Mechanisms for Defining Supercoiling Set Point of DNA Gyrase Orthologs

II. THE SHAPE OF THE GyrA SUBUNIT C-TERMINAL DOMAIN (CTD) IS NOT A SOLE DETERMINANT FOR CONTROLLING SUPERCOILING EFFICIENCY*□·

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DNA topoisomerases are essential enzymes that can overwind, underwind, and disentangle double-helical DNA segments to maintain the topological state of chromosomes. Nearly all bacteria utilize a unique type II topoisomerase, gyrase, which actively adds negative supercoils to chromosomes using an ATP-dependent DNA strand passage mechanism; however, the specific activities of these enzymes can vary markedly from species to species. *Escherichia coli* gyrase is known to favor supercoiling over decatenation (Zechiedrich, E. L., Khodursky, A. B., and Cozzarelli, N. R. (1997) *Genes Dev.* 11, 2580–2592), whereas the opposite has been reported for *Mycobacterium tuberculosis* gyrase (Aubry, A., Fisher, L. M., Jarlier, V., and Cambau, E. (2006) *Biochem. Biophys. Res. Commun.* 348, 158–165). Here, we set out to understand the molecular basis for these differences using structural and biochemical approaches. Contrary to expectations based on phylogenetic inferences, we find that the dedicated DNA wrapping domains (the C-terminal domains) of both gyrase are highly similar, both architecturally and in their ability to introduce writhe into DNA. However, the *M. tuberculosis* enzyme lacks a C-terminal control element recently uncovered in *E. coli* gyrase (see accompanying article (Tretter, E. M., and Berger, J. M. (2012) *J. Biol. Chem.* 287, 18636–18644)) and turns over ATP at a much slower rate. Together, these findings demonstrate that C-terminal domain shape is not the sole regulatory determinant of gyrase activity and instead indicate that an inability to tightly couple DNA wrapping to ATP turnover is why *M. tuberculosis* gyrase cannot supercoil DNA to the same extent as its γ-proteobacterial counterpart. Our observations demonstrate that gyrase has been modified in multiple ways throughout evolution to fine-tune its specific catalytic properties.

DNA topoisomerases are ubiquitous enzymes that help maintain chromosome superstructure and integrity by countering the topological consequences of nucleic acid transactions such as DNA replication and transcription. Topoisomerases fall into two general categories, type I and type II, depending on their respective ability to cleave either one or two DNA strands during catalysis. Although all bacteria sustain a steady-state level of DNA supercoiling by utilizing a combination of both topoisomerase types (1, 2), variations in topoisomerase specialization and composition can greatly affect DNA compaction and gene regulation between species (3–5).

Nearly all bacteria possess a distinct type IIA topoisomerase, termed gyrase, which introduces negative supercoils into DNA using an ATP-dependent DNA duplex strand passage mechanism (4, 6, 7). Gyrase is an $A_2B_2$ heterotetramer whose supercoiling activity requires a dedicated DNA binding domain (the CTD) that forms the C-terminal portion of the GyrA subunit (8). The GyrA CTD is thought to constrain a positive supercoil by wrapping a DNA duplex around its surface (9–12); this wrap is then converted into two negative supercoils upon strand passage (13). A highly conserved amino acid sequence motif, termed the GyrA box (14), maps to the N-terminal region of the CTD (15).

Gyrase is complemented in many bacteria by the presence of a closely related paralog, topo IV, whose ParC subunits bear a divergent variant of the GyrA CTD (4, 16, 17). In contrast to negatively supercoiling DNA, topo IV primarily relaxes positive DNA supercoils and unlinks entangled DNA segments such as...
Modulating Gyrase Activity–II

catenanes (18–20). Architectural differences between the ParC and GyrA CTDs, such as the deletion of the GyrA box and/or internal subdomains, have been shown to be at least partly responsible for the different activities displayed by gyrase and topo IV on DNA (9, 16, 21). Other considerations, such as CTD position, may also play a role in differentiating between the two enzymes. A number of structural studies in particular have shown that there exists a marked diversity of CTD shapes. Some of these differences, such as whether a CTD is a planar disc or lockwasher-shaped, have been suggested to control the degree to which DNA is wrapped by the GyrA CTD (11). Other features, such as the presence or absence of conserved amino acids at key positions (such as a subdomain interface), have in turn been implicated in controlling CTD shape (9). As yet, there has not been a systematic test of many of these proposals.

The genome of Mycobacterium tuberculosis encodes only a single gyrase ortholog, along with one type IA topoisomerase (22). Because topo IV is the principal agent responsible for unlinking newly replicated chromosomes in Escherichia coli (23), whereas topo IA primarily relaxes negatively supercoiled DNA (24, 25), this combination suggested that M. tuberculosis might employ one of its two topoisomerases in a nonconventional manner. Recent biochemical studies have provided support for this idea, indicating that the decatenation activity of M. tuberculosis gyrase is more robust relative to its supercoiling functions as compared with E. coli gyrase (26). The mechanism underlying this difference likewise has not been elucidated.

In the course of comparing gyrase functions, we noted that the CTD of M. tuberculosis GyrA (MtGyrA) contains a substitution at the site of a conserved proline that had been proposed to induce a spiral configuration in the domain. Hypothesizing that this change might lead to a new CTD structure that would wrap DNA less effectively, and hence, explain the depressed supercoiling capabilities of M. tuberculosis gyrase, we determined the structure of the M. tuberculosis CTD and characterized its biochemical properties. Surprisingly, we found that the domain adopts a spiral shape nearly indistinguishable from that exhibited by the E. coli GyrA CTD and that the M. tuberculosis CTD was also capable of robustly introducing writhe into DNA. Further investigation revealed that what instead differentiates M. tuberculosis gyrase is that it: 1) bears a naturally truncated version of the unstructured CTD tail that potentiates supercoiling in the E. coli enzyme (see accompanying article (27)) and 2) possesses a much lower DNA-stimulated ATPase activity. Together, these factors appear not to improve decatenation by M. tuberculosis gyrase per se, but rather to alter its supercoiling “set point,” such that the enzyme is incapable of underwinding DNA to the same degree as its E. coli ortholog. Thus, variations in gyrase activity and supercoil addition equilibrium are not solely determined by the CTD and its shape, but can be controlled by a variety of complex factors.

**EXPERIMENTAL PROCEDURES**

**Protein Purification**—The coding regions of the M. tuberculosis GyrA CTD (514–838), full-length M. tuberculosis gyrA (1–838), and full-length gyrB (1–714) were amplified from genomic DNA (ATCC) and cloned into a derivative of pET28b behind an N-terminal, tobacco etch virus protease-cleaveable hexahistidine tag using an in-house ligation independent cloning vector system (pLIC). The truncated E. coli GyrA CTD (531–853) and full-length E. coli gyrA (1–875) and gyrB (1–804) genes were cloned into pET28b. Proteins were expressed in E. coli BL21-CodonPlus(DE3)-RIL cells (Stratagene) by inducing log-phase cells with 0.25 mM isopropyl-β-D-thiogalactopyranoside either for 4 h at 37 °C or overnight at 18 °C. Full-length M. tuberculosis GyrB was expressed in BL21-CodonPlus(DE3)-pLysS cells by inducing log-phase cells with 1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 30 °C. Cells were harvested by centrifugation, resuspended in 20 mM Tris-HCl, pH 7.9, 800 mM NaCl, 30 mM imidazole, 10% glycerol, and protease inhibitors (1 μM leupeptin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), and frozen dropwise in liquid nitrogen for storage at −80 °C.

For purification, cells were sonicated and centrifuged, and the clarified lysate was passed over an Ni2+ affinity column (Amersham Biosciences). His-tagged protein was eluted with 20 mM Tris-HCl, pH 7.9, 100 mM NaCl, 500 mM imidazole, 10% glycerol, and protease inhibitors (1 μM leupeptin, 1 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), concentrated, and exchanged into the same buffer containing 30 mM imidazole, and then incubated overnight at 4 °C with 1–1.5 mg of hexahistidine-tagged tobacco etch virus protease (28). Following tobacco etch virus cleavage, the mixture was passed over an Ni2+ affinity column, and the flow-through was collected, concentrated (Millipore Amicon Ultra-10/30), and run over an S-200 or S-300 gel filtration column (Amersham Biosciences) in 50 mM Tris-HCl, pH 7.9, 500 mM KCl, 10% glycerol, and 2 mM 2-mercaptoethanol. Peak fractions were pooled and concentrated by centrifugal filtration (Millipore Amicon Ultra-10/30) and assessed for purity by SDs-PAGE (supplemental Fig. S1). M. tuberculosis GyrB was purified using the above protocol but with the following Ni2+ affinity column buffers: load/wash buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 5 mM imidazole) and elution buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, 250 mM imidazole).

**Crystallization, Data Collection, and Structure Solution**—Purified M. tuberculosis GyrA CTD at 12 mg/ml was dialyzed overnight at 4 °C against 50 mM NaCl and 10 mM Tris-HCl, pH 7.5. Crystals were grown in mosquito drop hanging drop format by mixing 100 nl of protein with 100 nl of well solution containing 0.2 M calcium acetate hydrate and 20% polyethylene glycol 3350. For harvesting, crystals were looped and flash-frozen in liquid nitrogen.

A single native dataset was collected at Beamline 8.3.1 at the Advanced Light Source at Lawrence Berkeley National Laboratory (29). Native data were indexed and reduced as P22,22,2 using HKL2000 (see Table 1) (30). Phasing by molecular replacement using the E. coli GyrA CTD (residues 532–853, Protein Data Bank (PDB) code 1ZI0) revealed one M. tuberculosis GyrA CTD protomer per asymmetric unit (11, 31). Density modification and initial model building were performed by PHENIX (32); subsequent cycles of manual rebuilding and refinement were performed using COOT (33). TLS parameters were analyzed with the TLSMD server (34), and 15 TLS groups were introduced in the later stages of refinement. The final model was refined to 1.65 Å resolution, with a final Rwork/Rfree of...
of 22.2/18.7%. Molprobit analysis (35) showed a total of 99% of residues in the most favored regions of Ramachandran space, with none in disallowed regions. Ribbons structures, electron density, and surface charge images were all prepared in PyMOL (36).

**DNA Binding Assays**—DNA binding was determined by fluorescence anisotropy. The DNA substrate for binding was a random, 37-bp segment with 40% GC content. Annealed oligonucleotides 5′-TAA AGT CTA GAG ACA CGC ATA GTC AAT GAC GGA GTT A-3′ and 5′-[56-FAM] TAA CTC CGT CAT TGA CTA TGC GTG TCT CTA GAC TTT A-3′ (where 56-FAM indicates the position of a carboxyfluorescein dye for visualization) were purchased from Integrated DNA Technologies and resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). For binding, varying amounts of truncated *E. coli* GyrA CTD or *M. tuberculosis* GyrA CTD were incubated with 20 nM of fluorescently labeled duplex oligonucleotide at room temperature in the dark in 20 mM Tris-HCl, pH 7.5, 70 mM KCl, 10% glycerol, and 1 mM MgCl₂. Fluorescence anisotropy measurements were performed using a Victor 3V (PerkinElmer Life Sciences) multilabel plate reader. Data points represent the average of three independent measurements on each plate, whereas error bars represent the S.D. between measurements (Figs. 2 and 7). Binding curves were fit to a simplified version of the single site binding equation that holds for our experimental conditions using KaleidaGraph version 4.0 (Synergy software)

\[
\theta = \frac{[L]}{K_{d,app} + [L]}
\]  
(Eq. 1)

where \( \theta \) represents the fraction of ligand binding sites filled, \( K_{d,app} \) is the apparent dissociation constant, and \( L \) is the ligand concentration.

**DNA Relaxation, Decatenation, and Supercoiling Assays**—pSG483, a derivative of pUC19 containing a unique Nb.BbvCI nicking site (New England Biolabs), was used for supercoiled and relaxed DNA substrates. Negatively supercoiled plasmid was purified from *E. coli* with a maxiprep kit (Macherey-Nagel). Relaxed plasmid was made using nick ligation by first nicking maxiprepped plasmid DNA with Nb.BbvCI and then ligating with T4 DNA ligase. Kinetoplast DNA (kDNA) from *Crithidia fasciculata* was purified in-house as described (37). DNA relaxation, decatenation, or supercoiling assays (30 μl) were performed in a buffer containing 15 mM Tris-HCl, pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, and 1 mM dithioerythritol, 2 mM ATP, and 300 ng (6 nM) of DNA substrate. To assess activity, reactions were supplemented with varying amounts of reconstituted holoenzyme, ATP was added to reaction mixtures, and reactions were allowed to proceed for 30 min at 37 °C. Time course reactions contained 4 mM ATP, with an additional 2 mM ATP added at 60 min. Reactions were stopped by the addition of SDS (1% final concentration) and EDTA (10 mM final concentration). Quenched reactions were analyzed by electrophoresis through 1.0% agarose gels with 1X TAE running buffer (40 mM Tris-acetate, 1 mM EDTA) and chloroquine gels containing 3 μg/ml chloroquine (Sigma) in the gel and running buffer. Gels were run at 1.7–5.0 volts/cm for 4–12 h at 25 °C, stained with ethidium bromide (EtBr), and visualized by UV transillumination.

**Topological Footprinting Assays**—DNA write assays (30 μl) were performed in a buffer containing 15 mM Tris-HCl, pH 7.5, 13% glycerol, 6 mM MgCl₂, 0.1 mg/ml BSA, 70 mM KCl, and 300 ng (6 nM) of nicked pSG483 as DNA substrate. Varying amounts of CTD were added to reaction mixtures, and the reactions were equilibrated at 37 °C for 20 min, after which 60 units of ligase and 1 mM ATP were added to each reaction mixture. Reactions were then allowed to incubate at 37 °C for an additional 20 min. Reactions were stopped by the addition of SDS (1% final concentration), EDTA (10 mM final concentration), and proteinase K (50 μg/ml final) and incubated at 37 °C for 30 min. Reactions were analyzed by electrophoresis through 1.0% agarose gels with 1X TAE running buffer. Gels were run at 1.7 volts/cm for 19–21 h at 25 °C, stained with ethidium bromide (EtBr), and visualized by UV transillumination.

To capture a gyrase holoenzyme “topology footprint,” we used an ATP-independent assay technique to prevent gyrase from supercoiling the plasmid DNA substrate. In these reactions, gyrase holoenzyme was incubated with relaxed plasmid and eukaryotic topoisomerase IB, which relaxes DNA supercoils in the absence of ATP (38, 39). Holoenzyme write assays were performed in the presence and absence of 2 mM AMP-PNP using relaxed pSG483 plasmid and topoisomerase IB purified in-house from wheat germ (40).

**ATPase Assays**—ATPase assays were performed using a malachite green/molybdate assay for free phosphate. Reactions, run under supercoiling assay conditions, contained 50 nM gyrase, 1 mM ATP, and 50 μM sheared salmon sperm DNA (Sigma). Reactions were initiated by the addition of enzyme and ATP and incubated for varying times at 37 °C. After incubation, 200 μl of malachite green reagent (0.034% malachite green, 10 mM ammonium molybdate, 1 N hydrochloric acid, 3.4% ethanol, and 0.01% Tween 20) was added to each reaction and incubated at room temperature in the dark for 5 min. The malachite green reagent was made fresh daily and sterile-filtered. Absorbance at 620 nm was measured using a PerkinElmer Life Sciences Victor 3V plate reader. All reactions were performed in triplicate.

**RESULTS**

**Structure of MtbGyrA CTD**—To first assess the physical basis for the activity differences seen between *M. tuberculosis* and *E. coli* gyrase, we determined the structure of the MtbGyrA CTD. Crystals of the isolated domain (residues 514–838) were refined to an excellent stereochemistry (Table 1). The structure was solved by molecular replacement using a polyserine model of the *E. coli* GyrA CTD (residues 532–853, PDB code 1Z1O) (Fig. 1A) (11). The final model, which comprises amino acids 514–538 and 547–819 of the *M. tuberculosis* CTD, was refined to an R_work/R_free of 18.76/22.21% with excellent stereochemistry (Table 1).

As seen in other GyrA and ParC CTDs, the *M. tuberculosis* gyrase CTD forms a “β-pinwheel” structure similar to, but topologically distinct from, canonical β-propellers (Fig. 1B). The *M. tuberculosis* CTD structure turns out to closely mimic that of the *E. coli* CTD (1.45 Å root mean square deviation) and
Modulating Gyrase Activity–II

TABLE 1

| Data collection and refinement statistics |
|------------------------------------------|
| **MtbGyrA CTD**                          |
| Resolution (Å)                           | 50.1-6.5 |
| Wavelength (Å)                           | 1.1000  |
| Space group                              | P2₁,2,1, |
| Unit cell dimensions (a, b, c) (Å)       | 38.87, 82.84, 83.43 |
| Unit cell angles (α, β, γ) (°)           | 90, 90, 90 |
| I/σ (last shell)                        | 27.1 (3.6) |
| Rmerge (last shell) (%)                  | 7.4 (5.6) |
| Completeness (last shell) (%)            | 100 (100) |
| Redundancy                               | 7.1 (7.0) |
| Unique reflections (%)                   | 33,301   |

**Refinement**

| Resolution (Å)                           | 41.72-1.65 |
| No. of reflections                       | 33,225     |
| Rwork (%) (last shell)                   | 18.76 (25.13) |
| Rfree (%) (last shell)                   | 22.21 (28.18) |

| Structure and stereochemistry            |
|------------------------------------------|
| No. of atoms                             | 2733      |
| Protein                                  | 2309      |
| Water                                    | 303       |
| Acetate                                  | 1         |
| Calcium                                  | 1         |
| Glycerol                                 | 1         |
| B-factor (Å2)                            | 20.72     |
| Protein                                  | 29.02     |
| Water                                    | 0.009     |
| r.m.s.d. bond lengths (Å)                | 1.182     |
| r.m.s.d. bond angles (°)                 | 90, 90, 90 |
| Ramachandran plot (%)                    | 99.0      |
| Favored region                           | 99.0      |
| Allowed region                           | 1.0       |
| Outliers                                 | 0         |

*The values in parentheses indicate the highest resolution shell.
*Rmerge = Σ [Fobs( hkl) - |Fcalc( hkl)|]/Σ |Fobs( hkl)|, where I( hkl) is the intensity of an observation and |Fcalc( hkl)| is the mean value for its unique reflection. Summations cover all reflections.

*Naobs/Nave. 
*Rwork = Σ [Fobs( hkl) - |Fcalc( hkl)|]/Σ |Fobs( hkl)|, Rfree was calculated same way as Rwork, but with the reflections excluded from refinement. The Rfree set was chosen using default parameters in PHENIX (32).

**D. Bacterial gyrase—Because biochemical properties are not necessarily evident from structure alone, we next asked whether the M. tuberculosis and E. coli CTDs might associate with DNA differently. We first compared the relative affinities of two CTDs for short DNA substrates using fluorescence polarization. In this assay, a fluorescently labeled, 37-bp duplex oligonucleotide was incubated with varying amounts of purified E. coli or M. tuberculosis gyrase CTD. In the course of conducting these studies, we noted that the E. coli CTD constructs used for biochemical and structural studies by other groups actually employed a variant that lacked some or all of a nonconserved acidic tail located at the C terminus of the domain (11). These constructs bound DNA robustly in our hands, whereas a full-length M. tuberculosis CTD bound DNA with an affinity similar to that of the tail-truncated E. coli domain (Kd,app = 80 ± 16 nM and 90 ± 21 nM, respectively) (Fig. 2). An inspection of GyrA amino acid sequences shows that the M. tuberculosis CTD naturally lacks ~15 residues from its C-terminal tail as compared with E. coli (see Fig. 1B of the accompanying article [27]). Thus, although the innate DNA binding properties of the core CTD are similar between E. coli and M. tuberculosis gyrase, their CTD tails and the regulatory capacities of these elements are distinct.

DNA wrapping by the CTD is essential for supporting supercoiling by gyrase (8). Reasoning that binding affinity might not necessarily imply an equal proclivity to introduce writhe into DNA, we set out to compare the wrapping potential of the M. tuberculosis and E. coli CTDs using “topology footprinting” (10, 11). In this assay, each CTD was first individually mixed with nicked plasmid DNA, after which the DNA was ligated, trapping any writhe constrained by the protein. Bound CTD
molecules were removed by SDS/proteinase K, and the supercoiling state of the DNA was resolved by native agarose gel electrophoresis. Inspection of the topoisomers resulting from this treatment shows that both the full-length *M. tuberculosis* and the tail-truncated *E. coli* CTDs were able to constrain a similar amount of writhe with respect to one another (Fig. 3A). Consistent with its binding defect, the full-length *E. coli* CTD did not show any ability to wrap DNA (see accompanying article (27)).

Because the *E. coli* gyrase holoenzyme (41), but not the isolated GyrA subunit (10), can constrain writhe, we were curious whether *M. tuberculosis* gyrase and its GyrA subunit, which naturally lack a portion of the *E. coli* CTD tail, would behave similarly. When assayed by topology footprinting, *M. tuberculosis* GyrA was able to wrap DNA to a similar degree as its isolated CTD, whereas *E. coli* GyrA failed to exhibit any wrapping (Fig. 3B). By contrast, both the *E. coli* and the *M. tuberculosis* holoenzymes wrapped DNA nearly equivalently (Fig. 3C). To probe these differences further, we next assessed whether DNA wrapping by *M. tuberculosis* gyrase could be blocked by the nonhydrolyzable ATP analog AMP-PNP, a behavior seen for *E. coli* gyrase (42, 43). Strikingly, the ability of *M. tuberculosis* gyrase to introduce writhe was unaffected by the presence of AMP-PNP, whereas wrapping by *E. coli* gyrase was all but abolished (Fig. 3D). Taken together, these results demonstrate that the extent of DNA wrapping induced by *E. coli* and *M. tuberculosis* gyrase is not appreciably distinct; rather, only *E. coli* gyrase appears capable of regulating wrapping in an ATP-dependent manner.

Decatenation by *M. tuberculosis* Gyrase—Having established that an ability to switch between wrapped and unwrapped states, rather than an inherent structural or biochemical feature of the GyrA CTD, is a point of divergence for the *M. tuberculosis* and *E. coli* orthologs, we turned our attention to other properties of the two enzymes. We first examined DNA decatenation, which has been reported to be more efficient with *M. tuberculosis* gyrase than with the *E. coli* protein (26). Decat-
Decatenation was quantified by incubating each reconstituted holoenzyme with kinetoplast DNA (kDNA) and ATP and then resolving the products using native agarose gel electrophoresis. The large, catenated network of DNA minicircles that comprises kDNA is unable to enter the gel unless individual minicircles are liberated by decatenation by gyrase. Contrary to our expectations, we found that the observed decatenation efficiency of *E. coli* gyrase was comparable with, or even slightly better than, that seen for the *M. tuberculosis* enzyme (Fig. 4). Although the basis for this discrepancy was not immediately apparent, it did not arise from the presence of a significant portion of inactive enzyme in our *M. tuberculosis* gyrase preparations. Indeed, the specific activities of both our gyrase preparations were sufficient to nearly fully supercoil a target plasmid at a >50-fold molar excess of substrate DNA over enzyme under standard published conditions (Fig. 5A). This level of activity, which may arise as a consequence of a more extensive purification procedure than that used in prior studies, is ~5–10-fold greater than previously observed for the *M. tuberculosis* enzyme.

**DNA Supercoiling by *M. tuberculosis* Gyrase**—If *M. tuberculosis* and *E. coli* gyrase decatenate DNA similarly, then why might *E. coli* require a topo IV for chromosome segregation, whereas *M. tuberculosis* does not? To address this question, we re-examined the DNA supercoiling activity of the two proteins in detail. One clue came from inspection of our kDNA decatenation data (Fig. 4), which indicated that the minicircles liberated by *E. coli* gyrase were supercoiled more readily than those produced by the *M. tuberculosis* enzyme. Because kDNA is an unusual substrate, we further compared the activities of both gyrases on relaxed circular DNA in the presence of ATP.

Analysis of the resultant products by agarose gel electrophoresis at first appeared to show comparable supercoiling activities between the two orthologs (Fig. 5A). However, we noticed that the band containing the supercoiled DNA species produced by *M. tuberculosis* gyrase often appeared somewhat
“fuzzy,” or less condensed. Because native gels have an innate limit on their ability to resolve topoisomers that are significantly underwound with respect to one another (44), we turned to chloroquine gels to determine whether the degree of DNA supercoiling introduced by \textit{M. tuberculosis} gyrase might differ from that of \textit{E. coli}. Chloroquine is a weak intercalating agent that separates supercoiled topoisomers and allows for the resolution of negatively supercoiled DNA bands that would otherwise run together as a single high mobility band on a native gel (44). A portion of each reaction used in our native gel studies was run on an agarose gel in the presence of 3 \mu g/ml chloroquine, after which the chloroquine was soaked out and replaced with ethidium bromide for visualization. Notably, this analysis revealed that at comparable concentrations, \textit{M. tuberculosis} gyrase is unable to introduce as many negative supercoils into DNA as \textit{E. coli} gyrase (Fig. 5B).

Although this experiment suggested that \textit{M. tuberculosis} and \textit{E. coli} gyrase differ in yet another fundamental property, it did not rule out the possibility that strand passage by the \textit{M. tuberculosis} enzyme might simply be slow, and hence, failed to go to completion during the course of our assay (30 min). To assess this possibility, we assayed supercoiling over a range of times. The reconstituted \textit{E. coli} and \textit{M. tuberculosis} gyrase enzymes were added to relaxed plasmid DNA and ATP at two concentrations (5 and 20 nM), both of which completely supercoiled the substrate in our enzyme titration experiments, and incubated at 37 °C for 1, 5, 10, 30, and 90 min. Time points that exceeded 30 min were supplemented with additional ATP to prevent ATP-independent relaxation events from occurring as the nucleotide cofactor was depleted, and the results were again visualized using agarose gel electrophoresis in the absence and presence of 3 \mu g/ml chloroquine. We found that supercoiling for both enzymes is complete within 30 min and that even at longer times, \textit{M. tuberculosis} gyrase still does not underwind DNA to the same degree as \textit{E. coli} (Fig. 6).

\textbf{ATPase Activity of \textit{M. tuberculosis} Gyrase—}Because \textit{M. tuberculosis} gyrase is able to robustly bind and wrap DNA, but lacks a CTD control element (the tail) that permits the coordination between nucleotide turnover and strand passage in its \textit{E. coli} counterpart, we hypothesized that the slower and less extensive supercoiling seen for this mycobacterial enzyme might arise from a similar coupling inefficiency. To test this idea, we examined the ATPase activity of \textit{M. tuberculosis} and \textit{E. coli} gyrase using a malachite green colorimetric assay in which ATP hydrolysis is measured by phosphate release (45). We conducted these measurements by incubating reconstituted gyrase in our supercoiling assay conditions with salmon sperm DNA, which is known to dramatically stimulate the ATPase activity of the enzyme (46). The reactions were incubated for different amounts of time using a fixed starting concentration of DNA and ATP. Surprisingly, we found that although both orthologs exhibited ATPase activity, \textit{M. tuberculosis} gyrase turned over ATP at a significantly lower rate than \textit{E. coli} gyrase (Fig. 7). Taken together, these data suggest that strand passage is closely linked to ATP hydrolysis in \textit{M. tuberculosis} gyrase, but that two factors, a lack of coupling between DNA wrapping and ATP binding, as well as an intrinsically slow rate of

![FIGURE 6. Negative supercoiling time course assay using 5 and 20 nM holoenzyme. Time points are listed in minutes. A portion of each sample was run on a 1% agarose gel in the absence (top) and presence (bottom) of 3 \mu g/ml chloroquine. Protein concentrations are listed in nM holoenzyme, and DNA topoisomers are labeled with graphic representations across the bottom of the chloroquine gel. Relaxed and supercoiled DNA species are labeled with graphic representations on the right of the native gel. Mtb, \textit{M. tuberculosis}.](image-url)
Intrigued as to whether the CTD of *M. tuberculosis* might differ from its well studied counterpart in *E. coli*, we reasoned that the domain might adopt a more flattened conformation that would impair DNA wrapping and thereby the lower the supercoiling propensity of *M. tuberculosis* gyrase as a whole. Indeed, we found that the *M. tuberculosis* CTD was highly similar structurally to that of *E. coli* GyrA that controls DNA wrapping is another (see accompanying article (27)). Despite extensive study, it is often unclear from phylogenetic information alone which modifications impact gyrase function and how.

*M. tuberculosis* gyrase expands further upon these distinctions. Not only is gyrase the sole type IIA topoisomerase of *M. tuberculosis* (22), its ability to decatenate DNA is elevated as compared with its supercoiling activity (26) (Fig. 8). Because decatenation is a function generally associated with topo IV, we set out to structurally and biochemically define which elements and characteristics of *M. tuberculosis* gyrase might differ from its well studied counterpart in *E. coli*. We were particularly intrigued as to whether the CTD of *Mtb*GyrA might play a role because this domain has been implicated in the controlling the specificity of the gyrase and topo IV strand passage mechanism in numerous contexts (8, 16).

We began examining these issues by first determining the structure of the *Mtb*GyrA CTD. Phylogenetic and structural analyses had suggested that a buried proline (Pro-636 in *E. coli* GyrA) imparts a nonplanar, spiral shape to the domain in certain CTDs (9); this lockwasher configuration, when present, has been suggested to enhance DNA wrapping and supercoiling by the gyrase holoenzyme (11). As the *M. tuberculosis* CTD lacks the proline, we reasoned that the domain might adopt a more flattened conformation that would impair DNA wrapping and thereby lower the supercoiling propensity of *M. tuberculosis* gyrase as a whole. Instead, we found that the *M. tuberculosis* CTD was highly similar structurally to that of *E. coli* GyrA (Fig. 1) and that the domain could robustly bind and introduce writhes into DNA (Figs. 2 and 3). Thus, the difference in shape between relatively planar CTDs (as with *Borrelia burgdorferi* GyrA) (12) and more skewed forms is not due to solely to a single amino acid. Likewise, the *M. tuberculosis* CTD did not contain a particularly distinct shape that might have been responsible for the altered supercoiling functions exhibited by *M. tuberculosis* gyrase.

The emergence of this result led us to probe the specific activities of *M. tuberculosis* gyrase further. Comparative biochemical studies with isolated domains and subunits, as well as the reconstituted holoenzyme, eventually highlighted several points of divergence between *M. tuberculosis* and *E. coli* gyrase. One was an ability of full-length *Mtb*GyrA and GyrA CTD to wrap DNA (Fig. 3). This activity is exhibited by mutants of *E. coli* GyrA that lack a nonconserved C-terminal tail (see accompanying article (27)), but not by the full-length *E. coli* proteins (10). A second difference was that *M. tuberculosis* gyrase failed to show any coupling between DNA wrapping and nucleotide binding (Fig. 3), an established property of the *E. coli* enzyme (42). A third variance was the finding that *M. tuberculosis* gyrase does not possess an enhanced ability *per se* to decatenate DNA relative to *E. coli*, but rather exhibits a diminished capacity to underwind DNA to the same extent (Figs. 4–6). Finally, we found that *M. tuberculosis* gyrase hydrolyzes ATP at a much slower rate than *E. coli* (Fig. 7).

**FIGURE 7.** ATPase activity. *E. coli* and *M. tuberculosis* (*Mtb*) gyrase ATPase activities are plotted as a function of nmol of phosphate produced (x axis) and time in minutes (y axis). The graph in the inset provides a magnified version of the *M. tuberculosis* gyrase ATPase data.

**FIGURE 8.** Schematic comparing relative supercoiling set points of a select number of bacterial type IIA topoisomerases. Topo IV relaxes DNA. *E. coli* gyrase can negatively supercoil DNA both rapidly and extensively. *M. tuberculosis* (*Mt*) and *S. typhimurium* gyrase, as well as an *E. coli* gyrase lacking the C-terminal tail of the GyrA subunit, can all negatively supercoil DNA, but to a lesser degree than wild-type *E. coli* gyrase. Some of the mechanical properties that appear to mediate these differences are listed at bottom.
Together, these distinguishing features have important implications for the mechanism of both *M. tuberculosis* gyrase and gyrase in general. The ability of gyrase to supercoil DNA derives in part from its DNA wrapping activity (8, 48), which juxtaposes two DNA segments in *cis* prior to strand transport. This property in turn disfavors the passing of two distal DNA segments through each other, impeding DNA decatenation and unknotting. *M. tuberculosis* gyrase can wrap DNA efficiently, thereby setting the stage for a single round of supercoiling. However, it does not appear to link the formation or dissolution of this wrapped state to the ATPase cycle (Fig. 3), which in *E. coli* gyrase occurs through the action of the tail appended to the C terminus of GyrA (see accompanying article (27)). Indeed, removal of the GyrA tail reduces the supercoiling set point of *E. coli* gyrase significantly, to a level close to that observed here for *M. tuberculosis* gyrase, yet does not alter the innate decatenation properties of the enzyme (supplemental Fig. S2); these findings suggest that part of the reason *M. tuberculosis* gyrase does not possess as robust a supercoiling capacity as *E. coli* is in part because it lacks this evolutionary modification. By failing to stabilize or release a wrapped DNA state at the correct point in time, the *M. tuberculosis* enzyme may be more prone to “slippage,” particularly as more energy becomes required to supercoil DNA to higher and higher levels. This characteristic would in turn favor strand passage events that do not have as great an energetic consequence, such as those needed to support chromosome disentanglement.

However, an inability to connect DNA wrapping to ATP binding or to supercoil DNA extensively does not imply that *M. tuberculosis* gyrase is an inefficient enzyme. In *E. coli*, removal of the GyrA tail dramatically slows the rate of supercoiling, but has little effect on the ATPase activity of gyrase as whole, resulting in a large increase of apparently futile cycles. By contrast, *M. tuberculosis* gyrase hydrolyzes ATP at a much lower rate, but is also slow to supercoil DNA (Figs. 6 and 7). Thus, ATP turnover rates and strand passage appear to be linked in *M. tuberculosis* gyrase, although the enzyme lacks the tail found in *E. coli* GyrA. Why *M. tuberculosis* gyrase is slow is not clear, but conceivably could be an adaptation to the sluggish life cycle of its host organism (23 h average generation time) (49). This concept is line with recent findings in other bacterial systems; for example, *E. coli* and *Salmonella typhimurium* gyrase appear to have evolved different supercoiling set points as a means to accommodate different rates of transcription (5), whereas the gyrase of *Aquifex aeolicus* appears to have undergone a relatively recent conversion into a topo IV, possibly to accommodate life at hyperthermophilic temperatures (21, 50). The slow rate of turnover exhibited by *M. tuberculosis* gyrase may also play a role in controlling the likelihood of sampling *cis* and *trans* strand passage events; a long dwell time in an inactive state where DNA wrapping and ATPase status are uncoupled would favor unwrapping, and thus loss of the transported segment in *cis*, disfavoring supercoiling as a nucleic acid substrate becomes progressively more unwound. Further analysis of gyrase orthologs from a range of bacterial species with distinct physiologies and environmental niches will be needed to more fully grasp the myriad of means by which evolution appears to have specialized type IIA topoisomerase function.

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