows a higher rate of ATP hydrolysis than the full-length enzyme and an uncoupling of ATP hydrolysis from strand passage (*40*). Evidence in that study suggested that the deleted region helps to ensure proper interaction with the uncleaved DNA during strand passage. It is tempting to speculate, by analogy, that the latch of reverse gyrase interacts with the passed strand.

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