GyrI: a counter-defensive strategy against proteinaceous inhibitors of DNA gyrase

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Received October 22, 2001; revised December 21, 2001; accepted January 3, 2002

INTRODUCTION

DNA gyrase is the target of two plasmid-encoded toxins CcdB and microcin B17, which ensure plasmid maintenance. These proteins stabilize gyrase–DNA covalent complexes leading to double-strand breaks in the genome. In contrast, the physiological role of chromosomally encoded inhibitor of DNA gyrase (GyrI) in Escherichia coli is unclear and its mechanism of inhibition has not been established. We demonstrate that the mode of inhibition of GyrI is distinct from all other gyrase inhibitors. It inhibits DNA gyrase prior to, or at the step of, binding of DNA by the enzyme. GyrI reduces intrinsic as well as toxin-stabilized gyrase–DNA covalent complexes. Furthermore, GyrI reduces microcin B17-mediated double-strand breaks in vivo, imparting protection to the cells against the toxin, substantiating the in vitro results. Thus, GyrI is an antidote to DNA gyrase-specific proteinaceous poisons encoded by plasmid addiction systems.

DNA gyrase is distinct from all other topoisomerases in its ability to introduce negative supercoils into DNA in the presence of ATP (Gellert et al., 1976; Wang, 1998; Champoux, 2001). The enzyme functions by making a transient break in the DNA and resealing it after passing another duplex through it. The active enzyme is a heterotetramer of GyrA and GyrB with A₂B₂ subunit composition. The N-terminal two-thirds of GyrA harbours the cleavage-religation activity while the C-terminal one-third wraps DNA around itself (Reece and Maxwell, 1991). On the other hand, the ATPase activity of the enzyme resides in the N-terminal half of GyrB (Ali et al., 1993), while the C-terminal half is involved in binding to DNA and GyrA (Brown et al., 1979; Tingey and Maxwell, 1996; Chatterji et al., 2000). Being an essential enzyme and present only in prokaryotes, DNA gyrase is an ideal target for antibacterials. A number of diverse chemical entities, both natural and synthetic in nature, have been found to inhibit the enzyme (Lewis et al., 1996; Maxwell, 1999). A group of synthetic inhibitors, fluoroquinolones, are used clinically as antibacterials.

Two proteinaceous inhibitors of DNA gyrase are known: CcdB and microcin B17 (Liu, 1994; Couturier et al., 1998). Genes coding for both of these proteins are present on plasmids specific to Gram-negative eubacteria. These inhibitors stabilize gyrase–DNA covalent complexes, which are converted to double-strand breaks leading to cell death (Vizan et al., 1991; Bernard et al., 1993). Another protein has been found to be associated with Escherichia coli DNA gyrase during purification of the enzyme (Nakanishi et al., 1998). Notably, the fractions containing DNA gyrase along with this protein were unable to supercoil DNA and therefore it was named the gyrase inhibitory protein, GyrI. Surprisingly, the gene coding for GyrI was found to be present on the E. coli genome itself. Moreover, in an independent study, the same protein (then called SbmC) was picked up in a screen for host factors imparting resistance to microcin B17 (Baquero et al., 1995).

Presence of a chromosomally encoded inhibitor of DNA gyrase raises many questions: (i) what is the mode of inhibition of DNA gyrase by GyrI?; (ii) why has an inhibitor of an essential enzyme been maintained through evolution?; and (iii) how does an inhibitor of DNA gyrase render protection against another? The mechanism underlying this apparent dual function of GyrI is puzzling. Moreover, the precise role of the protein in vivo has been elusive. In this study, we demonstrate that the mechanism of inhibition of DNA gyrase by GyrI is unique. We also observe that GyrI acts as an antidote for CcdB and microcin B17, and propose an interplay between the proteinaceous toxins and GyrI in vivo.
RESULTS

Mode of inhibition of DNA gyrase by GyrI

The gyrI ORF was amplified from E. coli genomic DNA, cloned and over-expressed. The protein was purified to apparent homogeneity as described in Methods (Figure 1A). The purified protein, of expected size, was able to inhibit supercoiling activity of DNA gyrase (Figure 1B). Interestingly, GyrI also inhibited the relaxation activity of DNA gyrase at similar concentrations (Figure 1C). The difference in the molar ratios of GyrI to gyrase required to inhibit supercoiling versus the relaxation reactions might be due to the differential affinity of the enzyme for the two forms of substrate DNA, namely relaxed and supercoiled closed-circular DNA (Higgins and Cozzarelli, 1982). Moreover, mechanistically the two reactions are also distinct (Williams and Maxwell, 1999). Inhibition of the ATP-independent relaxation reaction suggests that the primary mode of action of GyrI is not by interference with the ATPase activity. In agreement with such a mechanism, the intrinsic ATPase activity of GyrB was unaltered in the presence of GyrI (Figure 1D). Thus, GyrI appears to inhibit a step common to both supercoiling as well as the relaxation reactions.

The protein–DNA covalent adduct is a transient intermediate in every topoisomerization reaction cycle. The low steady-state levels of this intermediate are visualized in the cleavage reaction by the addition of SDS followed by proteinase K digestion. Poisons of DNA gyrase like CcdB and microcin B17, which alter the cleavage–religation equilibrium, show an accumulation of the linear product in such reactions. As opposed to these toxins, GyrI did not cause any increase in the cleavage product. Instead, there was a reduction in the gyrase–DNA covalent complexes in the presence of the inhibitor (Figure 2A). To assess whether GyrI interfered with the cleavage activity of the enzyme per se or with a preceding step, its effect on the binding of substrate DNA by DNA gyrase was assessed. Electrophoretic mobility shift assays (EMSAs) were performed with a labelled fragment containing the strong gyrase site from pBR322 as the substrate, in the absence and presence of GyrI (Figure 2B). A concentration-dependent decrease in DNA binding by gyrase was observed in the presence of GyrI (Figure 2B). Irrespective of the order of
Inhibition of DNA gyrase by GyrI

addition of gyrase, DNA and GyrI, a similar reduction in binding of DNA by the enzyme was seen (data not shown). It should be noted that GyrI itself did not interact with DNA (Figure 2B, lane I). Moreover, there was no additional complex seen in the presence of GyrI, ruling out the possibility of a ternary complex between DNA, gyrase and GyrI. The interaction between GyrI and Gyr subunits was assessed by surface plasmon resonance. The dissociation constants obtained from such an experiment revealed that GyrI was able to interact with both GyrA and GyrB, albeit with different affinities. GyrI has 6-fold higher affinity for GyrA as compared to GyrB (Table I). We have considered the possibility that GyrI might interfere with GyrA–GyrB interaction and thus inhibit the formation of active holoenzyme. However, gel filtration experiments appear to rule out this possibility. Taken together, these results imply that GyrI-mediated inhibition is prior to, or at the step of, DNA binding by the enzyme. In agreement with the above conclusion, although GyrI does not affect the intrinsic ATPase of GyrB, it completely abolishes the DNA-dependent stimulation of ATPase activity (Figure 1D).

Effect of GyrI on microcin B17 and CcdB stabilized gyrase–DNA covalent complexes in vitro

Since GyrI was able to reduce the steady-state levels of gyrase–DNA covalent complex in the absence of any inhibitor, we tested its effect on CcdB and microcin B17 stabilized complexes. Cleavage reactions were carried out wherein CcdB or microcin B17 was added along with GyrI. Cleavage reactions were carried out wherein CcdB or microcin B17 was added along with GyrI. CcdB and microcin B17 both alter the cleavage-religation equilibrium leading to the accumulation of a gyrase–DNA covalent complex. However, in the presence of GyrI, there was a net decrease in the steady-state levels of cleavage product (Figure 3). Such a reduction of gyrase–DNA covalent complex indicates that GyrI is able to nullify the effect of CcdB and microcin B17 in vitro. Order of addition experiments were carried out where CcdB or microcin B17 was incubated with gyrase and DNA prior to the addition of GyrI. Such reactions

Table I. Interaction between GyrI and Gyr subunits

<table>
<thead>
<tr>
<th>Protein</th>
<th>K_D (M)</th>
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<tbody>
<tr>
<td>GyrA</td>
<td>4.52 × 10^{-7}</td>
</tr>
<tr>
<td>GyrB</td>
<td>29.40 × 10^{-7}</td>
</tr>
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revealed that GyrI was able to revert even a preformed CcdB stabilized gyrase–DNA complex. However, under similar experimental conditions it was unable to revert a microcin-stabilized complex probably due to the extremely slow kinetics of action of microcin B17 (Heddle et al., 2001).

Action of GyrI in vivo

To test the ability of GyrI to counteract microcin B17 inside the bacterial cell, we analysed the sensitivity of cells expressing GyrI to the toxin. Microcin B17 was spotted on a lawn of DH5α cells harbouring either vector or plasmid producing GyrI and the zone of inhibition was monitored. Uninduced expression levels of GyrI imparted an 8-fold protection to the cells against the microcin B17, and this was further enhanced when its expression was induced with IPTG (Table II). Furthermore, the growth of AP1-200-9 transformed with pTrc99C-DraI or pTrc99C-gyrI was monitored in the presence of microcin B17. Cells lacking the GyrI over-expressing plasmid appeared to die 2 h after the addition of the toxin (Figure 4A). On the other hand, cells expressing GyrI continue to grow in the presence of microcin B17, suggesting that GyrI was also able to negate the effect of the toxin in vivo. Thus, GyrI is a defence mechanism of the cell against proteinaceous poisons targeting DNA gyrase.

Action of microcin B17 on its target, DNA gyrase, ultimately leads to generation of double-strand breaks in the genome and cell death. To show that the resistance imparted by GyrI was due to decrease in the double-strand breaks, an AP1-200-9 strain harbouring the lacZ gene under the SOS inducible dinD promoter was used (Piekarowicz et al., 1991). Treatment of the cells with microcin B17 leads to double-strand breaks. The consequent SOS response in these cells was visualized by increase in β-galactosidase activity (Figure 4B). The β-galactosidase activity peaked after 2 h of treatment; however, prolonged exposure led to lower levels of induction probably due to cell death. In contrast, in cells producing GyrI, there was no significant induction of β-galactosidase, suggesting a suppression of double-strand breaks. Thus, these results demonstrate that the resistance imparted by GyrI is by inhibiting the formation of lethal double-strand breaks in the cell.

**DISCUSSION**

DNA gyrase is the only enzyme that introduces negative supercoils into DNA. In addition to supercoiling, DNA gyrase catalyses catenation/decatenation as well as knotting/unknotting reactions in vitro (Reece and Maxwell, 1991). Furthermore, in the absence of ATP the enzyme catalyses relaxation of negatively supercoiled DNA. Each of these topoisomerization reactions involves a complex series of steps. The enzyme binds to DNA and introduces a break with concomitant formation of a protein–DNA covalent complex. This is followed by passage of another duplex through this molecular gate and finally resealing of the break. Although the actual covalent protein–DNA link is formed with GyrA, recent work shows that both subunits contribute to the cleavage-relinking catalysis (Berger et al., 1998; Liu and Wang, 1999). It is not surprising because GyrB not only harbours the ATPase domain but is also involved in interacting with DNA including the transfer segment (Tingey and Maxwell, 1996; Chatterji et al., 2000).

Mechanistically, all inhibitors of DNA gyrase can be classified into two groups (Lewis et al., 1996; Maxwell, 1999). The first class, exemplified by coumarins and cyclothialidines, prevent the binding of ATP to the enzyme and, as a result, inhibit supercoiling. The second class, e.g. quinolones, CcdB and microcin B17, act by stabilizing enzyme–DNA covalent intermediates. These protein–DNA adducts act as roadblocks for
inhibitory activity, GyrI is useful to combat the dire situation of breaks in the DNA even at the cost of partial inhibition of an essential function.

By virtue of its ability to reduce gyrase-mediated double-strand breaks, GyrI has the potential to play a more general role. Although Gyr seems to have evolved as an antidote to toxins, any signal that is amplified by gyrase action leading to DNA damage, e.g. quinolones and intrinsic gyrase-mediated lesions, can be combated by production of this protein by the cells. In addition, in a recent study, it was observed that gyrf when present in a multicopy plasmid imparted resistance against mitomycin C (Wei et al., 2001). Mitomycin C is a DNA damaging agent that intercalates into DNA and alkylates it. Bacterial cells turn on the SOS regulon in response to the toxic effects of this compound. Resistance to mitomycin C by GyrI might be due to reduced intercalation as a result of reduced negative supercoiling of DNA. Molecular details of a wider role of GyrI as an arsenal of cellular rescue machinery need further investigation.

**METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* DH10B was used for all cloning experiments. Over-expression plasmids pPH3, pAG111 (Hallett et al., 1990) and pJW312-SaII (Lynn and Wang, 1989) were employed for *E. coli* GyrA, GyrB and topoisomerase I purification, respectively. Microcin B17 was purified from ZK650 cells (Yorgey et al., 1993). API-200-9 was used for the detection of SOS response in the cells (Piekarowicz et al., 1991).

**Cloning of gyrI.** gyrI was PCR amplified using *E. coli* genomic DNA as template and primers P7 (5'-ATCAATCGATCCGTTCAT-GAACTACG) and P8 (5'-CCTGAGATCTATTGATGTATTTG) containing Rcal and BglII sites, respectively. The reaction was carried out using Phu polymerase. Rcal- and BglII-digested PCR product was ligated to *Ncol*-BamHI cut pTrc99C-Dral. pTrc99C-Dral is a derivative of pTrc99C, which contains a Dral site downstream of the *P* trc promoter.

**Enzyme and substrate preparation.** GyrA and GyrB were purified as described previously (Maxwell and Howells, 1999). Specific activity of purified DNA gyrase was calculated with 1 U defined as the amount of enzyme required to completely supercoil 500 ng of relaxed pUC18 DNA at 37°C in 30 min. Supercoiled pUC18 and pBR322 were prepared by standard DNA purification protocols (Sambrook et al., 1989). *Escherichia coli* topoisomerase I and relaxed pUC18 were prepared as described by Lynn and Wang (1989).

**Purification of proteinaceous inhibitors of DNA gyrase.** Microcin B17 was purified from ZK650 cells as described previously (Chatterji et al., 2001). The unit strength of microcin B17 was
determined as described previously (Davagnino et al., 1986), except that DH5α was used as the tester/sensitive strain. Purified CcdB was a kind gift from R. Varadarajan. For purification of GyrI, DH5α cells harbouring pTrc99C-gyrI were grown until an OD_{600} of 0.6 was reached, and induced with 0.05 mM IPTG for 4 h. The cells were harvested, resuspended in 30 mM Tris–HCl pH 7.5 containing 30 mM NaCl and sonicated. After centrifugation at 10 000 g for 90 min, the supernatant (S_{90}) was subjected to an ammonium sulfate fractionation (55% saturation). The pellet was dissolved in 10 mM Tris–HCl pH 7.5, 1 mM EDTA and 1 mM DTT, and resolved on a high-resolution Superdex S-200 column (150 ml). More than 99% homogenous preparation of protein was obtained as detected by silver staining.

**Enzyme assays.** Supercoiling and cleavage assays were performed as described previously (Chatterji et al., 2000). Relaxation reactions were carried out in supercoiling buffer except spermine and ATP were omitted and supercoiled pUC18 was used as substrate. The reactions were performed at 37°C for 30 min. ATPase and EMSAs were carried out as described previously (Chatterji et al., 2000). Surface plasmon resonance experiments were performed on a BIACore 2000 system (BIACore). GyrI was immobilized on the CMS sensor chip via amine coupling in acetate buffer (pH 3.0). The surface was blocked with ethanolamine hydrochloride. The interaction was assessed in 10 mM HEPES–NaOH pH 7.4 containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT. Different concentrations of GyrA and GyrB were passed over the immobilized GyrI and the subsequent changes in resonance units were recorded. Proteins used for the experiment were dialysed against running buffer prior to the experiment. Bovine serum albumin was used as the negative control.

**In vivo assays.** The effect of GyrI on the in vivo toxicity of microcin B17 was tested by spotting various concentrations of the microcin B17 on a lawn of DH5α cells containing either pTrc99C-Dral or pTrc99C-gyrI in the presence and absence of IPTG. The presence of a zone of inhibition and its size is indicative of the sensitivity of the cells to microcin B17. Furthermore, exponentially growing cultures transformed with GyrI containing plasmid or plasmid alone were treated with microcin B17 (2.7 U/ml) and their OD_{600} were recorded at different time intervals. As negative control, cells were treated with equivalent amounts of ethanol, and used as a solvent for microcin B17. AP1-200-9, harbouring the lacZ gene under the SOS inducible dinD promoter, was used to monitor double-strand breaks induced by gyrase in the presence of microcin B17. Cells harbouring either pTrc99C-Dral or pTrc99C-gyrI were grown to an OD_{600} of 0.6 and treated with 0.76 U/ml of microcin B17 for varied time and β-galactoside activity of the cells were measured by a standard procedure (Miller, 1992).

**ACKNOWLEDGEMENTS**

We thank J.C. Wang and A. Maxwell for over-expressing constructs of *E. coli* topoisomerase I and DNA gyrase, respectively, R. Kolter for ZK4 and ZK650 strains, A. Piekarowicz and D.C. Stein for AP1-200-9, and R. Varadarajan for CcdB. S. Unniraman is acknowledged for discussion and critical reading of the manuscript, and J. Jacob for technical assistance. This work is supported by research grants to V.N. from the Department of Science and Technology, Government of India.

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Inhibition of DNA gyrase by GyrI


DOI: 10.1093/embob reports/kvf038