Locking the DNA Gate of DNA Gyrase: Investigating the Effects on DNA Cleavage and ATP Hydrolysis†

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ABSTRACT: Supercoiling by DNA gyrase involves the passage of one segment of double-stranded DNA through another. This requires a DNA duplex to be cleaved and the broken ends separated by at least 20 Å. This is accomplished by the opening of a dimer interface, termed the DNA gate, which is covalently attached to the broken ends of the DNA. After strand passage, the DNA gate closes allowing the reunion of the broken ends. We have cross-linked the DNA gate of gyrase using cysteine cross-linking to block gate opening. We show that this locked gate mutant can bind quinolone drugs and perform DNA cleavage. However, locking the DNA gate prevents strand passage and the ability of DNA to stimulate ATP hydrolysis. We discuss the mechanistic implications of these results.

DNA gyrase is a molecular machine comprising of a series of protein gates (*1*−*3*). These gates open and close guiding a segment of DNA through a double-stranded break elsewhere within the same DNA duplex. Strand passage can be ATP-dependent or -independent, resulting in the introduction or removal of negative supercoils, respectively.[1]

Escherichia coli DNA gyrase is active as an A_2B_2 tetramer, composed of two pairs of subunits, GyrA and GyrB (*4*). The subunits can be subdivided into domains each with specific functions (Figure 1A). GyrB (90 kDa) contains an N-terminal 43 kDa domain (GyrB43) and a C-terminal 47 kDa domain (GyrB47). The crystal structure of GyrB43 complexed with a nonhydrolysable ATP analogue, ADPNP, has been solved and reveals a GyrB43 dimer (*5*). The N-terminal subdomains of GyrB43 both bind a molecule of ADPNP and form extensive dimer contacts (Figure 1A). The C-terminal subdomains form the sides of a 20 Å wide cavity within the dimer, proposed to bind a DNA duplex (*5*, *6*). This structure constitutes an ATP-operated clamp within gyrase that closes upon the binding of ATP or ADPNP and reopens upon ATP hydrolysis (*1*). At the C-terminus of GyrB, a pair of GyrB47 domains is proposed to interact with a GyrA dimer, forming a second DNA binding channel (*7*).

GyrA (97 kDa) contains an N-terminal 64 kDa domain (GyrA64) and a C-terminal 33 kDa domain (GyrA33). The crystal structure of a 59 kDa fragment of GyrA64 (GyrA59) has been solved (*8*), revealing a heart-shaped dimer with a 30 Å central cavity (Figure 1B). A pair of N-terminal subdomains forms a dimer interface known as the "DNA gate" (Figure 1A). This gate is proposed to bind a segment of DNA, perform double-stranded cleavage, and then pull the broken ends of the DNA apart. A pair of C-terminal subdomains also form extensive dimer contacts (Figure 1A); this dimer interface is also proposed to open and close, constituting the "exit gate" of gyrase (*3*). At the C-terminus of GyrA, the GyrA33 domains are proposed to induce the wrapping of DNA around gyrase in a positive superhelical sense (*4*).

Both domains within GyrA have been cloned and are available as separate gene products (*9*, *10*). A functional gyrase can be reconstituted by mixing GyrA33 and GyrA64 with GyrB, to form $A64_2A33_2B_2$ (*10*). Alternatively, a truncated version of gyrase, lacking the ability to wrap DNA, can be formed by mixing GyrA64 and GyrB to generate the $A64_2B_2$ complex (*11*). This complex is unable to perform the normal supercoiling and ATP-independent relaxation activities of gyrase, due to the absence of GyrA33. However, unlike full-length gyrase, this complex binds preferentially to supercoiled DNA and can perform ATP-dependent relaxation.

Supercoiling by DNA gyrase involves the cleavage of a short segment of DNA (termed the "gate" or G segment) bound across the DNA gate. The DNA contiguous with the G segment is wrapped around gyrase via the GyrA33 domains, presenting at least one segment of DNA (termed the "transported" or T segment) to the open ATP-operated clamp (*11*). Upon ATP binding, the ATP-operated clamp closes capturing the T segment (*12*). The DNA gate opens, pulling the broken ends of the G segment apart, and facilitating the passage of the T segment through the gap. To complete the strand-passage cycle, the G segment is

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[1] Abbreviations: ADPNP, 5′-adenylyl β,γ-imidodiphosphate; GyrA, DNA gyrase A protein; GyrB, DNA gyrase B protein; GyrB43, N-terminal 43 kDa domain of GyrB.; GyrB47, C-terminal 47 kDa domain of GyrB.; GyrA64, N-terminal 64 kDa domain of GyrA.; GyrA59, N-terminal 59 kDa fragment of GyrA.; GyrA33, C-terminal 33 kDa domain of GyrA.; A_2B_2, DNA gyrase tetramer; $A64_2A33_2B_2$, DNA gyrase reconstituted from GyrA64, GyrA33 and GyrB.; $A64_2B_2$, truncated version of DNA gyrase reconstituted from GyrA64 and GyrB.; DTT, dithiothreitol; PK/LDH, pyruvate kinase and lactate dehydrogenase; PEP, phosphoenol pyruvate; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; CuP, copper^{II}(1,10-phenanthroline)$_3$; PDM, *N*,*N*′-*o*-phenylenedimaleimide; topo II, DNA topoisomerase II.; bp, base pairs; DiS, disulphide bond.

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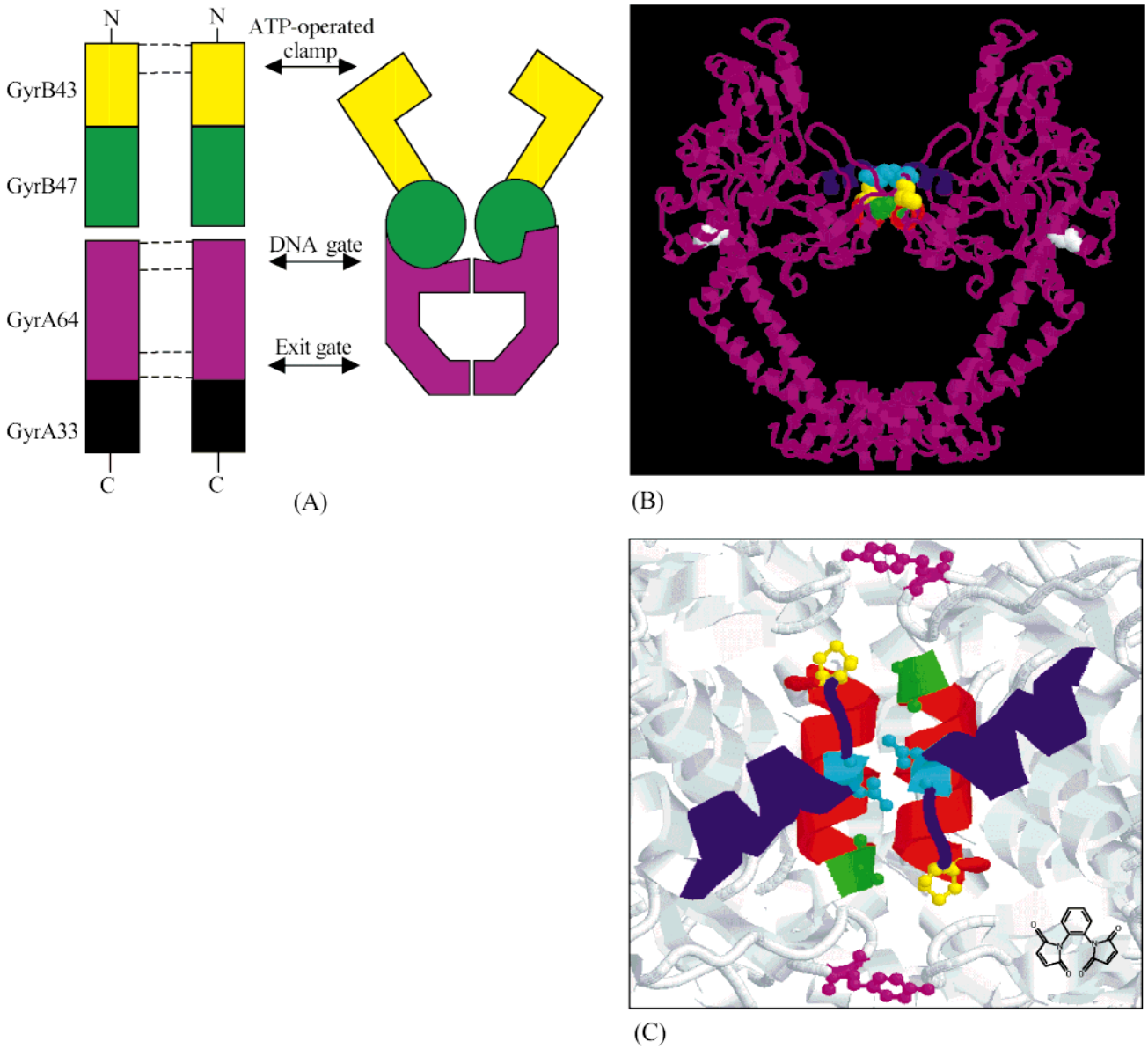

FIGURE 1: (A) Diagrammatic representation of the domain organization within the sequence and structure of DNA gyrase. Dashed lines enclose regions that form dimer contacts within the structure. Double-headed arrows identify the protein gates. The GyrA33 domains have been omitted from the structural representation since their precise location is unknown. (B) Ribbon representation of the crystal structure of GyrA59 (*8*). The location of the Asp^{82}, Pro^{79}, Ala^{67}, and Cys^{194} are shown as cyan, yellow, green, and white space-filling representations. The pair of antiparallel α-helices and the two recognition helices are shown in red and dark blue, respectively. (C) Detailed top view of the DNA gate. The location of the mutated residues and Tyr^{122} (magenta) are shown in ball-and-stick representations. The structure of PDM is represented approximately to scale (inset).

religated upon closure of the DNA gate, the T segment is expelled via opening of the exit gate, and the ATP-operated clamp re-opens upon ATP hydrolysis. The removal of negative supercoils by gyrase (DNA relaxation) is thought to be the reversal of supercoiling (*3*) and can proceed in the absence of ATP. In vitro, the exit gate can open allowing a T segment to enter the interior of the enzyme. The DNA gate can also open, pulling the broken ends of the G segment apart, and allowing the T segment to pass through. The T segment is then free to leave through the open ATP-operated clamp.

DNA cleavage is an essential part of the strand passage mechanism of gyrase. The hydroxyl groups of a pair of tyrosine residues, one on either side of the DNA gate, attack a pair of 5′ phosphodiester bonds within the G segment, that are 4 bp apart (*13*, *14*). This leads to the breakage of both DNA strands and the formation of a pair of 5′ phosphotyrosine linkages between each broken end of the DNA and either side of the DNA gate. Hence, opening of the DNA gate pulls the broken ends apart. Double-stranded cleavage is thought to be transient, since only low levels are revealed upon enzyme denaturation with SDS. Despite this, there are a number of ways to stabilize the cleaved conformation. Quinolone drugs target DNA gyrase and are thought to bind to the G-segment bound DNA gate, stabilizing DNA cleavage. Incubation of the drug with gyrase and DNA, in vitro, reveals extensive double-stranded cleavage upon the addition of SDS (*15*, *16*). In vivo, formation of the ternary complex is thought to block the translocation of enzymes along the DNA, ultimately leading to cell death (*17*, *18*). Quinolone-stabilized cleavage can also be performed by the truncated version of gyrase ($A64_2B_2$) lacking the DNA-wrapping

domains (*11*). This suggests that quinolone action does not necessarily require the presence of GyrA33 or the wrapping of the DNA around the enzyme. DNA cleavage by gyrase can also be revealed, in vitro, by incubation with Ca^{2+} or ADPNP followed by subsequent treatment with SDS (*19*, *20*). Unlike quinolone- and Ca^{2+}-stabilized cleavage, the extent of ADPNP-induced cleavage is determined by the topological state of the DNA, most cleavage being observed with negatively supercoiled substrates.

DNA gyrase has an intrinsic rate of ATP hydrolysis that is increased in the presence of DNA (*21*). Evidence suggests that the DNA must be of sufficient length to facilitate both DNA wrapping and T-segment presentation to the ATP-operated clamp. A mutation on the inner surface of the ATP-operated clamp of GyrB (Arg^{286} to Gln) has been shown to block the DNA stimulation of ATP hydrolysis (*6*). Together these results imply that the wrapped T segment must be captured by the ATP-operated clamp to enhance the rate of ATP hydrolysis. DNA cleavage activity is also required to stimulate ATP hydrolysis. Gyrase containing a mutation within the active site of GyrA (Tyr^{122} to Ser) is unable to cleave DNA (*22*) and is also unable to exhibit DNA-stimulated ATP hydrolysis (*20*).

In previous work, we have exploited the technique of cysteine cross-linking to investigate the role of the exit gate in DNA gyrase (*3*). Using similar techniques, we have now cross-linked the DNA gate. Cysteine residues have been introduced on either side of the DNA gate and have been subsequently cross-linked using disulfide bonds or a cysteine-specific cross-linking reagent. We report that locking the DNA gate does not block DNA binding or G-segment cleavage; however, strand passage and the DNA-stimulation of ATP hydrolysis is blocked. We also confirm that quinolones can bind to gyrase when the DNA gate is closed, stabilizing double-stranded cleavage. This suggests that DNA gate opening is not required for quinolone action.

EXPERIMENTAL PROCEDURES

Materials. 1,10 phenanthroline, trypsin and PDM were purchased from Sigma, DTT was from Melford Laboratories, and *E. coli* MAX Efficiency DH5αF′IQ competent cells were from GibcoBRL. DNA oligonucleotides used for site-directed mutagenesis and sequencing were obtained from PNACL (Leicester University). Negatively supercoiled and relaxed forms of plasmid pBR322 were gifts of A. J. Howells (University of Leicester). GyrB (gift of A. J. Howells, University of Leicester), GyrA33 and GyrA64 were purified as described previously (*9*, *10*, *23*), except that GyrA64 was expressed in *E. coli* DH5αF′IQ cells.

Site-Directed Mutagenesis. Amino acid changes within GyrA64 were performed by site-directed mutagenesis of plasmid pRJR242 (*9*), following the QuikChange protocol (Stratagene). Successful mutagenesis was confirmed by DNA sequence analysis performed by PNACL (Leicester University).

Protein Cross-Linking. Disulfide bonds were formed across the DNA gate in the absence of DTT with or without the addition of copper(II) phenanthroline and reduced in the presence of DTT as described previously (*3*). PDM cross-linking was also performed in the absence of DTT (*3*). Cross-linked GyrA64 was separated from uncross-linked GyrA64 and GyrA contamination using preparative SDS-PAGE as previously described (*3*), except that electro-elution was performed using a Mini Whole Gel Eluter (Biorad). After separating the cross-linked and uncross-linked GyrA64 using SDS-PAGE, all the bands were electro-eluted simultaneously into 50 mM Tris and 25 mM boric acid and collected as separate fractions. Hence, staining and excision of the cross-linked band was not required. In addition, electro-eluting into 50 mM Tris and 25 mM boric acid removes excess SDS, omitting the need to dialyze overnight against 2 M urea.

Enzyme Assays. Supercoiling, relaxation, quinolone- and Ca^{2+}-stabilized cleavage, and ATPase assays were performed as previously described (*3*). ADPNP-induced cleavage assays were performed as described previously (*20*). DNA binding was assessed by incubating DNA gyrase with an end-labeled 198 bp fragment of pBR322 (*12*) in 5 mM $MgCl_2$, 30 mM KCl, 1 mM EDTA, 35 mM Tris·HCl (pH 7.5), 6.5% glycerol, and 4 mM DTT at 25 °C for 1 h. Samples were loaded onto a 5% polyacrylamide gel (37.5:1 acrylamide/bis) containing 90 mM Tris, 90 mM boric acid, and 5 mM $MgCl_2$. Gels were run in the same buffer for 45 min at 150 V, transferred to Whatmann 3MM paper, dried under vacuum at 65 °C, and exposed to a PhosphorImager screen (Molecular Dynamics) overnight. Gel images were quantitated on a PhosphorImager (Molecular Dynamics) using Imagequant software. In all procedures described above involving disulfide cross-linked protein, DTT was omitted. If appropriate, DTT was also removed from buffers containing GyrB and GyrA33 by gel filtration (Nick spin columns, Pharmacia) immediately prior to use.

RESULTS

Design and Properties of the DNA Gate Mutants. In previous work, the exit gate of DNA gyrase was cross-linked using a disulfide bond or cysteine-specific cross-linking reagents (*3*). This technique has now been successfully applied to the DNA gate of gyrase. The high-resolution crystal structure of the 59 kDa fragment of GyrA has been examined in order to design DNA gate mutants for cross-linking (*8*). Pairs of amino acids separated by less than 6 Å between respective *beta*-carbons, ($C\beta-C\beta$ separation) on either side of the DNA gate, were considered for replacement by cysteine residues. GyrA contains four native cysteine residues that are known to participate in unwanted cross-linking, in conditions that favor disulfide bond formation (*3*). To overcome this problem, the novel cysteines were introduced into the N-terminal 64 kDa domain of GyrA (GyrA64). This domain contains a single native cysteine that was replaced with alanine (Cys^{194} to Ala) with no effect on supercoiling activity (*3*). Novel cysteine residues were introduced into this cysteine-less version of GyrA64. Replacement of Asp^{82} with Cys gave rise to a double mutant ($GyrA64^{C194A/D82C}$) that in the dimer, produced a single pair of cysteine residues in close proximity across the dimer interface ($C\beta$-$C\beta$ separation of ~5 Å, Figure 1, B and C). Replacement of Pro^{79} and Ala^{67} both with Cys gave rise to a triple mutant ($GyrA64^{C194A/P79C/A67C}$) that, in the dimer, produced two pairs of cysteine residues. The Cys^{67} and Cys^{79} residues on opposite sides of the dimer interface are located very close together ($C\beta-C\beta$ separation of ~5 Å, Figure 1, B and C). The GyrA64 mutants were reconstituted with GyrB

Table 1: Activities and Cross-Linking Efficiencies of Mutant Proteins

amino acids changed within GyrA64	C194A D82C	C194A P79C A67C
supercoiling activity[a]	50%	50%
ATP-independent relaxation activity[a]	10%	35%
ATP-dependent relaxation activity[b]	10%	25%
DNA binding affinity[a]	50%	50%
Ca^{2+}-stabilized cleavage activity[a]	10%	100%
quinolone-stabilized cleavage activity[b]	50%	10%
disulfide bond formation (−DTT)[c]	30%	50%
disulfide bond formation (+CuPhen)[c]	30%	95%
PDM cross-linking[c]	60%	90%

[a] Compared to wild-type $A64_2A33_2B_2$. [b] Compared to wild-type $A64_2B_2$. [c] Percentage of cross-linked dimer compared to total protein content determined by SDS−PAGE analysis.

with or without GyrA33 to form mutant versions of $A64_2A33_2B_2$ and $A64_2B_2$, respectively.

The two DNA gate mutants were tested for activity in the presence of 4 mM DTT, prior to cross-linking. In the absence of the DNA wrapping domains (GyrA33), $A64^{C194A/D82C}{}_2B_2$ exhibited a 10-fold reduction in ATP-dependent relaxation activity compared to wild-type (Table 1). This may reflect a reduction in G-segment binding. In contrast, $A64^{C194A/D82C}{}_2A33_2B_2$ exhibited only a 2-fold reduction in supercoiling activity and DNA binding compared to wild-type (Table 1). The adverse effects of poor G-segment binding may be compensated for by the more extensive DNA binding provided by the GyrA33 domains. $A64^{C194A/D82C}{}_2A33_2B_2$ also exhibits a 10-fold reduction in Ca^{2+}-stabilized cleavage and ATP-independent relaxation activity (Table 1), suggesting that the Asp^{82} to Cys mutation may also perturb DNA cleavage. Despite this, the proposed reduction in G-segment binding and cleavage is not reflected in the quinolone-stabilized cleavage activity, which is only reduced 2-fold (Table 1). However, this result is not unexpected since quinolones stabilize DNA binding to the complex (*24*). Taken together, these results suggest that the Asp^{82} to Cys mutation gives rise to an active form of gyrase, albeit with reduced G-segment binding and cleavage activity compared to wild-type.

In contrast, characterization of gyrase, containing the Pro^{79} to Cys and Ala^{67} to Cys mutations reflects a less significant reduction in G-segment binding and cleavage. $A64^{C194A/P79C/A67C}{}_2A33_2B_2$ exhibits full Ca^{2+}-stabilized cleavage, a 2-fold reduction in DNA binding, and a 2- and 3-fold reduction in supercoiling and ATP-independent relaxation, respectively (Table 1). In the absence of the DNA wrapping domains, $A64^{C194A/P79C/A67C}{}_2B_2$ exhibits a 4-fold reduction in ATP-dependent relaxation activity (Table 1). In contrast, quinolone-stabilized cleavage is reduced 10-fold compared to wild-type $A64_2B_2$ (Table 1), and is likely to reflect a reduction in quinolone binding. This was anticipated since one of the novel cysteines (Cys^{67}) is located at the same position as a quinolone resistance mutation (Ala^{67} to Ser) (*25*). Taken together, these results suggest that the combined Pro^{79} to Cys and Ala^{67} to Cys mutations give rise to an active form of gyrase, albeit with reduced quinolone-binding affinity.

Cross-Linking the DNA Gate of Gyrase using Disulfide Bond Formation or a Cysteine-Specific Cross-Linking Reagent. To promote disulfide bond formation, the reducing agent (DTT) was removed by gel filtration (Nick spin columns, Pharmacia) from buffers containing GyrA64. The formation of intersubunit disulfide bonds between the novel cysteines within GyrA64 was assessed using nonreducing SDS-PAGE. $GyrA64^{C194A/D82C}$ exhibited 30% disulfide cross-linking (Table 1). The use of the mild oxidizing agent, copper (II) phenanthroline, has been found to promote disulfide bond formation (*3*). However, mild oxidation of $GyrA64^{C194A/D82C}$ did not increase disulfide bond formation in this instance (Table 1). More efficient cross-linking was achieved using the cysteine-specific cross-linking reagent PDM (Table 1). PDM comprises two maleimide functional groups on adjacent carbons of a phenyl ring (Figure 1C) and reacts selectively and irreversibly with the thiol side chains of cysteine residues. In contrast, the cysteine-less mutant, $GyrA64^{C194A}$, exhibited no disulfide bond formation or PDM cross-linking (data not shown). This suggests that the observed cross-linking of $GyrA64^{C194A/D82C}$ involves the novel cysteine residues introduced on either side of the DNA gate.

Gel filtration experiments suggest that GyrA64 exists as a stable dimer in solution (data not shown). Hence, separation of cross-linked and uncross-linked GyrA64 dimers was not possible using native purification techniques. Hence, $GyrA64^{C194A/D82C}$ cross-linked using PDM was separated from uncross-linked GyrA64 using preparative SDS-PAGE (Figure 2A). The cross-linked band was excised and electro-eluted to extract the protein. The sample was then fully denatured in the presence of urea and allowed to refold following the removal of urea by dialysis. Similar gel purification and refolding of both $GyrA64^{wt}$ and uncross-linked $GyrA64^{C194A/D82C}$ did not affect supercoiling activity (data not shown).

In the absence of reducing agent, $GyrA64^{C194A/P79C/A67C}$ formed 50% disulfide cross-linked dimers, as assessed by nonreducing SDS-PAGE. However, upon mild oxidation with copper (II) phenanthroline 95% disulfide bond formation was observed (Table 1 and Figure 2C). Likewise, 90% cross-linking was observed upon incubation with the cysteine-specific cross-linking reagent PDM (Table 1 and Figure 2D). Further purification by preparative SDS-PAGE was not required due to the very high levels of cross-linking obtained.

Locking the DNA Gate Blocks Strand Passage. $A64^{C194A/P79C/A67C}{}_2A33_2B_2$ cross-linked via disulfide bonds or PDM exhibits a ~90% reduction in supercoiling activity (Table 2). The low level of supercoiling activity observed is most likely due to a population of uncross-linked GyrA64. $GyrA64^{C194A/D82C}$ that was gel-purified following PDM cross-linking, i.e., contains no uncross-linked GyrA64 contamination, exhibits complete abolition of supercoiling activity following reconstitution with GyrA33 and GyrB (Figure 2B and Table 2). These results confirm that the DNA gate must open to allow strand passage.

Quinolone-Stabilized Cleavage is Exhibited by Cross-Linked $A64_2B_2$. We have found that locking the DNA gate of $GyrA64^{C194A/P79C/A67C}$ via disulfide bond formation does not block quinolone-stabilized cleavage by $A64_2B_2$ (Figure 3A and Table 2). In contrast, locking the DNA gate with PDM does block this reaction (Figure 3A and Table 2). The low level of linear product observed is most likely due to the 10% un-cross-linked $GyrA64^{C194A/P79C/A67C}$ within the sample, as seen in Figure 2D. This finding may reflect the difference in size between the PDM cross-linker and a

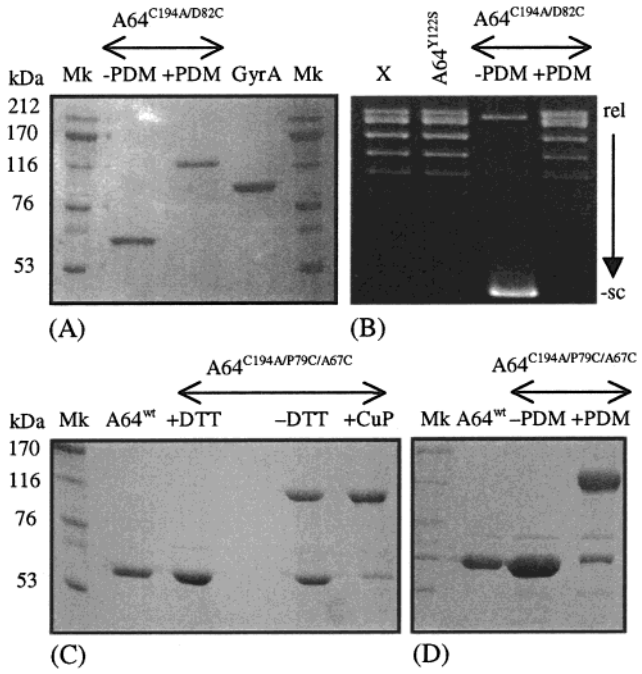

FIGURE 2: (A) GyrA64$^{C194A/D82C}$ (10 μM) was incubated in nonreducing conditions in the presence or absence of PDM (25 μM) for 1 h at 37 °C. The reaction was then quenched with DTT (4 mM). The cross-linked sample was purified as described in Experimental Procedures. Samples, including GyrA, were compared against a range of high molecular weight markers by SDS-PAGE. (B) Supercoiling assay using cross-linked gyrase. Relaxed pBR322 (7.2 nM) was incubated alone (X) or with GyrA33 (200 nM) and GyrB (300 nM), with the addition of GyrA64^{Y122S}, un-cross-linked GyrA64$^{C194A/D82C}$ or gel purified GyrA64$^{C194A/D82C}$ cross-linked with PDM, in the presence of 1.4 mM ATP at 37 °C for 1 h. Samples were analyzed on a 1% agarose gel. (C) GyrA64$^{P79C/A67C/C194A}$ (10 μM) was incubated in the presence of DTT (4 mM), the absence of DTT or the presence of copper (II) phenanthroline (125 μM) as indicated, for 1 h at 37 °C. Copper phenanthroline was quenched by the addition of EDTA to 1 mM. Samples, including GyrA64^{wt}, were compared against a range of high molecular weight markers (Mk) by nonreducing SDS-PAGE. (D) GyrA64$^{P79C/A67C/C194A}$ (10 μM) was incubated in nonreducing conditions in the presence or absence of PDM (25 μM) for 1 h at 37 °C. The reaction was then quenched with DTT (4 mM). Samples, including GyrA64^{wt}, were compared against a range of high molecular weight markers by SDS−PAGE.

disulfide bond. The presence of two relatively large PDM molecules may perturb the quinolone-binding pocket, resulting in the observed loss in quinolone-stabilized cleavage activity.

Gel-purified GyrA64$^{C194A/D82C}$, cross-linked with PDM and reconstituted with GyrB exhibits a slight reduction in quinolone-stabilized cleavage compared to the uncross-linked complex (Figure 3A and Table 2). In this location, the presence of a single PDM cross-linker does not seem to block quinolone action, as seen with the other mutant. To further investigate the effect of cross-linking the DNA gate, quinolone-stabilized cleavage by $A64_2B_2$ has been performed at a range of ciprofloxacin concentrations (Figure 4A). For both cross-linked and uncross-linked complex, the appearance of linear pBR322 increases as a function of ciprofloxacin concentration, exhibiting saturation at high drug concentrations. This resembles a simple binding isotherm. Since quinolone-stabilized cleavage is dependent upon quinolone binding, this can be interpreted in terms of the quinolone-binding affinity of the cross-linked and uncross-linked mutant. The drug-bound enzyme exhibits an apparent dissociation constant (K_d^{app}) of 3.8 ± 0.9 and 5.9 ± 0.9 μM, respectively, in the presence and absence of the PDM. This suggests that quinolone binding is not significantly perturbed by the presence of the cross-linker, indicating that quinolones can bind to the closed conformation of the DNA gate. The observed reduction in the maximal level of quinolone-stabilized cleavage in the presence of PDM may reflect a reduction in G-segment binding.

Ca^{2+}-Stabilized Cleavage by Gyrase Cross-Linked at the DNA Gate. GyrA64$^{C194A/P79C/A67C}$ reconstituted with GyrB exhibits Ca^{2+}-stabilized cleavage. However, cross-linking GyrA64$^{C194A/P79C/A67C}$ using disulfide bonds or PDM severely inhibits this reaction (Figure 3B and Table 2). In contrast, $A64^{C194A/D82C}{}_2B_2$ exhibits Ca^{2+}-stabilized cleavage in the presence or absence of the PDM cross-linker (Figure 3B and Table 2). This was further investigated by monitoring cleavage by full-length gyrase ($A64_2A33_2B_2$) at a range of $CaCl_2$ concentrations (Figure 4B). The extent of double-stranded cleavage increases with $CaCl_2$ concentration and approaches saturation. If these data are interpreted as a simple binding isotherm, the Ca^{2+}-bound gyrase complex exhibits a K_d^{app} of 1.7 ± 0.3 or 1.1 ± 0.2 mM in the presence or absence of PDM, respectively. These results show that opening of the DNA gate and separation of the broken ends of the G-segment are not required to stabilize DNA cleavage in the presence of Ca^{2+}. Moreover, although Ca^{2+} binding affinity is apparently reduced, the maximal level of double-stranded cleavage exhibited by gyrase is increased almost 2-fold in the presence of PDM (Figure 4B). This implies that the presence of the PDM cross-linker confers additional stabilization of DNA cleavage in the presence of Ca^{2+}.

ADPNP-Induced Cleavage is Exhibited by Cross-Linked Gyrase. DNA cleavage by gyrase, reconstituted with GyrA64$^{C194A/D82C}$, can be revealed upon the incubation with ADPNP and subsequent denaturation with SDS (Figure 5 and Table 2). Likewise, PDM cross-linked gyrase also exhibits an enhanced level of cleavage in the presence of ADPNP. It has been proposed that ADPNP-induced cleavage reflects T-segment capture by the ATP-operated clamp (*20*). Unexpectedly, a low level of cleavage by cross-linked gyrase can be observed upon SDS denaturation in the absence of ADPNP (Figure 5). This suggests that the presence of the cross-linker perturbs the cleavage-religation equilibrium in favor of DNA cleavage. A similar effect was observed by cross-linked gyrase when $MgCl_2$ was replaced by $CaCl_2$, as previously shown in Figure 4B.

Cross-Linking the DNA Gate Blocks DNA-Stimulated ATP Hydrolysis. The rate of ATP hydrolysis has been measured for $A64_2A33_2B_2$ in the presence and absence of DNA (Figure 6 and Table 2). Wild-type gyrase exhibits an ~3-fold stimulation of ATP hydrolysis in the presence of DNA. Gyrase reconstituted with GyrA64$^{C194A/D82C}$ exhibits slightly less DNA stimulation, perhaps reflecting a reduction in DNA binding. In contrast, gyrase reconstituted with GyrA64$^{C194A/D82C}$ that has been cross-linked with PDM does not exhibit a significant increase in ATPase rate upon the addition of DNA. A similar result is obtained for the active site mutant of gyrase reconstituted with GyrA64^{Y122S} (Figure 6).

Table 2: Properties of Cross-Linked Proteins

location of novel cysteines	D82C	P79C/A67C	P79C/A67C
type of cross-linker	PDM	PDM	DiS
SDS−PAGE purified	Yes	No	No
supercoiling activity[a]	None	10%	10%
quinolone-stabilized cleavage by $A64_2B_2$	Yes	No	Yes
Ca^{2+}-stabilized cleavage by $A64_2B_2$	Yes	No	No
ADPNP-induced cleavage by $A64_2A33_2B_2$	Yes	NT[b]	NT
DNA-stimulated ATP hydrolysis by $A64_2A33_2B_2$	No	NT	NT

[a] Compared to uncross-linked mutant $A64_2A33_2B_2$. [b] Not tested.

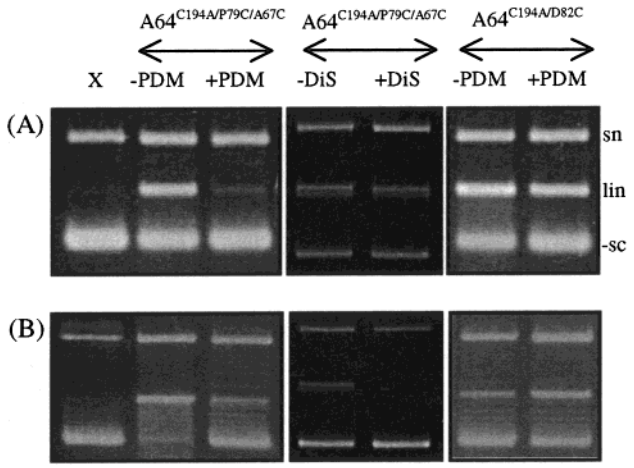

FIGURE 3: Quinolone- and Ca^{2+}-stabilized cleavage by cross-linked $A64_2B_2$. Negatively supercoiled pBR322 (7.2 nM) was incubated alone (X) or with GyrB (180 nM) and disulfide cross-linked $GyrA64^{C194A/P79C/A67C}$ (reduced or nonreduced; 180 nM) or PDM cross-linked or un-cross-linked $GyrA64^{C194A/P79C/A67C}$ or $GyrA64^{C194A/D82C}$ in the presence of (A) ciprofloxacin (50 μM) or (B) $CaCl_2$ (4 mM) for 1 h at 37 °C. Reactions were terminated upon incubation with SDS (0.2%) and proteinase K (0.1 mg/mL) for 30 min at 37 °C. Samples were analyzed on a 1% agarose gel (sn: singly nicked, lin: linear, -sc: negatively supercoiled). Note: The low level of relaxation exhibited in the presence of Ca^{2+} by $A64^{C194A/P79C/A67C}{}_2B_2$ cross-linked with PDM can be attributed to GyrA contamination of GyrB (data not shown). The observed doublets of linear DNA are a photographic artifact.

DISCUSSION

DNA gyrase catalyses the breakage of a DNA duplex and the passage of a second duplex through the break. The DNA gate of gyrase opens during the strand passage cycle, pulling the broken ends of one DNA duplex apart, allowing the second DNA duplex to pass through. We have investigated the opening and closing of the DNA gate by restricting it to the closed conformation. This has been achieved by the introduction and subsequent cross-linking of novel cysteine residues on either side of the DNA gate. The inability to open the DNA gate blocks strand passage and the DNA stimulation of ATP hydrolysis, although quinolone binding and quinolone- and Ca^{2+}-stabilized cleavage are still possible.

Location of the DNA Gate Cross-Links. Crystal structure information regarding the DNA gate is available (*8*), revealing a total buried surface area of ~1400 Å^2. The dimer interface is dominated by two antiparallel α-helices that pack very closely (mean center-to-center distance of 7 Å); Pro^{79} and Ala^{67} are located at either end of each helix (Figure 1C). Both residues have been mutated to cysteine, enabling the pair of α-helices to be cross-linked together at either end via a disulfide bond or a short cross-linking reagent, PDM (Figure 1C). At the top of the dimer interface, two "recognition" helices make a head-to-head antiparallel dimer contact at Asp^{82} (*8*). The two side chains may be bridged by hydrogen bonds or the participation of a cation. Asp^{82} has been mutated to Cys, and the two symmetry-related residues subsequently cross-linked by PDM (Figure 1C).

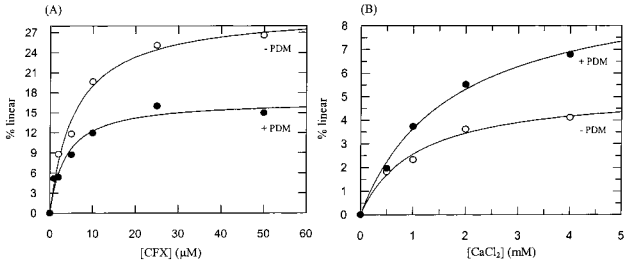

FIGURE 4: Quinolone- and Ca^{2+}-stabilized cleavage by PDM cross-linked $A64_2B_2$ and $A64_2A33_2B_2$, respectively. (A) Negatively supercoiled pBR322 (7.2 nM) was incubated with GyrB (60 nM) and $GyrA64^{D82C/C194A}$ (90 nM) or PDM cross-linked $GyrA64^{D82C/C194A}$ (90 nM) in the presence of ciprofloxacin (0 to 50 μM) for 30 min at 37 °C. Reactions were terminated upon incubation with SDS (0.2%) and proteinase K (0.1 mg/mL) for 30 min at 37 °C. Samples were analyzed on a 1% agarose gel. (B) Relaxed pBR322 (7.2 nM) was incubated with GyrB (60 nM), GyrA33 (90 nM) and $GyrA64^{D82C/C194A}$ (90 nM) or PDM cross-linked $GyrA64^{D82C/C194A}$ (90 nM) in the presence of $CaCl_2$ (0 to 4 mM) for 30 min at 37 °C. Reactions were terminated upon incubation with SDS (0.2%) and proteinase K (0.1 mg/mL) for 30 min at 37 °C. Samples were analyzed on a 1% agarose gel containing 3 μg/mL chloroquine. Quantitation of ethidium-stained bands was performed using Gene Tools (Syngene). The percentage of linear pBR322 compared to total DNA was determined and fitted to a simple binding isotherm.

Consequences of Gyrase Modification by the Introduction of Novel Cysteine Residues and Subsequent Cross-Linking. When interpreting the cross-linking results, it is important to distinguish the consequences due to the introduction of the novel cysteines and the presence of the cross-linkers from the inability to open the DNA gate. This is particularly important since the cross-linkers are situated very close to both the breakage-reunion site and proposed quinolone-binding pocket. It is likely that modifying this area with a cross-linker may perturb cleavage and/or the interaction of quinolones. Such effects have been encountered in this study. Introduction of novel cysteine residues at positions Pro^{79} and Ala^{67} significantly reduced quinolone-induced cleavage (Table 1). This was anticipated since position 67 is a site of a known quinolone-resistance mutation (Ala^{67} to Ser). However, the formation of two disulfide bonds between the two pairs of cysteine residues does not have an additional effect on the interaction with quinolones. In contrast, addition of the PDM cross-linking reagent blocks quinolone action completely. This highlights the sensitivity of this site to modification. Supercoiling activity and Ca^{2+}-stabilized cleavage are not significantly affected by the introduction of the novel cysteines, suggesting that DNA binding and cleavage are not greatly altered in the mutant (Table 1). However, all attempts to cross-link these residues leads to the abolition of Ca^{2+}-stabilized cleavage (Table 2). This most likely reflects perturbation of the cleavage−religation equilibrium caused by the presence of the cross-linker rather than the enzyme's inability to open the DNA gate.

The second mutant involves the replacement of Asp^{82} with Cys. This residue is situated on the end of the recognition

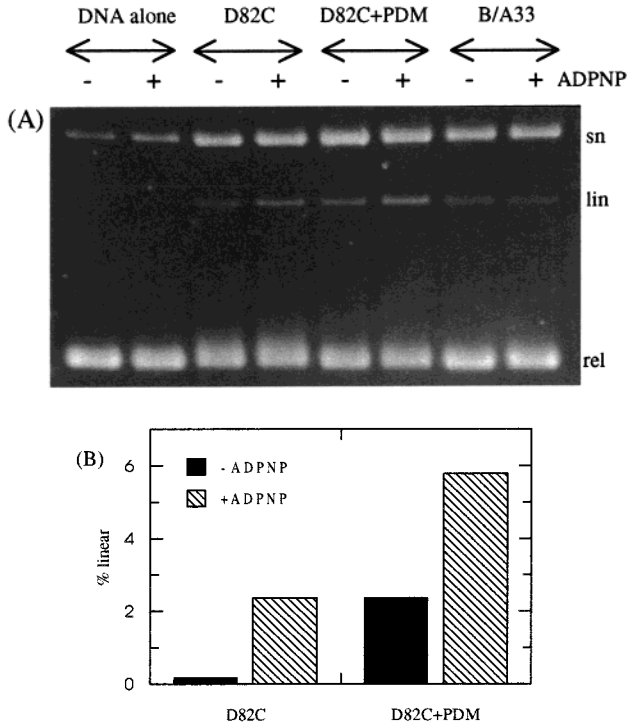

FIGURE 5: ADPNP-induced cleavage exhibited by PDM cross-linked gyrase. (A) Relaxed pBR322 (10 nM) was incubated alone or with GyrB (500 nM), GyrA33 (500 nM) with or without GyrA64$^{D82C/C194A}$ ± PDM (500 nM) at 37 °C for 20 min. Samples were further incubated in the presence or absence of ADPNP (2.5 mM) for 30 min at 37 °C. Reactions were terminated upon incubation with SDS (0.2%) and proteinase K (0.1 mg/mL) for 30 min at 37 °C. Samples were analyzed on a 1% agarose gel in the presence of 3 μg/mL chloroquine (sn: singly nicked, lin: linear, rel: relaxed). (B) Graphical representation of ADPNP-induced cleavage for cross-linked and uncross-linked gyrase. The intensity of the linear band was measured using Gene Tools (Syngene) and expressed as a percentage of the total DNA. The background level of cleavage exhibited by GyrB/GyrA33 alone (3.2%) has been subtracted to show the extent of ADPNP-induced cleavage performed by gyrase in the presence and absence of the cross-linker.

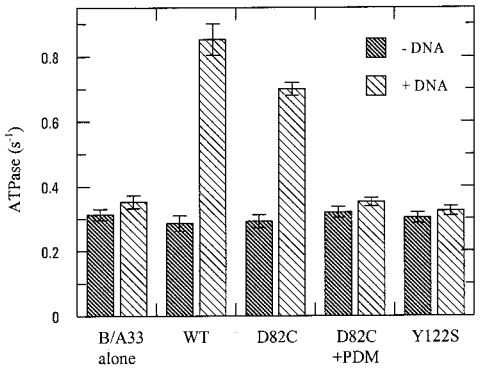

FIGURE 6: Analysis of the rate of ATP hydrolysis by cross-linked gyrase in the presence or absence of DNA. GyrB (50 nM) and GyrA33 (80 nM) were incubated alone or with GyrA64^{wt}, GyrA64$^{C194A/D82C}$±PDM, or GyrA64^{Y122S} (100 nM) with ATP (2 mM) in the presence or absence of linear pBR322 (5 nM) at 25 °C. Initial rates of ATP hydrolysis were monitored using the PK/LDH linked assay.

helix (Figure 1C), which is thought to interact directly with the bound G segment (*8*). As anticipated, this mutation does seem to reduce G-segment binding, although does not abolish it. It appears that DNA cleavage is also reduced by the mutation. It has been suggested that the product release step of ATP hydrolysis is normally the rate-limiting step in the gyrase supercoiling reaction (*26*). However, it is feasible that DNA cleavage is rate limiting in the ATP-independent relaxation of gyrase and that this may explain why A64$^{C194A/D82C}{}_2$A33$_2$B$_2$ shows only a 2-fold decrease in supercoiling activity compared to a 10-fold reduction in ATP-independent relaxation. Cross-linking this pair of novel cysteines with PDM does not seem to affect quinolone binding, although quinolone-stabilized cleavage is impaired (Figure 4A). In contrast, cleavage in the presence of both Ca^{2+} and Mg^{2+} is enhanced by the introduction of PDM (Figures 4B and 5). Again, this suggests that the presence of the cross-linker perturbs the cleavage−religation equilibrium, this time stabilizing the cleaved conformation.

The 59 kDa Crystal Structure of Gyrase is likely to Represent an Active Gate-Closed Conformation. To date, no high-resolution structural information is available for the gyrase-DNA complex. However, the crystal structure of a 59 kDa fragment of GyrA alone reveals a strongly basic concave surface at the top of the DNA gate, with the active-site tyrosines (Tyr^{122}) located on either side, 30 Å apart (*8*). This has allowed a DNA duplex to be modeled onto the DNA gate such that both "recognition" helices lie in the major groove of the G segment. The closed DNA gate brings together a cluster of conserved residues proposed to form the site for DNA cleavage-reunion. Tyr^{122} and Arg^{121} from one side of the dimer interface are very close to His^{80}, Arg^{32} and Lys^{42} from the other side. One unexpected consequence of this model is that the active site tyrosines (Tyr^{122}) are ~7 Å away from the phosphate backbone of the modeled G segment. This may suggest that the 59 kDa crystal structure represents a gate-closed conformation that is unable to perform DNA cleavage. It is possible that alternative dimer contacts form within active gyrase, which bring the active site residues closer together. However, this is now disfavored because cross-linked gyrase can bind and cleave DNA. Cross-linking the DNA gate using disulfide bonds or a short cross-linking reagent locks gyrase in the gate-closed conformation observed in the crystal structure. This is likely to restrict any large-scale conformational changes at the dimer interface. Hence, it is likely that the 59 kDa structure does provide a fairly accurate picture of the gate-closed conformation and the active site cluster. To accommodate this structure, the major groove in the G segment may widen, locating the scissile phosphodiester bonds closer to the active site tyrosines.

Quinolone-Stabilized Cleavage does not Require DNA Gate Opening. It is well-established that gyrase cleaves DNA via a transesterification reaction in which the hydroxyl group of Tyr^{122} attacks the phosphoryl backbone of the DNA. In a similar manner, religation involves the attack of the phosphotyrosine linkage by the hydroxyl group of the broken 3′ DNA strand. Hence, it is likely that a cleavage−religation equilibrium is established between these two reactions. Religation is thought to predominate, since only low levels of cleavage are revealed upon enzyme denaturation with SDS. Quinolone drugs perturb this equilibrium such that DNA cleavage is favored. Quinolone binding, close to the active site, may distort the bound DNA such that the 3′ hydroxyl group is moved away, thus blocking religation. Alternatively, quinolones may bind to an open conformation of the DNA gate, such that the broken DNA ends remain

separated and religation is blocked. Recent evidence disfavors the latter hypothesis. It has been shown that DNA cleavage is not necessary for quinolone binding (*22*); a mutation within the active site of GyrA (Tyr^{122} to Ser) blocks G-segment cleavage by gyrase but does not affect quinolone binding. In addition, the rate of quinolone binding is faster than the corresponding rate of quinolone-induced cleavage (*27*). Together, these observations strongly suggest that quinolone binding precedes cleavage, seemingly incompatible with the idea that quinolones bind to the open conformation of the DNA gate. Nevertheless, it is possible that although quinolones may initially bind to the closed-gate conformation, they may stabilize the subsequent DNA cleavage by preventing closure of the DNA gate. To address whether quinolones do block religation by stabilizing an open-gate conformation of gyrase, we have cross-linked the DNA gate, restricting it to a closed conformation. We have shown that cross-linked gyrase can still bind and cleave DNA, although opening of the DNA gate is not possible. Despite this, cross-linked gyrase can bind quinolones and perform quinolone-stabilized cleavage. This provides strong evidence that quinolones do not prevent religation by stabilizing the open conformation of the DNA gate. Instead, it is likely that quinolones bind to the gate-closed conformation and perturb the DNA cleavage-religation equilibrium. Since the active site tyrosine residues are ~30 Å apart (*8*), whereas a DNA duplex is approximately ~20 Å in diameter, this suggests that the G segment must bind in a distorted manner. Quinolone binding is likely to alter this distorted conformation, such that religation is disfavored.

Opening the DNA Gate Promotes ATP Hydrolysis. Gyrase exhibits a low level of ATP hydrolysis that can be stimulated in the presence of DNA (*21*). More specifically, it is proposed that DNA must bind across the DNA gate and within the ATP-operated clamp to promote ATP hydrolysis (*6*). It is also essential that the G segment is cleaved (*20*). However, it is difficult to imagine how simply breaking the phosphodiester backbone of the G segment can trigger the hydrolysis of ATP in a different subunit. It is more likely that the large conformational change required to pull the ends of the DNA apart after cleavage is the actual trigger. This hypothesis has been tested directly by cross-linking the DNA gate of gyrase. We have shown that gyrase, cross-linked by PDM, does not exhibit DNA-stimulated ATP hydrolysis. However, in the absence of DNA, the rate of ATP hydrolysis is unaffected by the cross-linking reagent. A similar result is obtained for the active site mutant of gyrase reconstituted with $GyrA64^{Y122S}$ (Figure 6). In both cases it is most likely that the abolition of DNA-stimulated ATP hydrolysis is due to the failure to open the DNA gate. However, it is possible that this may reflect the inability of cross-linked gyrase to capture a T segment within the ATP-operated clamp. This is disfavored because additional DNA cleavage by cross-linked gyrase can be revealed upon incubation with ADPNP and subsequent denaturation with SDS (Figure 5). Recent evidence suggests that the presence of the T segment inside the ATP-operated clamp may promote ADPNP-induced cleavage (*20*). Hence, the ability of cross-linked gyrase to exhibit ADPNP-induced cleavage suggests that T-segment capture is not blocked by the presence of the cross-linker.

Taken together, these results suggest that cross-linked gyrase can bind and cleave a G segment and capture a wrapped T segment upon closure of the ATP-operated clamp. Despite this, the rate of ATP hydrolysis is not enhanced. This suggests that opening of the DNA gate is an essential prerequisite for the DNA-stimulation of ATP hydrolysis by gyrase.

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