Studies of a Positive Supercoiling Machine

NUCLEOTIDE HYDROLYSIS AND A MULTIFUNCTIONAL “LATCH” IN THE MECHANISM OF REVERSE GYRASE*

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Reverse gyrase, the only topoisomerase known to positively supercoil DNA, has an N-terminal ATPase domain that drives the activity of a topoisomerase domain. This study shows that the N-terminal domain represses topoisomerase activity in the absence of nucleotide, and nucleotide binding is sufficient to relieve the repression. A “latch” region in the N-terminal part was observed to close over the topoisomerase domain in the reverse gyrase crystal structure. Mutants lacking all or part of the latch relax DNA in the absence of nucleotide, indicating that this region mediates topoisomerase repression. The mutants also show altered DNA-dependent ATPase activity, suggesting that the latch may be involved in coupling nucleotide hydrolysis to supercoiling. It is not required for this process, however, because the mutants can still positively supercoil DNA. Nucleotide hydrolysis is essential to the specificity of reverse gyrase for increasing the linking number of DNA. Although with ATP the enzyme performs strand passage always toward increasing linking number, it can increase or decrease the linking number in the presence of a nonhydrolyzable ATP analog. This suggests that the mechanism of reverse gyrase is best described by a combination of recently proposed models.

Topoisomerases participate in practically every DNA transaction, including transcription, replication, recombination, and chromosomal segregation (1). They change the topological state of DNA by altering its linking number, i.e. the number of times that one strand of the double helix crosses the other. They use a three-step mechanism of cleavage, strand passage, and religation. In the first step, a nucleophilic tyrosine attacks the phosphodiester backbone, cleaving the DNA and leading to a covalent intermediate of protein-DNA termed the “cleavage complex.” Type I topoisomerases cleave one strand of the duplex; type II enzymes cleave both strands. The enzyme, covalently attached to the cut DNA, separates the free ends of the cleaved strand(s) and allows the other strand of the duplex (type I), or another region of duplex (type II), to pass through this gap. The protein then reseals the backbone of the cleaved DNA and releases the product.

Although all topoisomerases can relax supercoiled DNA, only prokaryotic gyrase is able to introduce negative supercoils in DNA. This type II topoisomerase uses the energy of ATP hydrolysis to reduce the linking number, leading to an underwound, or negatively supercoiled, product. Until recently, gyrase was the only topoisomerase known to supercoil DNA. Later, another supercoiling enzyme was discovered and named reverse gyrase, because it increases the linking number, leading to overwound, or positively supercoiled, product (2, 3).

Reverse gyrase is the only topoisomerase known to overwind DNA. So far it has been found exclusively in hyperthermophiles, organisms that live above ~80 °C (4, 5). The role of the protein in vivo remains unclear (6); one suggestion is that it rewrinds the DNA strands in regions of the chromosome that have opened up to allow transcription or replication (7). Such strand separation at the growth temperatures of hyperthermophiles could be lethal if the strands do not re-associate efficiently.

Reverse gyrase belongs to the family of type IA topoisomerases (3, 8–10), which includes the well-studied topoisomerases I and III from Escherichia coli. Reverse gyrase differs from other type I enzymes in three key respects. First, whereas type I enzymes can only relax supercoiled DNA, reverse gyrase positively supercoils. Second, reverse gyrase requires nucleotide hydrolysis for this activity (2, 10). This property makes reverse gyrase akin to type II topoisomerases in general, all of which require ATP hydrolysis, and specifically to gyrase, which couples ATP hydrolysis to supercoiling. Lastly, in the presence of ATP, reverse gyrase performs the strand passage step unidirectionally: It can increase the linking number of DNA but not decrease it (3). In contrast, topoisomerase I can relax either negatively or positively supercoiled DNA (11), corresponding to an increase or a decrease in linking number, respectively.

The N-terminal domain of reverse gyrase provides the ATP energy to drive positive supercoiling by the topoisomerase domain. Understanding how the two domains interact is therefore critical for understanding the enzyme’s mechanism. The crystal structure of Archaeoglobus fulgidus reverse gyrase revealed a bimodular enzyme comprising an N-terminal domain with a pair of RecA-like folds homologous to the ATPase domains of helicases, and a C-terminal domain highly homologous to E. coli topoisomerase I (12). The structure immediately suggested a basis for the interaction between the two domains. Residues 352–427 of the N-terminal domain form a “latch” that clamps down over the topoisomerase module. We predicted that the latch would mediate part or all of the communication between the domains.

This report explores the role of the latch in the reverse gyrase mechanism. Although the N-terminal domain allows positive topoisomerization in the presence of ATP, it uses the latch to repress topoisomerization in the absence of nucleotide. This study also examines aspects of the protein’s DNA-binding behavior, its selectivity for nucleotide cofactor, and the rela-

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tionship between nucleotide hydrolysis and unidirectional strand passage.

**EXPERIMENTAL PROCEDURES**

**Cloning**—An *A. fulgidus* reverse gyrase expression clone (pRGY1N) was constructed as described previously (12). The clone contains two PCR-induced mutations, Pro-719→Leu and Leu-1046→Met. Expression clones containing just the Pro-719→Leu mutation or neither mutation were created by moving SacI-BamHI or NsiI-BamHI cassettes, respectively, from an error-free PCR clone into pRGY1N. The topoisomerase domain was subcloned from pRGY1N and inserted into pRET3a using NdeI and BamHI sites encoded in the primers. The subclone begins with a Met, followed by Glu-478 and extends through the natural end of the gene into a Gly-(His)6 sequence as in pRGY1N. Double mutant reverse gyrase clones were PCR amplified using a mutagenic primer to fuse, in-frame, the sequences immediately upstream and downstream of the region to be deleted. The mutated fragments were digested with appropriate restriction enzymes and inserted into pRGY1N cut in the same way. The Δlatch mutant (missing residues 360–418) and Δ570 mutant (missing residues 370–381) were created with a MunI-NsiI cassette, and the Δ575 mutant (missing residues 857–866) was created with an SfiI-BamHI cassette. The Tyr-809→Phe mutant of reverse gyrase was created by the megaprimer method (13). The mutant PCR product was digested with SacI and BamHI and inserted into pRGY1N. The Δlatch-Y809F mutant was created by moving the Tyr-809→Phe cassette (SacI-BamHI) into the Δlatch background. All mutant constructs were checked by DNA sequencing.

**Protein Overexpression and Purification**—All proteins were overexpressed in *E. coli C41(DE3)* (14). Freshly transformed colonies were cultured overnight, then inoculated into 2×YT containing 100 μg/ml ampicillin. Cells were induced with isopropyl-1-thio-β-D-galactopyranoside (1 mM Tris-Cl, pH 8.0; 200 mM NaCl; 10 mM MgCl2; 0.02% NaN3). The filtration column (Amersham Biosciences) equilibrated in buffer B (20 mM Tris-Cl, pH 8.0; 300 mM NaCl), and stored at room temperature. The gel was run in one dimension for 2.5 h, soaked in running buffer supplemented with 4.5 mM Tris-HCl, 10 mM EDTA, and 5% glycerol, but these components can be omitted. The reactions were set up in 1× standard reaction buffer containing 8 mM dATP, 8 mM dTTP, 8 mM dCTP, 8 mM dGTP, 2.5 mM of the indicated NTP. The NaCl concentration was reduced to 18 mM, then halted by adding SDS to a final concentration of 1% (w/v) and placing the tubes on ice. When all time points had been processed, 700 μl of this supernatant was added to 5 ml of FluoranSafe 20 mM Tris-Cl, pH 8.0; 200 mM NaCl; 10 mM MgCl2; 0.02% NaN3), and stored at −80 °C. The following procedures were performed at 4 °C, unless otherwise noted. Cells were thawed rapidly at room temperature, sonicated, and spun for 30 min at 40,000 rpm in a Beckman Ti-45 rotor. The supernatant was purified on nickel-nitrilo-triacetic acid-agarose (Qiagen) equilibrated in buffer A containing 10 mM imidazole. The protein was eluted with 150 mM imidazole. Protein fractions were pooled and concentrated on YM-30 concentrators (Amicon). To maintain solubility during concentration of the protein, buffer A containing 5 mM NaCl was added to the Ni2+ column pool until the final NaCl concentration was 1 M. Alternatively, MgCl2 was added to a final concentration of 10 mM. The concentrate was applied to a 26/60 Sephacryl S300 gel filtration column (Amersham Biosciences) equilibrated in buffer B (20 mM Tris-Cl, pH 8.0; 200 mM NaCl; 10 mM MgCl2; 0.02% NaN3). The reverse gyrase peak was pooled, concentrated in YM-30 concentrators, aliquoted, and stored at −80 °C. Protein concentrations were estimated using the BCA assay (Pierce). Protein dilutions for experiments were made in buffer B.

**Positive Supercoiling Assays**—Unless stated otherwise, 30-μl reactions were set up in “standard reaction buffer” (50 mM Tris-Cl, pH 8.0; 30 mM NaCl; 10 mM MgCl2; 0.02% NaN3) containing 2 μg of negatively supercoiled pBR322 and 1 mM of the indicated NTP. The relaxed supercoiled pBR322 substrate was subjected to two-dimensional electrophoresis in 1.2% agarose gels at 20 mA, using the BCA assay (Pierce). Protein dilutions for experiments were made in buffer B.

**Nucleotide Hydrolysis Assays**—Reactions (30 μl) were set up with 7.8 pmol of the indicated protein in standard reaction buffer containing 8 mM dX174 single-stranded DNA (New England BioLabs) and 100 μM of the indicated dNTP spiked with 2.5 μCi of the same dNTP radiolabeled on either the α- or γ-phosphate (−3000–6000 Ci/mmol, Amersham Biosciences).

**RESULTS**

**Overexpression and Properties of Recombinant Reverse Gyrase**—The full-length reverse gyrase gene from *A. fulgidus* was overexpressed in *E. coli* (12). This clone contains two mutations created during PCR: Pro-719→Leu, and Leu-1046→Met. The protein with or without the β-h darn mutations behaves similarly in positive supercoiling assays (data not shown), with changing Leu-719 to the native Pro creates a clone extremely toxic to the cells and reduces expression levels 30-fold. The “wild-type” and mutant reverse gyrase described in this report carry both mutations.

In the absence of divalent metals, *A. fulgidus* reverse gyrase aggregates at NaCl concentrations below 600 mM, similar to

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1 The abbreviations used are: DTT, dithiothreitol; ADPNP, adenylyl-imidodiphosphate; P/D, protein-to-DNA molar ratio; FL, full-length reverse gyrase; Δlatch, a reverse gyrase mutant lacking residues 360–418; Δ370, a reverse gyrase mutant lacking residues 370–381; Δ575, a reverse gyrase mutant lacking residues 857–866; pdDT, polydeoxythymidine.
**Sulfolobus acidocaldarius** and **Methanopyrus kandleri** reverse gyrases (16, 17). However, in the presence of Mg²⁺/H₁₁₀₀₁ or Mn²⁺/H₁₁₀₀₁, the protein remains soluble down to 200 mM NaCl. In the presence of excess DNA, no salt is needed to keep the protein in solution.

*A. fulgidus* reverse gyrase shows a marked dependence on temperature for carrying out its positive supercoiling reaction (Fig. 1), similar to the **S. acidocaldarius** and **Desulfurococcus amylolyticus** enzymes (10, 18, 19). Numerous studies indicate that this temperature dependence reflects the need for single-stranded regions in the plasmid substrate (7, 9, 20). At elevated temperature, the DNA strands of a duplex tend to separate locally, especially when the DNA is negatively supercoiled, and this allows reverse gyrase to gain access to the DNA. To understand this preference for single-stranded DNA in more detail, the binding of *A. fulgidus* reverse gyrase to various types of DNA substrates was studied in bandshift assays.

**DNA Binding Behavior**—Fig. 2A shows the binding of reverse gyrase to single-stranded poly(dT) oligonucleotides. At least three protein-DNA species are visible: two bands that penetrate into the gel (marked with arrows in Fig. 2A) and a species that remains trapped in the wells. The nucleophilic Tyr-809 in the active site of the topoisomerase domain is indicated in space-filling representation. This image was generated with Molscript (27) and rendered with POV-Ray (www.povray.org).

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interacting protein and DNA. Reduced to 20 bases, however, complex formation is very low.

S. acidocaldarius (d)UTP was detected (7). In contrast, the positive supercoiling is observed in the absence of ATP. This correlated with the fact that (d)ATP was the only (d)NTP (d)NTPs allowed the enzyme to relax DNA but not supercoil it. To determine the minimal duplex region necessary for efficient relaxation (data not shown), suggesting that the duplex region contacted by the protein is small.

Because these bandshift studies were performed at room temperature, they indicate that the protein does not require elevated temperature for DNA binding per se. Together with previous studies using plasmids (7, 9, 20), these experiments with oligonucleotides further support the idea that the temperature dependence of the positive supercoiling reaction reflects the need for reverse gyrase to bind to locally denatured regions of the substrate.

Selectivity for Nucleotide Cofactor—Reverse gyrase is unique among type I topoisomerases in its requirement for nucleotide hydrolysis, which it couples to the overwinding of DNA. In the following discussion, “(d)NTP” refers to a dNTP, together with the corresponding NTP. Previous work found that, of the four natural NTPs and dNTPs, only (d)ATP could support positive supercoiling by S. acidocaldarius reverse gyrase (7). The other (d)NTPs allowed the enzyme to relax DNA but not supercoil it. This correlated with the fact that (d)ATP was the only (d)NTP hydrolyzed by the enzyme; no hydrolysis of (d)GTP, (d)CTP, or (d)UTP was detected (7). In contrast, the positive supercoiling activity of the A. fulgidus enzyme can be powered by all four standard (d)NTPs (Fig. 4A). Maximal positive supercoiling occurs at a concentration around 10 μM for (d)ATP, as reported for S. acidocaldarius reverse gyrase (3), contrasting with about 100 μM for the other (d)NTPs. The results observed for the S. acidocaldarius enzyme (7) could be reproduced by repeating the assays in Fig. 4A using less protein; saturating amounts of (d)ATP supported a modest degree of positive supercoiling, whereas the other nucleotides supported only quantitative relaxation (data not shown).

The selectivity of reverse gyrase for (d)ATP was confirmed in hydrolysis assays performed in the presence of single-stranded DNA (Fig. 4B). The enzyme hydrolyzes ATP and dATP with a turnover number of −0.1 s−1 (corresponding to a specific activity of 1 nmol s−1 mg−1), whereas it hydrolyzes CTP and dTTP at a rate only slightly above background. The crystal structure of the enzyme with the nonhydrolyzable ATP analog adenylylimidodiphosphate (ADPNP) suggests that the selectivity for (d)ATP stems from an interaction between the N6 amino group of the adenine ring with the Oε1 atom of the invariant residue Gln-61 (12).

The N-terminal Domain Represses Topoisomerase Activity in the Absence of Nucleotide—The homology between the topoisomerase domain of reverse gyrase and topoisomerase I suggests that reverse gyrase, like its E. coli homologue, might act as a DNA relaxing enzyme in the absence of nucleotide. However, no significant topoisomerization by reverse gyrase is observed without ATP (Fig. 5A), even at high molar ratios of protein to DNA (P/D). The topoisomerase domain expressed on its own does weakly relax DNA in the absence of ATP (Fig. 5D), as reported for the topoisomerase domain of S. acidocaldarius reverse gyrase (21). These results indicate that the N-terminal domain represses the activity of the topoisomerase domain in the absence of nucleotide. Nucleotide binding is sufficient to “de-repress” topoisomerase activity, because reverse gyrase relaxes DNA at low P/D ratios in the presence of ADPNP (Fig. 5B).

The Latch Communicates Nucleotide Binding to the Topoisomerase Domain—How might the N-terminal domain repress topoisomerase activity? The reverse gyrase crystal structure revealed that subdomain H3 of the N-terminal module closes like a latch over the topoisomerase gate, preventing the gate opening thought to be required for strand passage and topoisomerization (22). We proposed that binding of both nucleotide and DNA cause the latch to pull away from the topoisomerase domain (12). To test this hypothesis, reverse gyrase deletion mutants were studied. Mutants were constructed lacking essentially the entire latch (Latch, residues 360–418) or just the part interacting with the topoisomerase in the crystal structure (Δ370, residues 370–381) (Fig. 1D).

The supercoiling activity of the ΔLatch mutant is shown in Fig. 6. The enzyme, as predicted by our model, shows weak relaxation activity in the absence of nucleotide. Hence the latch is critical for the repression of topoisomerase activity by the N-terminal domain. At higher P/D ratios, nicked (form II) and linearized DNA molecules accumulate; this effect is addressed later in this section. The Δ370 mutant behaves similarly to ΔLatch (Fig. 7), suggesting that topoisomerase repression is mediated principally in the region of residues 370–381. Both mutants retain positive supercoiling activity in the presence of ATP, indicating that the latch is not required for coupling nucleotide hydrolysis to positive supercoiling. The latch is also dispensable for efficient substrate binding, because both mutants behave similarly to the full-length protein in bandshift assays like those in Fig. 3 (data not shown).

That deletion of residues 370–381 allows “de-repression” of the topoisomerase activity suggests that the interface between the latch and topoisomerase domain in the crystal structure is functionally relevant. The sides of this interface are formed chiefly by residues 370–381 of the latch and residues 857–866 of subdomain T3 (Fig. 1D). To examine the other side of the putative interface, a mutant lacking residues 857–866 (Δ857) was produced and checked for supercoiling activity (Fig. 8).

Like the full-length enzyme, the Δ857 mutant does not show significant relaxation activity in the absence of nucleotide. This suggests that the latch need not interact directly with residues 857–866 to repress the topoisomerase module.

When the ΔLatch, Δ370, and Δ857 mutants are used in excess in the supercoiling assay, especially in the absence of nucleotide hydrolysis, nicked or linearized DNA products accumulate.

FIG. 2. Reverse gyrase binding to single-stranded and duplex DNA. Titrations were performed with single-stranded poly(dT) oligonucleotides (A) or with a 52-bp duplex (B). The lanes correspond to the addition of 0, 1.5, 3.0, 5.8, and 11.8 ng of reverse gyrase. The “W” indicates the position of the wells; the arrows mark protein-DNA complexes.
It is possible that the form II and III bands reflect nuclease contamination, but this seems unlikely for two reasons. The bands accumulate to a significant extent only with the mutant enzymes, yet all the proteins in this study were purified from the same E. coli strain in a similar manner and to a similar degree of purity (Fig. 1C). Moreover, the form II and III products require the formation of a covalent intermediate of reverse gyrase and DNA, because they are not observed with a Δ latch mutant carrying a Phe in place of the active-site Tyr-809 (Fig. 9). These products may therefore reflect formation of cleavage complex that does not undergo religation but instead suffers hydrolytic attack. Normally, topoisomerases perform

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the religation step efficiently such that DNA hydrolysis by the solvent is not a competing reaction, but the reverse gyrase mutants may be compromised in the religation step, allowing hydrolysis to become observable. Further studies are needed to test this hypothesis.

The Latch May Be Involved in Coupling Nucleotide Hydrolysis to Positive Supercoiling—That removal of the latch allows nucleotide-independent relaxation indicates that the latch communicates nucleotide binding to the topoisomerase domain. Does it also communicate nucleotide hydrolysis? It is not required for this, because the mutants still positively supercoil DNA in the presence of ATP, but it may still play a role. To investigate this question, ATP hydrolysis activity of the full-length and mutant proteins was assessed in the presence of single-stranded DNA. This DNA acts as an effector to stimulate ATP hydrolysis but not as a bona fide supercoiling substrate like a plasmid. Therefore any rate differences between the proteins should reflect differences in the rate of hydrolysis rather than differences in rates of supercoiling.

Relative to the full-length enzyme, the Δlatch mutant shows an enhancement in ATPase rate of ~13-fold, and the Δ370 and Δ857 mutants an enhancement of ~3-fold (Fig. 10). Thus per-
turbation in the region of the latch affects the rate of DNA-dependent ATPase activity, raising the possibility that hydrolysis depends on the conformational state of the latch. In fact, the faster ATPase rate of the mutants may mean that interaction of the latch with the topoisomerase domain is required for keeping hydrolysis tightly coupled to supercoiling. The rate enhancement with the Δ857 mutant may mean that this region of the enzyme does interact with the latch during catalysis, although in a way different from the interaction required for topoisomerase repression (see above).

**Nucleotide Hydrolysis Forces Unidirectional Strand Passage**—It is puzzling that above a P/D of ~0.5–2 in the presence of ADP, reverse gyrase switches from giving partially relaxed products to giving negatively supercoiled ones (see the B panels of Figs. 5–7). This corresponds to a switch in the direction of strand passage from increasing linking number to decreasing linking number. Such a switch is never observed in the presence of ATP (see the C panels of Figs. 5–8). This may indicate that, in the absence of hydrolysis, the superhelical density of the DNA can influence the direction in which reverse gyrase performs strand passage. This is an important possibility to explore, because it bears directly on the question of why reverse gyrase always performs strand passage toward increasing linking number in the presence of ATP (3). From the crystal structure we proposed a model of controlled strand passage (12), stipulating that the unidirectionality results from the fact that reverse gyrase binds the DNA strands in a specific manner to allow strand passage in only one direction. A similar model had been proposed earlier based on biochemical studies (20). A contrasting explanation for the unidirectionality is provided by the “domain model” (6). Reverse gyrase is known to unwind DNA locally upon binding (9, 21), and the domain model stipulates that the enzyme can isolate this unwound DNA topologically from the rest of the substrate. In DNA that is topologically constrained as a whole, such as a plasmid, the creation of unwound DNA at the protein binding site creates compensatory overwinding elsewhere. If reverse gyrase increases the linking number of the unwound domain without affecting the overwound domain, the net result is to introduce positive supercoils into the plasmid. Thus, the domain model attributes the enzyme’s unidirectionality to its specificity for performing strand passage in an unwound domain. Up to this point, the controlled strand passage model is compatible with the domain model. However, unlike the former, the latter model allows for the possibility that the topoisomerase domain could perform strand passage in either direction. So what if reverse gyrase were forced to bind to an overwound domain of DNA: would it increase or decrease the linking number?

To examine this question, the following experiment was performed. A small amount of reverse gyrase was incubated in a standard supercoiling assay at 80 °C in the presence of ADP to yield partially relaxed product (Fig. 11). This was the end point of the reaction, because extending the incubation beyond 30 min did not lead to further relaxation (data not shown). The reaction was cooled on ice, causing the dissociation of protein from the plasmid (9). Then an excess of inactive reverse gyrase mutant was added to the reaction, and the 80 °C incubation resumed. The inactive mutant carries a Phe in place of the catalytic Tyr-809, reducing DNA cleavage to very low levels (data not shown), without affecting the enzyme’s ability to bind and unwind DNA (21). The inactive reverse gyrase should bind to the partially relaxed plasmid and create overwinding elsewhere, such that the active reverse gyrase would rebind, on average, to overwound regions.

If the active molecules performed strand passage toward increasing linking number, the DNA would remain as it was at the end of the first 80 °C incubation or perhaps become slightly more relaxed. If instead the active molecules performed strand passage in the opposite direction, the DNA would become more negatively supercoiled. Fig. 11 shows the latter result, indicating that the active reverse gyrase molecules performed strand passage toward increasing linking number before the addition of inactive enzyme, and toward decreasing linking number afterward.

The ability of reverse gyrase to work bidirectionally without ATP is consistent with the domain model but not with the controlled strand passage model. The two models can be reconciled if we assume that the enzyme binds DNA as described by the domain model, and nucleotide hydrolysis then creates a situation of controlled strand passage.

**DISCUSSION**

The bandshift assays described here show that reverse gyrase binds to single-stranded, duplex, and mixed DNA substrates. The enzyme senses both the single- and double-stranded parts of mixed substrates, as does topoisomerase I. In fact, topoisomerase I prefers to cleave near the single-strand/double-strand DNA junction (23). Cleavage is most efficient when the single-stranded overhang is at least 26 bases long (23), similar to the minimum overhang length of ~30 bases seen here.

Reverse gyrase combines a topoisomerase domain with the same ATPase domain used by helicases. Not surprisingly, like a number of helicases, reverse gyrase can power its reaction using all of the four standard NTPs and dNTPs, although it prefers ATP and dATP. T7 DNA helicase can hydrolyze all of the standard dNTPs and NTPs but is selective for dTTP (24). PcrA helicase from *Bacillus stearothermophilus* works equally well with all of the standard dNTPs and NTPs except dTTP (25). In contrast, the NS3 helicase from hepatitis C virus can hydrolyze all of the standard nucleotides, but only hydrolysis of ATP and dATP is strongly stimulated in the presence of RNA substrate (26).

The N-terminal domain not only confers on the topoisomerase module the ability to positively supercoil DNA using ATP but also represses its activity in the absence of nucleotide. The latch subdomain, specifically the region around residues 370–381, mediates this repression. The fact that reverse gyrase

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**Fig. 9. Accumulation of nicked and linear DNA by the ΔLatch mutant requires formation of the cleavage complex.** ΔLatch and ΔLatch(Y809F) mutants were overexpressed and purified in parallel and brought to the same concentration. The proteins were analyzed in parallel in supercoiling assays at P/D = 20. The far left panel shows a control reaction incubated in the absence of protein.
relaxes DNA in the presence of ADPNP, yet the latch remains in the “closed” position in the ADPNP cocrystal structure, provides further evidence that latch opening depends on binding of both DNA and nucleotide, as suggested from the precedent of PcrA helicase (12).

Even without the latch, reverse gyrase can positively supercoil DNA in the presence of ATP, but it shows a much higher rate of DNA-dependent ATP hydrolysis than when the latch is present. Therefore the latch may be involved in, but not required for, coupling nucleotide hydrolysis to topoisomerization. Presumably the coupling is primarily mediated by other regions of the protein. The crystal structure revealed the possibility of extensive interactions between subdomain H1 in the N-terminal domain and subdomain T1 in the topoisomerase domain (12). These two regions are connected covalently by a disulfide bond between Cys-35 and Cys-650, and noncovalently in a β-sheet where three of the strands come from the first –30 residues of the N terminus and the fourth strand comprises T1 residues 575–579. Perhaps movements of H1 upon nucleotide binding and hydrolysis are communicated to the topoisomerase via T1. Within T1 lies the zinc finger motif, which we predict to be important for binding the DNA and perhaps helping to unwind it (12).

The crystal structure suggested that the interaction between the latch and the topoisomerase domain occurs principally through residues 370–381 of the former and residues 857–866 of the latter. However, deletion of residues 857–866 does not destroy the ability of the latch to repress topoisomerase activity. The interface between the latch and topoisomerase domain in the crystal structure should therefore be interpreted with caution. It may be that the latch interacts with the topoisomerase gate in multiple ways during catalysis, and the crystal structure captures only one of these modes. It could be that for topoisomerase repression, the latch and topoisomerase domain interact largely independently of residues 857–866 but that for coupling of hydrolysis to supercoiling, the interaction mode does involve these residues. This could explain why the Δ857 mutant shows an enhancement in ATPase rate identical to that seen with the Δ370 mutant. The conformational differences in the latch between the structures of the apoenzyme and the enzyme complexed with ADPNP are consistent with the possibility of multiple interaction modes (12).

Like topoisomerase I (11), reverse gyrase can perform strand passage in either direction. Nucleotide hydrolysis, however, forces reverse gyrase to work only toward increasing linking number. Such a fundamental role for hydrolysis was not predicted by mechanistic models invoking DNA domains (6) or controlled strand passage (12, 20). I postulate that each of the two types of model describes a different stage in the reverse gyrase mechanism and nucleotide hydrolysis drives the transition between the stages. As stipulated in the domain model, and supported by the bandshift experiments described here, the enzyme binds initially to a region of single-stranded DNA, while also making contact with the duplex regions upstream and downstream. These contact points with the duplex allow the enzyme to unwind the DNA locally (9, 21), partitioning it into an unwound domain at the binding site and an overwound domain elsewhere (6). When both nucleotide and DNA are bound, the latch subdomain releases its grip on the topoisomerase gate, allowing strand passage to occur. Nucleotide hydrolysis commits the enzyme to perform controlled strand passage toward increasing linking number, regardless of the superhelical density of the substrate.

A critical question is at what stage(s) hydrolysis occurs in the
mechanism. Does hydrolysis precede strand passage, follow it, or both? Answering this question will require working out how many ATP molecules are consumed per catalytic cycle. Knowing this stoichiometry will then permit the identification of mutants impaired in the coupling between hydrolysis and supercoiling. Such studies should reveal whether reverse gyrase uses nucleotide hydrolysis in a similar way as DNA gyrase, the only other topoisomerase known to supercoil DNA. Perhaps the most difficult, yet most intriguing, question is how nucleotide hydrolysis forces reverse gyrase to work unidirectionally. For this a crystal structure of the enzyme complexed with both DNA and nucleotide will be essential.

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