The key DNA-binding residues in the C-terminal domain of *Mycobacterium tuberculosis* DNA gyrase A subunit (GyrA)

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**ABSTRACT**

As only the type II topoisomerase is capable of introducing negative supercoiling, DNA gyrase is involved in crucial cellular processes. Although the other domains of DNA gyrase are better understood, the mechanism of DNA binding by the C-terminal domain of the DNA gyrase A subunit (GyrA-CTD) is less clear. Here, we investigated the DNA-binding sites in the GyrA-CTD of *Mycobacterium tuberculosis* through site-directed mutagenesis. The results show that Y577, R691 and R745 are among the key DNA-binding residues in *M.tuberculosis* GyrA-CTD, and that the third blade of the GyrA-CTD is the main DNA-binding region in *M.tuberculosis* DNA gyrase. The substitutions of Y577A, D669A, R691A, R745A and G729W led to the loss of supercoiling and relaxation activities, although they had a little effect on the drug-dependent DNA cleavage and decatenation activities, and had no effect on the ATPase activity. Taken together, these results showed that the GyrA-CTD is essential to DNA gyrase of *M.tuberculosis*, and promote the idea that the *M.tuberculosis* GyrA-CTD is a new potential target for drug design. It is the first time that the DNA-binding sites in GyrA-CTD have been identified.

**INTRODUCTION**

DNA gyrase (EC 5.99.1.3) is a predominantly prokaryotic enzyme which has enzymatic properties distinct from other type II topoisomerases, and has diverged significantly during evolution. It is an A2B2 tetramer that catalyzes the relaxation of supercoiled DNA, catenation and decatenation of DNA rings, and knotting and unknotting of duplex DNA (1–3). It is also the only topoisomerase capable of introducing negative supercoiling into closed-circular DNA in an ATP-dependent manner (4,5). As an essential enzyme, DNA gyrase is involved in crucial cellular processes such as replication, transcription and recombination (2,6), and is the target of the quinolone drugs and aminocoumarin antibiotics (7). These discoveries about gyrase have led to the expansion of interest in its basic and clinical research.

The reaction mechanism of DNA gyrase is relatively well understood and the crystal structures of the N-terminal domain of GyrB (GyrB-NTD) (8–12), the N- and C-terminal domains of GyrA (GyrA-NTD and GyrA-CTD) have already been solved (13–15). The functions of different domains of the enzyme have also been identified. It is known that the GyrB-NTD harbors the ATPase activity and binds to coumarins; the C-terminal domain of GyrB (GyrB-CTD) is the domain that interacts with GyrA and forms a complex capable of catalyzing DNA breakage and reunion (6,13). GyrA-NTD is the catalytic center of gyrase, which harbors the DNA cleavage–reunion activity (16,17), and GyrA-CTD is involved in binding and wrapping the DNA (18–20). Furthermore, the active sites for the ATPase activity, the coumarin-binding site in GyrB-NTD (10,11,17–27) and the sites of interaction of the quinolones with GyrA and GyrB-CTD (28–31), have also been identified. However, the active sites of DNA-binding in GyrA-CTD and the mechanisms of DNA binding are largely unknown.

It is known that GyrA-CTD is a non-specific DNA-binding protein and has a unique role in DNA-.wrapping and T-segment presentation by gyrase (14,18,19). The GyrA-CTD is catalytically inactive on its own, but can complement the supercoiling activity of GyrA-NTD upon mixing (18). GyrA-CTD has been shown to bind DNA independently

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and constrain positive supercoils, and is necessary for gyrase-mediated supercoiling (18,32). DNA wraps around gyrase in a manner dependent upon the presence of the GyrA-CTD, producing superhelicity, and that wrapping plays a crucial role in controlling the directionality of supercoil induction (33–35). Removal of the C-terminal domain abolishes the wrapping of DNA around gyrase, eliminates the introduction of supercoiling and converts gyrase into a conventional topoisomerase II (33). On the contrary, the addition of GyrA-CTD results in restoration of the capacity to introduce negative supercoils (36). The binding of gyrase to DNA can be shown to involve the positive wrapping of DNA, and GyrA-CTD is also capable of bending DNA by >180° over a 40 bp region (14). The GyrA-CTD was predicted previously to have a six-bladed β-propeller structure and it was believed that the opposite side of the propeller is involved in interaction with DNA (37). However, the first published structure of GyrA-CTD derived from the spirochete Borrelia burgdorferi reveals an intriguing new fold designated a β-pinwheel (14). Recently, the X-ray structure of Escherichia coli GyrA-CTD was published (15). The E.coli GyrA-CTD adopts a circular-shaped β-pinwheel fold, similar to the B.burgdorferi GyrA-CTD; however, the B.burgdorferi GyrA-CTD is flat, whereas the E.coli GyrA-CTD is a spiral. How does GyrA-CTD affect supercoiling activity in the catalytic cycle? Which residues are involved in interacting with DNA in the bending proposed to occur with GyrA-CTD? Are there other roles for the GyrA-CTD in the activities of gyrase? We have investigated the active DNA-binding sites in Mycobacterium tuberculosis GyrA-CTD using site-directed mutagenesis, in an attempt to understand the role of GyrA-CTD in the function of gyrase. The results showed that the mutations of Y577A, D669A, R691A, R745A and G729W led to loss of supercoiling, relaxation activities, but had a little effect on drug cleavage and decatenation activities and had no effect on ATPase activity, and showed that the GyrA-CTD is essential to DNA gyrase of M.tuberculosis. It is the first time that the DNA-binding sites in GyrA-CTD have been identified.

MATERIALS AND METHODS

Bacterial strains and plasmids

Escherichia coli DH5α was used as the host for cloning purposes. Strain BL21 (DE3) and strain AD494 (DE3) were used for protein expression. Plasmids pET-20b and pET-32a (Novagen) were used to construct vectors for overexpression of M.tuberculosis GyrA and GyrB proteins, respectively. Supercoiled plasmid pBR322 DNA was extracted using the PureYield™ Plasmid Midiprep System (Promega Biological Products, Ltd. Joint Venture, China), and relaxed plasmid pBR322 DNA was purchased from John Innes Enterprises Ltd, UK.

Construction of GyrA and GyrB expression vectors

The gyrA and gyrB genes were both amplified from the genomic DNA from M.tuberculosis H37Rv, and ligated to pET20b and pET32a, respectively. The resulting plasmids, pET20b-gyrA and pET32a-gyrB, were sequenced and the sequences of the gyrA and gyrB genes were identical respectively to the corresponding regions of the sequence with the GenBank accession number NC 000962.

In vitro mutagenesis

To identify the DNA-binding sites, site-directed mutations were introduced into the selected sites in gyrA gene by overlap PCR (38,39). All fragments were ligated into pET20b, and were completely sequenced to confirm the presence of the site-directed mutations.

Protein overexpression and purification

Wild-type GyrA, all the mutants of GyrA and GyrB proteins were overexpressed and purified by the same procedure. E.coli BL21(DE3)/pET20-gyrA and E. coli AD494(DE3)/pET32a-gyrB were induced by final concentrations of 0.4 mM and 1 mM IPTG, respectively, at OD600 = 0.6 for 4 h at 25°C. Cells were harvested by centrifugation, resuspended in Binding Buffer [20 mM Tris–HCl (pH 7.9), 500 mM NaCl and 5 mM imidazole], and then disrupted using an Ultrasonic Cell Disruptor. The disrupted suspension was centrifuged and the supernatant was added to a column of nickel chelate (Amersham Bioscience, USA) equilibrated with Binding Buffer. The column was washed initially with Washing Buffer [20 mM Tris–HCl (pH 7.9), 500 mM NaCl and 60 mM imidazole] and the histidine-tagged protein was eluted with Elution Buffer [20 mM Tris–HCl (pH 7.9), 500 mM NaCl and 250 mM imidazole]. According to the purity in the SDS–PAGE, the peak fractions were pooled, and concentrated by ultrafiltration with Storing Buffer [50 mM Tris–HCl (pH 7.9), 30% glycerol and 5 mM dithiothreitol]. The proteins were centrifuged at 20 000 g for 15 min and then stored at −20°C or −80°C in aliquots. The purity of the protein was examined by SDS–PAGE and the concentration of proteins was measured by BCA protein assay kit (Beyotime Biotechnology, China). To test the enzyme activities of the holoenzyme of gyrase A2B2, equal molar amounts of GyrA and GyrB were mixed together at 25°C for 20 min before use in assays.

Gel-retardation assay of DNA binding

Gel-retardation assays were performed using a PCR-amplified 240 bp DNA fragment containing the strong gyrase site (SGS) from pBR322 (40) using specific primers (forward primer, 5’-CAAGCGGTCGCATGCTGCAGC-3’; reverse primer, 5’-CGCAGGGAGGCCCTTGTAGC-3’). The 240 bp DNA (75 ng) was incubated with the proteins in 20 μl DNA-binding buffer containing 20 mM Tris–HCl (pH 7.5), 55 mM KCl, 5 mM DTT, 5% glycerol and 4 mM MgCl2 for 30 min at 37°C (18). The samples were then loaded onto a 9% native polyacrylamide gel (29:1 acrylamide/N,N’-methylenebisacrylamide). Electrophoresis was performed in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA) containing 10 mM MgCl2 for 2–3 h at 4°C (18,40). Gels were stained with ethidium bromide (0.7 μg/ml) for 20 min, and scanned using a Bio-Rad gel documentation system. All assays were performed at least three times.
DNA supercoiling and relaxation activity assays
DNA supercoiling activity was assayed with recombinant *M. tuberculosis* GyrA and GyrB proteins, and relaxed pBR322 DNA as a substrate. The assay reaction mixture (20 µl) contained 20 mM–Tris·HCl (pH 7.5), 4 mM MgCl₂, 50 mM KCl, 3 mM DTT, 1.5 mM ATP, 1.8 mM spermidine, 30 mg/l *E. coli* tRNA, 20 mg/l BSA, 125 ng relaxed pBR322, and GyrA and GyrB proteins (32,41). Various DNA gyrase concentrations from 0.01 to 10 µM were tested to determine the optimal enzyme concentration and the enzyme reaction condition was also optimized. The buffer, DNA and enzyme mixtures were incubated for 3 h at 30°C. The reaction was terminated by the addition of 5 µl Stop-Dye Buffer [5% (w/v) SDS, 25% glycerol and 25 µg/ml bromophenol blue] and 2 µl solution of 20 mg/ml proteinase K at 37°C for 30 min (40). The samples were loaded onto a 0.8% agarose gel containing no ethidium bromide and run for 2.5 h at 90 V in TAE (40 mM Tris acetate and 2 mM EDTA) running buffer at 4°C. The gels were stained with ethidium bromide (0.7 µg/ml) for 30 min and were then visualized and quantified by using the Bio-Rad gel documentation system. One unit of enzyme activity was defined as the concentration of enzyme that catalyzes the conversion of 1 µg relaxed pBR322 DNA into the completely supercoiled form in 3 h at 30°C (42).

The DNA relaxation assays were performed in the supercoiling buffer without ATP and using supercoiled DNA as substrate. The reaction mixtures (20 µl) were incubated for 6 h at 37°C, and processed as described above for the DNA supercoiling reaction. One unit of enzyme activity was defined as the concentration of enzyme that catalyzes the conversion of 1 µg relaxed pBR322 DNA into the completely supercoiled form in 3 h at 30°C (42).

DNA cleavage assays
DNA cleavage assays were carried out in the same buffer as for DNA relaxation. The substrate was 75 ng supercoiled pBR322 (33). Various norfloxacin (Sigma) concentrations from 0.1 to 60 µg/ml were tested to determine the optimal concentration. These assays were performed at 37°C for different reaction time. The reactions were terminated by the addition of 5 µl Stop-Dye Buffer and 2 µl of 20 mg/ml proteinase K at 37°C for 30 min. The samples were loaded onto a 0.8% agarose gel containing no ethidium bromide and run for 2.5 h at 90 V in TAE running buffer (40 mM Tris acetate and 2 mM EDTA) at 4°C. The DNA bands were stained with ethidium bromide (0.7 µg/ml) for 30 min and were then visualized and quantified by using the Bio-Rad gel documentation system.

Decatenation of kDNA
Decatenation of kDNA was performed at 30°C for 30 min using 100 ng of kDNA (John Innes Enterprises Ltd, UK) as substrate (33). The reaction was terminated by adding 5 µl Stop-Dye buffer [1% (w/v) SDS, 25% glycerol and 25 µg/ml bromophenol blue] and 2 µl at 20 mg/ml protease K at 37°C for 30 min. The DNA products were analyzed by agarose gel (1.0%) electrophoresis (2 h at 90 V in TAE running buffer containing ethidium bromide). The DNA bands were visualized and quantified by using the Bio-Rad gel documentation system.

ATPase assays
ATPase activity was tested with the Micro-ATPase Assay Kit (Nanjing Jiancheng research Institute, China) according to the operation manual. Each GyrA mutant was mixed with GyrB at an equal molar concentration at 25°C for ~20 min. A 240 bp DNA, amplified from pBR322 plasmid, was added to the reaction mixture at a final concentration of 1 µg/ml. The ATP was hydrolyzed by gyrase at 37°C and the reaction was stopped after 10 min. The reaction mixture was centrifuged at 2000 g for 10 min, a color reaction was carried out with the supernatant, and 20 nM inorganic phosphate was used as a control. The OD value was determined at 636 nm after 5 min. One unit of ATPase activity is defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of ATP at 37°C in 1 h.

Surface plasmon resonance
Interactions of gyrase and DNA were investigated using surface plasmon resonance (SPR) with the help of a BIACore 3000 instrument (BIACore AB, Uppsala, Sweden). A short double-stranded DNA (dsDNA) was obtained by annealing two synthesized complementary 30 bp ssDNA fragments, of which one ssDNA was modified with biotin at its 5’ end. A 260 bp dsDNA was amplified from the 1–260 bp region of *rpoB* gene of *M. tuberculosis* by PCR. These two DNA fragments, 30 bp dsDNA and 260 bp dsDNA, were ligated to produce a 290 bp dsDNA which was then coupled to a streptavidin-coated chip (SA sensor chip, BIACore). This coupling generated ~400 response units (RU) on the SPR machine. The running buffer containing 50 mM Tris–HCl (pH 7.5), 55 mM KCl, 5 mM DTT, 4 mM MgCl₂ and 0.005% (v/v) Tween-20 (43) was filtered through Millipore Film (pore size 0.22 µm) and degassed before use. Samples containing the gyrase proteins (100 nM) were applied to flow across the chip surface at a rate of 20 µl/min.

RESULTS

Selection of the conserved residues for site-directed mutagenesis and construction of the mutants
The sequence of *M. tuberculosis* H37Rv GyrA was compared with that from *B. burgdorferi* and *E. coli* and the conserved residues in the GyrA-CTD (between residues 499 and 810 of *B. burgdorferi* GyrA) were chosen (Figure 1). However, the number of conserved residues chosen in this step is large because gyrase itself is rather conserved in this region. Therefore, we selected the residues that are likely to participate in DNA binding and DNA bending that are on the surface of the structure of *B. burgdorferi* GyrA-CTD and are polar (especially positively charged) (14,15,44). Based on these criteria, 10 residues were chosen for further study (Table 1). Residue R581 was not considered as it is absent from the three-dimensional structure of *B. burgdorferi* GyrA-CTD. Based on the structure of *B. burgdorferi* GyrA-CTD, the ten conserved residues can be broadly divided into two highly conserved groups, according to their locations (Figures 1 and 2): the first one is mostly located in the first blade, including E506, K519, H556 and Y574, and the other is mostly located at the third blade, including D647.
D660, R681 and R735. Y569 is located at the second blade and K534 is located at the sixth blade. Considering that the first blade of GyrA-CTD is connecting and interacting with GyrA-NTD, and the conserved residues of the third blade could potentially contribute to a DNA-binding and DNA-bending site in GyrA/ParC/RCC1 (14), our studies are focused on the four conserved residues in the second conserved domain, as they are all located in loops likely to bind DNA (14,15,37). In the first conserved domain, only the E506 was chosen for mutation as a comparison. Ala was introduced into full-length GyrA by site-directed mutagenesis. E514A, Y577A, D669A, R691A and R745A in \textit{M. tuberculosis} GyrA-CTD were constructed successfully and verified by sequencing. G729W was introduced accidentally in the cloning process and used as a comparison.

Production of the GyrA protein and GyrB protein

The expression levels of the mutated GyrA proteins were similar to that of the wild-type GyrA protein (data not shown). All the GyrA proteins and GyrB protein were in the soluble fraction of the cell extract and were purified by nickel-chelate chromatography. These proteins were assessed for purity by SDS–PAGE, and were kept in storing buffer at −20°C or −80°C in aliquots.

Substitutions of Y577A, D669A, R691A and R745A lead to total loss of DNA-binding activity of GyrA in the absence of GyrB

The GyrA subunit is known to have DNA-binding activity in the absence of GyrB (45). Six GyrA mutants were tested for DNA binding. As shown in Figure 3, GyrA with the substitutions G729W and E514A could bind DNA, whereas Y577A, D669A, R691A and R745A did not have such activity. Both the wild-type and the mutant G729W had about the same binding activity, and the binding activity of E514A was slightly less than that of the wild-type. These results suggest that residues Y577, D669, R691 and R745 may not be involved in DNA binding.

Mutants Y577A, D669A, R691A and R745A also lose DNA-binding activity in the presence of GyrB

Gyrase is composed of GyrA and GyrB subunits; only when GyrA is combined with GyrB the enzyme is able to carry...
out supercoiling and relaxation reactions. DNA binding of the GyrA mutants in combination with GyrB was tested. As shown in Figure 4, Y577A, R691A and R745A still had no DNA-binding activity, but E514A and G729W had. The results confirmed that residues Y577, R691 and R745 not only participate in DNA binding, but also have a key role in this process; whereas residues E514 and G729 are not key DNA-binding residues. D669A, in the presence of GyrB, had weak DNA-binding activity, although alone it had no DNA-binding activity (Figure 3), indicating that D669 may have some role in DNA binding, although it is not vital. These results imply that, as described previously (46,47), GyrB cooperates with GyrA in DNA binding. We found that the DNA-binding activity of wild-type M. tuberculosis GyrA is nearly equal to that of the holoenzyme (A2B2).

Mutants Y577A, D669A, R691A, G729W and R745A have no supercoiling activity in the presence of GyrB

The supercoiling activity of the GyrA mutants was tested in the presence of GyrB. As indicated in Figure 5, no mutants showed supercoiling activities, except for E514A. The latter had supercoiling activity but lower than that of the wild-type, reflecting the results of the DNA-binding assays. Interestingly, in the presence of GyrB, G729W could bind DNA (Figure 4), but had no supercoiling activity.
Mutants Y577A, D669A, R691A and R745A, in the presence of GyrB, show no relaxation activity

In the presence of GyrB, all mutants showed nearly no relaxation activity (Figure 6) with the exception of E514A and G729W, but the relaxation activity of E514A was less than that of the wild-type and that of G729W was weak. The results show that residues Y577, D669, R691 and R745 are also involved in the process of relaxing supercoiled DNA, whereas E514 had some role in relaxing DNA. G729W retains DNA-binding activity, but shows very little relaxation activity.

Mutants D669A, R691A and R745A show less cleavage activity than the wild type in the presence of GyrB

The drug dependent DNA cleavage activity of the GyrA mutants was tested by incubating the GyrA mutants with norfloxacin. As shown in Figure 7, the drug cleavage activity of E514A was similar to the wild-type A2B2, those of Y577A and G729A were nearly similar to that of the wild-type, and those of D669A, R691A and R745A are less than that of the wild-type. In the presence of GyrB, mutants D669A, R691A and R745A could still cleave DNA completely by increasing reaction time. This suggests that the GyrA-CTD is less important for drug-dependent cleavage activity.

All mutants except for E514A, in the presence of GyrB, showed less decatenation activity than the wild type

The decatenation activity of the GyrA mutants was tested in the presence of GyrB. As shown in Figure 8A and B, all mutants remained decatenation activity when the gyrase concentrations was 1 μM or 0.1 μM. However, lowering gyrase concentration to 0.01 μM, mutants of D669A, R691A, G729A and R745A showed no decatenation activity, whereas the wild-type enzyme and E514A still had (Figure 8C). The data suggests some facts that decatenation activity of E514A is similar to that of the wild-type, TB gyrase is a strong decatenase (40) and GyrA-CTD is less important for decatenation reaction.

Six mutants have ATPase activity

It is known that GyrB alone or the gyrase holoenzyme (A2B2) have ATPase activity, and that the activity of A2B2 can be stimulated greatly by adding DNA (48). Such DNA-dependent ATPase activity is likely to involve DNA wrapping. The results (Figure 9) showed that all six mutants have ATPase activity, nearly equal to that of the wild-type A2B2, even in the existence of DNA.

Figure 5. The supercoiling activity of the GyrA mutants in the presence of GyrB. All GyrA mutants were mixed with GyrB in an equal molar concentration (1.0 μM) at 25°C for ~20 min prior to the assay. Lane 1, relaxed pBR322 only; lanes 2–8, relaxed plasmid pBR322 treated with the wild-type gyrase, E514A, Y577A, D669A, R691A, G729W and R745A, respectively.

Figure 6. Relaxation activity of the GyrA mutants in the presence of GyrB. GyrA and GyrB were mixed together at an equal molar concentration (1.0 μM) at 25°C for ~20 min prior to the assay. Lane 1, supercoiled pBR322 only; lanes 2–8, supercoiled pBR322 treated with wild-type A2B2, E514A, Y577A, D669A, R691A, G729W and R745A, respectively.

Figure 7. The cleavage activity of GyrA mutants in the presence of GyrB. GyrA and GyrB were mixed together at an equal molar concentration (1.0 μM) at 25°C for ~20 min before the assay. The concentration of norfloxacin was 60 μg/ml in the reaction buffer. Lane 1, supercoiled pBR322 only; lanes 2–8 are supercoiled pBR322 treated with wild-type A2B2, E514A, Y577A, D669A, R691A, G729W and R745A, respectively, in (A), (B), (C) and (D). The reaction time was (A) 5 min, (B) 10 min, (C) 15 min and (D) 30 min, respectively.
Mutants Y577A, R691A and R745A lose the capability of interaction with DNA in the absence or presence of GyrB

Although the DNA-binding activity of the six mutants was measured by gel-retardation assay, it was not clear whether the mutants were impaired just in wrapping or whether they are unable to bind DNA at all. SPR technology, a sensitive means to measure the molecular interactions, was employed to study interaction between DNA and the GyrA mutants either in the absence or presence of GyrB. The SPR 3000 system contains a dual-channel measuring cell. Both reference and sample channels have the same streptavidin-modified sensor chip, but the chip in the sample channel was modified with a 290 bp dsDNA through streptavidin–biotin interaction. GyrA samples were repeatedly injected in the flow-through carrying buffer. Response signal (response unit, RU) represents the binding of GyrA proteins to the bound DNA. As shown in Figure 10, no response was observed for the mutants Y577A, D691A and R745A with or without GyrB, indicating that these mutants have completely lost the capability of DNA binding. D669A did not bind DNA in the absence of GyrB, but had a weak response signal when GyrB was present, which agrees with the results of the gel-retardation assay (Figure 4). Both E514A and G729W could interact with DNA in the absence or presence of GyrB with significantly lowered binding signal, comparing with the wild-type GyrA or A2B2. The sensorgram shows that the binding mode of the G729W had changed from rapid to slow association and dissociation kinetics.

DISCUSSION

As far as we know, gyrase is essential in all bacteria and has not been found in humans or more generally in eukaryotes, except recently in plant organelles (49,50). So gyrase is a good therapeutic target and effective antibiotics have been developed. However, increasing drug resistance is now a serious problem and new agents against such resistance are urgently needed (51). To date, most drugs act on GyrA-NTD and GyrB-NTD, and quinolones probably act on GyrB-CTD as well (1,27); mutations in these domains are found in clinical isolates of different types of bacteria leading to high resistance to existing antibiotics, including M.tuberculosis. There are huge number of people with tuberculosis in the world and it is urgent to get more effective drugs for treating this disease.

Since the 3D structure of M.tuberculosis GyrA is not yet available, the homologous structures of GyrA-CTD from both B.burgdorferi and E.coli were taken as references in this study. There is a positively charged region around the surface of the first, fourth, fifth and sixth blades in the
structure of B. burgdorferi GyrA-CTD, apparent from the electrostatic surface (14), and a positively charged strip also extending along the midriff of the structure of E. coli GyrA-CTD (15). So their midriff is the obvious locus for interaction with a contiguous stretch of duplex DNA, and the arginines and lysines on the long loops could contact DNA (15). B. burgdorferi GyrA-CTD can bind and bend DNA over four of its six blades, and E. coli GyrA-CTD can bind DNA containing complementary superhelicity (14,15). However, there are two highly conserved regions in GyrA-CTD based on homology mapping and the structure of B. burgdorferi GyrA-CTD. The substitutions of D669A, R691A, R745A in the second conserved region caused loss of DNA-binding activity (Figures 3 and 4), so the third blade of GyrA-CTD should be the main DNA-binding domain, which is consistent with the previous work (37). Except for these sites, other sites in other blades such as Y577, are also involved in DNA binding. According to the structure of B. burgdorferi GyrA-CTD, it can be expected that R691 and R745 are the DNA-binding sites as they are positively charged, lying on the loop and in the midriff. However, Y577 and D669 are not lying in the midriff region but still participate in DNA binding. At the same time, previous work showed that there are two main DNA-binding domains in DNA gyrase. The first is the region of the catalytic center, lying in the GyrA-NTD, and the other is in the GyrA-CTD (1,6). However, GyrA-NTD alone has no DNA-binding activity, but GyrA-CTD alone can still bind DNA (18). Our data show that mutations of some residues in GyrA-CTD can lead to complete loss of the DNA-binding activity of the GyrA subunit or the A 2B 2 holoenzyme (Figures 3 and 4). There must be some relation between the two main DNA-binding domains, and maybe it is related to the substrate preference of gyrase. Furthermore, the results of gel-retardation assays were confirmed by SPR (Figure 10). It was also confirmed that mutants of D669A, R691A, R745A can not bind DNA at all by SPR. Thus, it seems that the third blade is the main DNA-binding region in the GyrA-CTD, and the GyrA-CTD is the main DNA-binding domain in M. tuberculosis gyrase. These results are helpful to explain the DNA-binding mechanism of gyrase. GyrA-CTD was speculated to be involved in stabilizing the DNA–protein complex (18). Previous work proved that the removal of GyrA-CTD from E. coli gyrase results in loss of the ability to supercoil DNA, a gain of the ability to relax DNA, and a 30-fold increase in decatenation activity (18,33). This suggests that GyrA-CTD is required for supercoiling and has no role in relaxation and decatenation (18,33). However, in our experiments, mutations of the proposed DNA-binding sites in GyrA-CTD not only caused loss of supercoiling activity, but also led to the loss of relaxation (Figures 5 and 6). This suggested that, in M. tuberculosis, GyrA-CTD has also a role in the process of relaxing supercoiled DNA. But mutations of the proposed DNA-binding sites in GyrA-CTD not only caused loss of supercoiling activity, but also led to the loss of relaxation (Figures 5 and 6). This suggested that, in M. tuberculosis, GyrA-CTD has also a role in the process of relaxing supercoiled DNA. But mutations of the proposed DNA-binding sites in GyrA-CTD not only caused loss of supercoiling activity, but also led to the loss of relaxation (Figure 8), which indicates that GyrA-CTD is less important to these activities. This is consistent with the previous report (33). It is known that the supercoiling DNA activities of Mycobacterium bovis BCG is also much less than that of E. coli (42), and Mycobacterium smegmatis gyrase exhibits a stronger decatenase activity than E. coli gyrase (40). What is the cause of the difference? The protein sequences of

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<th>Mutants</th>
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<td>G729W</td>
<td>4.72×10$^6$</td>
<td>1.47×10$^3$</td>
<td>3.20×10$^7$</td>
<td>3.12×10$^8$</td>
</tr>
<tr>
<td>WT (A$_B$B$_b$)</td>
<td>3.56×10$^5$</td>
<td>6.58×10$^3$</td>
<td>5.41×10$^7$</td>
<td>1.85×10$^8$</td>
</tr>
<tr>
<td>E514A+GyrB</td>
<td>1.32×10$^6$</td>
<td>0.11</td>
<td>1.20×10$^3$</td>
<td>8.34×10$^8$</td>
</tr>
<tr>
<td>D669A+GyrB</td>
<td>2.82×10$^3$</td>
<td>5.82×10$^3$</td>
<td>4.85×10$^7$</td>
<td>2.06×10$^6$</td>
</tr>
<tr>
<td>G729W+GyrB</td>
<td>4.83×10$^5$</td>
<td>3.27×10$^3$</td>
<td>1.48×10$^3$</td>
<td>6.78×10$^8$</td>
</tr>
</tbody>
</table>

Figure 10. SPR experiment of DNA-GyrA bindings. (A) Sensorgram of the GyrA and its mutants in absence of GyrB. (B) Sensorgram of the GyrA and its mutants in presence of equal molar concentration (0.1 μM) of GyrB. The inserted table lists the kinetic constants derived from the sensorgrams.
GyrA-CTD are not as highly conserved as those of GyrA-NTD, and the percent sequence similarities of \textit{M. tuberculosis} GyrA-CTD compared with \textit{B. burgdorferi} GyrA-CTD and \textit{E. coli} GyrA-CTD only are 25.9% and 37.8%, respectively. The β-pinwheel of \textit{B. burgdorferi} GyrA-CTD is flat, whereas that of \textit{E. coli} GyrA-CTD is a spiral (15). The \textit{E. coli} GyrA-CTD wraps DNA inducing substantial positive superhelicity, whereas \textit{B. burgdorferi} GyrA-CTD introduces a more modest positive superhelicity (15). In other words, the diversity of GyrA-CTD may cause the differences in structure and function. It also indicates that the GyrA-CTD is essential to enzyme activities of \textit{M. tuberculosis} DNA gyrase and the precise catalytic mechanism of \textit{M. tuberculosis} gyrase may be different from that of other bacterial gyrase. It is very interesting to find that, although G729W still kept high-DNA-binding activity, its supercoiling activity was almost lost completely and its relaxation activity was much weak. From the association and dissociation responses in SPR (Figure 10), G729W has a different kinetic performance from other mutants: G729W showed slowed association and dissociation modes in the absence or presence of GyrB, whereas the other two (the wild type and E514A) showed fast association and dissociation process. This suggests that binding DNA by G729W largely reduces enzyme activities.

It has been shown that GyrB has no DNA-binding activity on its own (18), and consistent results were seen in this study (Figure 4). However, a double mutation of S759R and R760C in the C-terminal region of \textit{E. coli} GyrB significantly reduced the DNA-binding activity of gyrase (46), and the deletion of additional 165 residues in \textit{E. coli} GyrB also caused the almost complete loss of DNA-binding activity (47). Thus GyrB also has an important role in binding DNA by gyrase, despite its incapability of binding DNA by itself in gel-retardation assays. GyrB should have effects on the conformation of active sites of GyrA by interaction (28), for GyrA only when combined with GyrB, has enzyme activities. In our study, the D669A mutant of GyrA combined with GyrB turned out to have weak DNA-binding activity, compared with the D669A mutant alone, which had no DNA-binding activity at all (Figures 3, 4 and 10). However, the mutants of Y577A, R691A and R745A can not interact with DNA at all, even combined with GyrB (Figures 3, 4 and 10). Despite this, GyrB has the same effect on the constants, although it is much weaker: the A$_2$B$_2$ holoenzyme of WT, E514A and G729W has a lower association constant and a higher dissociation constant than those alone (Figure 10). In other words, GyrB retards the A$_2$B$_2$ holoenzyme to bind DNA, and accelerates the bound DNA to deviate from A$_2$B$_2$, which may have an important role in the catalytic mechanism of gyrase.

As shown in Figure 9, the mutations have no obvious effect on the ATPase activity. Either in the presence or absence of DNA, all mutations exhibited ATPase activities similar to that of the wild-type A$_2$B$_2$, implying that the DNA-dependent ATPase is not dependent on DNA binding by the GyrA-CTD.

Data reveal the DNA-stimulated ATPase activity is ~2-fold as high as the activity without stimulating by DNA. This increase is much lower than that observed with \textit{E. coli} gyrase (48). The reason remains unknown.

GyrA-CTD is conserved among gyrases and exhibits significant sequence diversity from the other type II topoisomerases. Eukaryotic topo IIs possess a CTD, but it appears to be completely different in structure and function from that of the prokaryotic CTD (1,2,6). Substitutions in GyrA-CTD can lead to the loss of DNA-binding activity, supercoiling activity and relaxation activities of \textit{M. tuberculosis} DNA gyrase, which stop crucial cellular processes in cells and lead to cell death. It shows that the GyrA-CTD is essential to \textit{M. tuberculosis} gyrase, and that drugs could be devised more specifically to act on \textit{M. tuberculosis} GyrA-CTD more selectively, based on the divergence of different bacterial GyrA-CTD. Thus, \textit{M. tuberculosis} GyrA-CTD is a potential new target for drug design. It also is the first time that the DNA-binding sites in GyrA-CTD have been identified.

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