Direct observation of topoisomerase IA gate dynamics

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Type IA topoisomerases cleave single-stranded DNA and relieve negative supercoils in discrete steps corresponding to the passage of the intact DNA strand through the cleaved strand. Although type IA topoisomerases are assumed to accomplish this strand passage via a protein-mediated DNA gate, opening of this gate has never been observed. We developed a single-molecule assay to directly measure gate opening of the Escherichia coli type IA topoisomerases I and III. We found that after cleavage of single-stranded DNA, the protein gate opens by as much as 6.6 nm and can close against forces in excess of 16 pN. Key differences in the cleavage, ligation, and gate dynamics of these two enzymes provide insights into their different cellular functions. The single-molecule results are broadly consistent with conformational changes obtained from molecular dynamics simulations. These results allowed us to develop a mechanistic model of interactions between type IA topoisomerases and single-stranded DNA.
**Fig. 1** Proposed mechanism of type IA topoisomerase activity. 

a. Crystal structures of the *E. coli* type IA topoisomerases, topo III (PDB 1I7D) and topo I (PDB 1MW8). DNA bound to the active site is shown in orange, and the catalytic tyrosines are highlighted in green. For simplicity, the C-terminal zinc-finger domains of topo I are not shown.  

b. Proposed model of type IA topoisomerase protein-mediated DNA-gate mechanism for strand passage.

**Fig. 2** Magnetic tweezers measurements of topoisomerase binding and gate opening. 

a. Experimental design (not to scale). Hairpin DNA was unfolded at high force in the presence of topoisomerase. Gate opening was detected as an increase in DNA extension beyond the fully extended state. Binding was detected as pauses in refolding after the force was decreased.  

b. Example experimental traces for topo III, topo I, and topo I Y319F. Black lines indicate DNA extension. Blue lines represent the force, which alternated between ~9 pN and ~22–24 pN. Green arrows indicate gate-opening events. Blue arrows indicate binding. Differences in the baseline extension of the unfolded state are due to variation in the applied force.  

c. Expanded examples of gate opening events for topo III and topo I. Extension increases were 6.6 ± 0.7 nm for topo III and 6.6 ± 1.0 nm for topo I; force, ~22–24 pN (Supplementary Fig. 1). There is a difference in time scales, as indicated.  

d. Correlation between gate opening and topoisomerase binding events. The average number of opening events ($N_{\text{open}}$) for a given number of bound proteins ($N_{\text{bound}}$) is plotted. Topo III is shown in cyan ($n_{\text{tethers}} = 4$, $n_{\text{cycles}} = 58$), topo I in dark blue ($n_{\text{tethers}} = 5$, $n_{\text{cycles}} = 25$), and topo I catalytic mutant Y319F in red ($n_{\text{tethers}} = 5$, $n_{\text{cycles}} = 18$). Error bars, s.d. Dashed lines indicate linear fits to the data with slope 0.16 ± 0.05 for topo III, 0.9 ± 0.5 for topo I, and 0.0 for topo I Y319F.
at high force. Protein-mediated cleavage associated with increases in extension was confirmed by a rapid loss of DNA tethers that had lengthened in the presence of topoisomerase after denaturation of the protein by the addition of SDS, which has previously been used to confirm topo IA–mediated DNA cleavage (Supplementary Note 1 and Supplementary Fig. 1g).

A comparison of the number of ~6.6 nm extension increases (N_open) with the number of binding events (N_binding) for each unfolding–refolding cycle indicated that these numbers are linearly related (Fig. 2d). Interestingly, nearly all the bound topo I molecules cleaved and opened the DNA, whereas only a small fraction of topo III molecules did. We observed no opening events in the absence of bound protein. From these results, we concluded that the 6.6 nm extension increases correspond to the opening of a topoisomerase-mediated DNA gate.

**Force dependence of gate dynamics.** To accurately characterize the conformational changes and the kinetics of the gate dynamics, we developed an assay that allowed us to measure the gate dynamics of a single topoisomerase bound to ssDNA as a function of force. Force selectively affects the kinetic transitions involving motion by altering the underlying free-energy profile. For these experiments, a 2.5 kb dsDNA with a 37 nt gap was attached to a coverslip on one end and a magnetic bead on the other (Fig. 3a).

Opening and closing of the topoisomerase gate was observed as transient increases in the DNA extension (Fig. 3b) \( (n_{	ext{onset}} = 8 \) for topo III; \( n_{	ext{onset}} = 7 \) for topo I). The average size of the extension change was 5.5 ± 0.4 nm for topo III and was independent of the applied force (8–16 pN) (Supplementary Fig. 2 and Supplementary Table 1). For topo I, the extension change increased slightly with applied force (12–18 pN) (Supplementary Fig. 2 and Supplementary Table 1) and had an average value of 5.9 ± 0.6 nm. Unexpectedly, the topoisomerases were able to close the gate and religate DNA against forces of 18 pN.

To obtain kinetic states from the extension data, we used the program vbFRET \( ^{40} \) (Fig. 4), which applies an unbounded hidden Markov model to determine the number of states in single-molecule time traces. The program reliably found two extension states for both topo III and topo I. However, analysis of the lifetime distributions revealed a third state; in addition to the open state and a long-lived closed state, there is a short-lived closed state (Fig. 4a,b).

This transient closed state is consistent with a three-state model in which the protein–ssDNA complex is in a ligated state (L), a closed cleaved state (C), or an open cleaved state (O) (Fig. 4b), thus resulting in the following kinetic scheme:

\[
\begin{align*}
\text{k}_{\text{cleavage}} & \quad \text{k}_{\text{open}} \\
\text{L} & \quad \text{C} \quad \text{O} \\
\text{k}_{\text{ligation}} & \quad \text{k}_{\text{close}}
\end{align*}
\]

In this model, the short-lived closed state corresponds to state C, which the protein may visit multiple times before religiating the DNA and returning to the longer-lived closed state L. Although we assigned three states to our data, there are likely to be additional states that cannot be directly observed.

The gate kinetics as a function of force for both topo III and topo I is plotted in Fig. 4c. For topo III, the lifetimes of the long- and short-lived closed states are well separated, and the individual lifetime distributions were fit to single exponentials to determine \( k_{\text{open}} \) and \( k_{\text{close}} \) (Supplementary Fig. 3a,b). For topo I, the short and long closed-state lifetimes were comparable, and the closed-state-lifetime distribution was best fit with a double exponential (Supplementary Fig. 3c). The distribution of open-state lifetimes was fit to an exponential to determine \( k_{\text{close}} \) (Supplementary Fig. 4a,b).

To estimate the ligation rate, we considered the kinetic competition between ligation and opening from the closed state, C. The probability of opening from state C depends on the rates of these two competing pathways:

\[
P_{\text{open}} = \frac{k_{\text{open}}}{k_{\text{open}} + k_{\text{ligation}}}
\]

We determined \( P_{\text{open}} \) from the number of opening events (short-lived closed states) relative to the total number of closed-state events.

For both topoisomerases, \( k_{\text{ligation}} \) and \( k_{\text{close}} \) were insensitive to the applied force (Fig. 4c and Supplementary Table 2). Unexpectedly, the opening rates were also unaffected by the applied force (Fig. 4c and Supplementary Table 2). This lack of force dependence indicates that the opening rate reflects a rate-limiting step that precedes the mechanical opening of the gate. The closing rates exhibited an exponential force dependence (Fig. 4c, \( k(F) \approx k_0 \exp(-F\Delta x/k_B T) \)), where \( k_0 \) is the zero-load closing rate, \( \Delta x \) is the distance from the open state to the transition state, and \( k_B T \) is the thermal energy. The distances to the transition state, \( \Delta x_{\text{close}} \), obtained from exponential fits, were 1.10 ± 0.01 nm for topo III and 2.2 ± 0.1 nm for topo I. The zero-load closing rates were 1.8 ± 0.3 s\(^{-1}\) for topo III and 1.642 ± 549 s\(^{-1}\) for topo I (Table 1).

The lifetime of the open state was several orders of magnitude longer for topo III than topo I (Fig. 4c and Table 1). Biochemical and single-molecule studies have demonstrated that topo I is generally more effective at relaxing negatively supercoiled DNA than topo III \( ^{57,58} \), whereas topo III is more effective at unlinking DNA catenanes \( ^{57,58} \). Relaxation of negative supercoils requires passage of a single DNA strand through the protein-mediated DNA gate. In contrast, decatenation requires passage of dsDNA. The more stable open state of topo III may facilitate capture of duplex DNA required for decatenation, whereas the faster dynamics of topo I is compatible with capturing a local ssDNA segment. The fast closing rate also explains the difficulty in capturing the open conformation of topo I; only by slowing gate closing through the application of substantial opposing forces could the open state configuration be observed.

The estimated rate of a full catalytic cycle on the basis of extrapolated zero-force kinetics of the individual steps for topo I was 0.64 ± 0.38 s\(^{-1}\), a value comparable to previous relaxation-rate measurements (Table 1 and Supplementary Table 3). Conversely, the estimated rate of the topo III catalytic cycle was 0.0011 ± 0.0004 s\(^{-1}\). This unexpectedly slow rate, which was dominated by the cleavage rate, is much slower than the measured relaxation rates. The slow cleavage rate is consistent with previously observed long lag times (up to ~129 s for negatively supercoiled DNA) between relaxation events for topo III \( ^{57,58} \). However, these experiments...
also demonstrated much faster processive relaxation by topo III (between ~19 s⁻¹ and 129 s⁻¹; Table 1 and Supplementary Table 3). One possible explanation for this discrepancy is the absence of a second DNA strand in our experiments. If topo III cleavage and ligation rates were stimulated by the presence of a second ssDNA or dsDNA strand, then the passage of an initial strand might lead to the previously observed fast bursts of processive activity. The requirement of a second strand of DNA would align with kinetic studies of *Sulfolobus solfataricus* topo III, in which ligation of cleaved ssDNA is facilitated by annealing of a complementary strand.

**Magnesium-dependent kinetics.** Although magnesium is necessary for the activity of type IA topoisomerases, the distinct roles that magnesium plays in cleavage and religation remain under debate. Previous studies have shown that increasing magnesium concentration increases the cleavage product, but magnesium is not required for cleavage. Religation requires magnesium, but the magnesium dependence of the religation rate is unknown. We determined the effect of magnesium on the cleavage and religation rates by measuring the gate kinetics as a function of magnesium concentration (Fig. 4d and Supplementary Table 4).

The cleavage rate of both proteins was independent of the magnesium concentration (Fig. 4d and Supplementary Table 4). The ligation rate of topo III depended strongly on the magnesium concentration (Fig. 4d and Supplementary Table 4). The magnesium-concentration dependence of ligation and cleavage rates for topo III at 8-pN force (n tether = 3, 8, and 2 for 1, 3, 5, and 10 mM Mg²⁺, respectively) and topo I at 12-pN force (n tether = 6, 4, 3, 5, and 2 for and 0, 0.1, 0.3, 0.5, and 3 mM Mg²⁺). The topo III magnesium-dependent ligation rate was fit with a sigmoid with a Kₘ of 8.3 ± 1.2 mM Mg²⁺ and a Vₘₐₓ of 17 ± 5 s⁻¹.

### Table 1 | Equilibrium rates and barriers

| Protein | kₑ原则上 (s⁻¹) | kᵣ原则上 (s⁻¹) | kₑopen (s⁻¹) | kᵣclose (s⁻¹) | Δxₑopen (nm) | Δxᵣclose (nm) |
|---------|----------------|----------------|--------------|---------------|--------------|---------------|
| Topo III | 0.0012 ± 0.0004 | 1.5 ± 0.7 | 1.07 ± 0.17 | 1.8 ± 0.3 | 1.10 ± 0.04 | 1.3 ± 0.4 |
| Topo I | 0.9 ± 0.2 | 4.6 ± 1.0 | 4.1 ± 1.0 | 1.642 ± 549 | 2.2 ± 0.1 | 2.9 ± 0.4 |

Zero-load values (associated with Fig. 6) for kₑ原则上, kᵣ原则上 and the rate-limiting conformation changes associated with gate opening, kₑopen, and closing, kᵣclose. Δxₑopen values calculated from experiments and simulations are also shown. In the case of Topo III, this corresponds to Δxᵣclose, the distance between the fully open state and the barrier associated with formation of an open-state-stabilizing salt bridge.
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and a third acidic residue in the acidic loop, D251, are also involved in loop

contacts with both E253 and E252, and Q508 interacts with the backbone

catch are highlighted. Dashed lines indicate specific contacts. K513 forms

in blue, and the acidic loop is shown in red. Key residues in forming the

transition from L to C* is independent of force (Supplementary Fig. 6).

The simulations reveal that domain III initially moves along domain I rather than outward, first breaking contacts with domain IV (Fig. 5a, structure C) before breaking contacts with domain I (structure C*). As a result of this sliding motion, the protein gate is not large enough to accommodate ssDNA until the distance between the cleavage site and the catalytic tyrosine exceeds 3.8 nm. For dsDNA to pass through the DNA break, a separation of ~5.8 nm is required (Fig. 5a, structure O), in close agreement with our experimental results.

The umbrella-sampling method can be used to calculate equilibrium free-energy profiles along any reaction coordinate, provided that the conformational space of the coordinate is fully sampled during the simulations45. In this way, we were able to estimate the free-energy profile along the experimental coordinate defined by the applied force (Fig. 5b, Supplementary Note 1 and Supplementary Fig. 6a–c). The initial barrier to opening was defined by breaking of contacts between domain III and domains IV and I. Once these contacts are broken, the free-energy profile is relatively flat except for a small barrier between ~3.5 and 4.5 nm that corresponds to interactions between the decatenation loop of topo III and a second smaller loop on domain II. Both loops are absent in topo I (Fig. 5c and Supplementary Video 2). The decatenation loop has a cluster of conserved basic residues46, which form contacts with acidic residues in the second loop. The interaction presumably stabilizes the open state, thus suggesting a structural role for the decatenation loop, which is required for the unlinking activity of topo III47. Stabilization of the open state explains why the closing rate was slower for topo III in our experiments (Fig. 4c and Supplementary Table 2). The distance of this barrier from the open-state well is 1.3 ± 0.4 nm, a finding consistent with the experimental topo III Δxclose value of 1.10 ± 0.01 nm. The experimental Δxclose for topo I, 2.2 ± 0.1 nm, agrees with the distance between the open-state well and state C*, 2.9 ± 0.4 nm (Fig. 5b).

Mechanistic model of type Iα topoisomerase–ssDNA interactions.

Whereas the three-state model is consistent with the number of observed states, analysis of the force-dependent rates, combined with simulation results, suggests the presence of at least one additional kinetic state in the gate dynamics pathway. The lack of force dependence of the experimental opening rate (Fig. 4c and Supplementary Table 2) indicates that the opening rate is dominated by a rate-limiting step that precedes the mechanical opening of the gate. This additional rate-limiting kinetic transition is supported by the finding that the initial barrier associated with breaking contacts between domains III and I in the simulations is too high to be readily thermally accessible. This high energy barrier suggests that opening is limited by a conformational change that lowers the transition-energy barrier but that was too slow to be sampled in our simulations.

To accommodate these results, we extended the model of topoisomerase–ssDNA interactions (Fig. 6 and Table 1) to include an additional state, C* that was inferred but not directly observed.

\[
k_{\text{c}} \quad k_{\text{c-c*}} \quad k_{\text{open}}
\]

\[
L \quad C \quad C^* \quad O
\]

\[
k_{\text{ligation}} \quad k_{\text{c-c*}} \quad k_{\text{close}}
\]

Cleavage and ligation rates in this scenario correspond to the chemical reactions, but the opening and closing rates each represent

to play a role in positioning the 3′-hydroxyl group relative to the phosphotyrosine linkage for DNA religation48. The potential role of a second Mg2+ remains to be determined.

For topo III, we observed a decrease in binding affinity and decreased religation below 1 mM Mg2+. Topo I showed decreased binding below 0.3 mM but was able to cleave and religate DNA, even without magnesium in the buffer. Only when EDTA was added to the reaction to chelate trace amounts of metal was ligation by topo I suppressed (Supplementary Fig. 5). These results suggest that magnesium-dependent DNA binding may contribute to the observed effects of magnesium on the ratio of cleavage to religation in ensemble measurements.

Simulations of gate dynamics. Our experimental results indicated a separation between the cleaved DNA ends of 5.5–6.6 nm, which was unexpectedly large. ssDNA requires a gap of only ~1 nm to pass through the break, whereas duplex DNA requires ~2 nm. Given the relatively high forces used in our experiments, the enzyme might normally undergo a much smaller conformational change in the absence of force on the DNA. To test this possibility, we conducted umbrella-sampling64 simulations of topo III bound to ssDNA8 (Fig. 5, Supplementary Video 1 and Supplementary Fig. 6a–d). In these simulations, the distance between the center of mass of domain III (shown in red in Fig. 5a) and the center of mass of domain IV (shown in blue) was restrained by using a harmonic potential, \[ V = \frac{1}{2} k (r - r_0)^2 \], where \( r \) is the restrained distance, and \( r_0 \) is the center of the potential well for a given window. To open the gate, \( r_0 \) was gradually increased over a series of windows. Domains III and IV must move away from each other for the gate to open, but the direction of this motion may not have been aligned with our experimentally imposed force and displacement axis. Restraining the distance between the domains rather than pulling along a vector allowed the system to follow an energetically unbiased pathway.

Fig. 5a Molecular dynamics simulations of topo III gate opening. a. Topo III structures from molecular dynamics umbrella-sampling simulations. Representative structures are shown for the closed state (L), the state in which contacts between domains III and IV are broken (C), the state in which contacts between domains III and I are broken (C*), and the fully open structure (O). x (dashed black line) is the change in distance between the oxygen of the catalytic tyrosine, Y328, and the cleavage site of the DNA backbone. Blue scale bar, 2 nm. b. Relative free-energy change as a function of gate opening (x). Arrows indicate the locations of the states represented in a. The distance between the closed state and C*, \( \Delta x_{\text{open}} \), is 2.2 ± 0.3 nm. \( \Delta x_{\text{open}} \) is 2.9 ± 0.4 nm. The dashed line is the estimated free energy for an applied force of 22 pN under the assumption that the transition from L to C* is independent of force (Supplementary Fig. 6). c. Structure of the decatenation latch. The decatenation loop is shown in blue, and the acidic loop is shown in red. Key residues in forming the latch are highlighted. Dashed lines indicate specific contacts. K513 forms contacts with both E253 and E252, and Q508 interacts with the backbone of P248. Two other basic residues in the decatenation loop, K510 and K514, and a third acidic residue in the acidic loop, D251, are also involved in loop contacts at other points in the simulations (Supplementary Video 2).

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\[
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\[
L \quad C \quad C^* \quad O
\]

\[
k_{\text{ligation}} \quad k_{\text{c-c*}} \quad k_{\text{close}}
\]

Cleavage and ligation rates in this scenario correspond to the chemical reactions, but the opening and closing rates each represent...
a convolution of two rates from C to O via C* and vice versa. Accordingly, the measured $k_{\text{open}}$ is dominated by the rate of a conformational change ($k_{\text{C-C*}}$) that allows domain III to escape contacts with domain I. The actual mechanical opening rate ($k_{\text{open}}$) is force dependent but too fast to observe. Similarly, $k_{\text{C-C*}}$ is assumed to be faster than $k_{\text{close}}$ and $k_{\text{open}}$, such that the measured closing rate is dominated by $k_{\text{close}}$, and the equilibrium between C* and C is biased toward state C, from which religation can occur. In this scenario, the gate can undergo multiple transitions between states C and C* before opening, in which case $k_{\text{open}}$ would represent a multiple of $k_{\text{C-C*}}$, rather than $k_{\text{C-C}}$. The measured force dependence of the closing rate supports the hypothesis that the transition from state C* to C is fast (much faster than the fastest closing rate of $2.7 \pm 0.1 \text{s}^{-1}$). Furthermore, the lack of force dependence of the religation and opening rates is consistent with the hypothesis that the rate $k_{\text{C-C}}$ exceeds all other rates.

Topo III has an additional state, $O^*$, between C* and O, in which the decatenation loop forms a bridge that creates a barrier to closing. This additional barrier observed in the simulated free-energy profile is supported by the significantly slower gate-closing rate and by the shorter distance between the open state and the transition state obtained from the force-dependent closing rate of topo III in comparison to topo I. The long lifetimes of the topo III–ligated state also suggest the possibility that the protein might open and close while being bound to ligated DNA (Fig. 6), thus contributing to the slower apparent cleavage rate.

Of note, this model is based exclusively on interactions with a single strand of DNA and lacks the second strand that is presumably passed through the gate during relaxation. This strand is likely to affect the gate kinetics. This possibility is especially true for topo III, which has a faster relaxation rate than our gate-dynamics measurements would predict.

**Discussion**

Since the first structure of a type IA topoisomerase was solved, these enzymes have been predicted to use a protein-mediated DNA-gate mechanism for strand passage, similarly to type II topoisomerases. In the intervening 24 years, no structures of the protein in an open state have been solved, though crystal structures of a fragment suggest that domain II can bend, separating domains I and III(3) (Supplementary Fig. 6e–f). Biochemical studies have shown that domains I and III move relative to each other in order for strand passage to occur(4). The same studies have demonstrated that circular DNA can be trapped in a complex with the protein by cross-linking domains I and III, thus suggesting that the passed strand resides within the protein cavity. Ensemble fluorescence quenching assays have similarly linked strand passage to a conformational change between domains I and III(5). More recent combined magnetic tweezers–fluorescence experiments also indicate a conformational change before strand passage(6), although the results have been interpreted as domain closure rather than domain opening. Attempts to estimate the gate opening of topo I from force-dependent relaxation kinetics have resulted in a Δx value of ∼5 nm, too large to be reflective of gate opening(7). These studies are consistent with, but do not conclusively demonstrate, the existence of the putative protein-mediated DNA gate or the gate dynamics.

By measuring the extension of individual DNA molecules containing ssDNA regions, we observed conformational changes corresponding to the separation of cleaved DNA by 5.5–6.6 nm in two bacterial type IA topoisomerases. These experiments provide direct evidence for the protein-gate model of topoisomerase IA activity. Although the magnitude of gate opening was unexpectedly large, molecular dynamics simulations indicate that a 5.8 nm separation is required to pass duplex DNA through the gate. The finding that the gate opening and ssDNA cleavage are reversible and occur with kinetics comparable to that of the strand-passage reaction suggests that the observed open state is biochemically relevant. This conclusion is bolstered by the close agreement between the experimental results and simulations, and the observation that topo III and topo I remained catalytically active under applied loads as high as 12 pN (Supplementary Note 1 and Supplementary Fig. 7).
These results offer new insight into type IA topoisomerase activity. The differences in gate dynamics between topo III and topo I suggest a possible mechanism for their different biochemical activities and roles in the cell. Whereas topo I removes negative supercoils, topo III functions primarily as a decatenase, an activity that requires passing duplex DNA through the break in a single strand. The fast dynamics of topo I may favor efficient relaxation of negatively supercoiled DNA, whereas the slower closing rate of topo III may facilitate capture of dsDNA. This possibility is supported by the gate-stabilizing interaction between the decatenation loop of topo III and an acidic loop in domain II observed in our simulations. A similar role in promoting the open state has been proposed for the eukaryotic protein RM11, which is required for the decatenation activity of topo III (refs 16,22). After DNA has entered the cavity, it may disrupt this interaction, thereby leading to fast closing of the gate and potentially facilitating the processive bursts of relaxation activity observed by Terekhova et al.

Because of their importance in genome maintenance and replication, topoisomerases are a potential target for both antibacterial and anticancer drugs. Topo I is the only type IA topoisomerase present in mycobacteria. M. tuberculosis topo I has been validated genetically and chemically as a tuberculosis drug target. Poison inhibitors targeting type IA topoisomerases in other bacterial pathogens are also expected to be bactericidal, because trapping of a small number of the covalent cleavage complexes on the chromosomal DNA would be sufficient for bacterial cell killing. Therefore, identifying poisons that can inhibit DNA ligation after the DNA-cleavage step is highly desirable. Currently no drugs are in use that target type IA topoisomerases. The design of such drugs has been hampered by the inability to experimentally probe the details of the full strand-processing reaction. Key residues that drive the conformational changes for the opening and closing of the DNA gate have not yet been identified. The magnetic tweezers assays described here have the potential to be powerful tools in testing the effects of mutations and small-molecule interactions on this class of proteins.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0158-x.

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Author contributions

M.M. and K.C.N. conceived the experiments. M.M. conducted the experiments and analyzed the data. Y.-C.T.-D. provided materials. M.M., K.C.N., and Y.-C.T.-D. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Methods

Protein purification. Recombinant topoisomerase III was purified as previously described in Seol et al.\(^6\). E. coli topo III containing a histidine tag and tobacco etch virus–cleavage site was expressed in Rosetta (DE3) competent cells (EMD Biosciences). Cells were grown at 37 °C in LB medium containing 50 μg/ml carbenicillin and 34 μg/ml chloramphenicol and induced with 1 mM IPTG for 20 h. Cells were harvested by centrifugation and resuspended in 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 10% (vol/vol) glycerol. Cells were emulsified with a high-pressure cell homogenizer (Avestin). lysates were centrifuged at 40,000 r.p.m. at 4 °C for 1 h in a Ti45 rotor (Beckman). The supernatant was loaded onto a 12 ml Ni–nitrilotriacetic acid (NTA) column (Qiagen) and eluted with an imidazole gradient (buffer A, 50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 1 mM 0.1% Tween, 0.03% BSA, and 10% (vol/vol) glycerol). Eluted protein was concentrated and buffer exchanged into buffer A. The protein was incubated with 0.5 U/μg tobacco etch virus protease for 6 h to remove the histidine tag. Digested protein was loaded onto a His-Trap FF 5 ml Ni–NTA column (GE) and collected in the flow through. The cleaved protein was concentrated and loaded onto a Hiloald 16/60 Superdex 200 column (GE) and eluted with buffer A. Size and purity were confirmed by SDS–PAGE gel electrophoresis.

Recombinant E. coli topoisomerase I and the Y319F mutant were purified as previously described\(^6\). Topo I was overexpressed in E. coli strain MV1190 (Bio-Rad). Cells were grown at 37 °C in LB medium containing 100 μg/ml ampicillin and induced with 400 μM IPTG for 3 h. Cells were harvested by centrifugation and resuspended in 10 mM Tris-HCl, pH 8.0, 400 mM KCl, and 1 mg/ml lysozyme. Cells were lysed by freezing and thawing three times. Lysates were centrifuged at 5,080 g in a Ti45 rotor (Beckman) at 4 °C for 3 h. The extract was dialyzed overnight against 20 mM potassium phosphate, pH 7.4, 200 mM KCl, 1 mM DTT, and 0.1% Tween.20 The supernatant was loaded onto a phosphocellulose column (Whatman) and eluted with an imidazole gradient. Measurements were performed at room temperature on a commercial Picotest instrument. An average force calibration versus magnet position for 1 μM MyOne magnetic beads was obtained with Brownian-motion analysis of 11 kb DNA tethers. Force-calibration measurements over many beads indicated that relying on the average force calibration introduced an error in force of ~10%. The bead position in three dimensions was acquired at 60 Hz.

Hairpin assays. Topo III or topo I was diluted to 1 μM in reaction buffer lacking magnesium (50 mM Tris-HCl, pH 8.0, 30 mM monopotassium l-glutamate, 0.1% Tween-20, and 0.03% BSA) and incubated at 37 °C for 30–60 min. Samples were then diluted to 500 pM in 1 mM magnesium reaction buffer. Hairpin-DNA tethers were characterized before the addition of protein by application of a range of forces to determine the critical melting force of each individual tether, because slight differences in bead magnetization could affect this value. After hairpin characterization, 200 μl of topoisomerase sample was flowed into the experimental sample cell. A force of 8–10 pN was applied to determine a baseline for the folded state of the hairpin, and then a force or 22–24 pN was applied to mechanically melt the DNA. The DNA was held at high force for 30 s to 5 min, and then the force was returned to the baseline level. After the force was again raised to 22–24 pN, and the measurement was repeated. Hairpin-unfolding experiments were also conducted in buffer containing no magnesium l-glutamate to determine the magnesium dependence of binding.

Gapped-DNA assays. Topoisomerase was diluted to 1 μM in no-magnesium reaction buffer and incubated at 37 °C for 30–60 min, then diluted to 250 pM in reaction buffer containing 0–10 mM magnesium l-glutamate, and 200 μl of sample was introduced to the experimental sample cell. Data were collected at 3 mM Mg\(^{2+}\) for topo III and 0.5 mM (n=15) and 3 mM (n=2) for topo I. Forces of 8, 10, 12, 14, 16, and 18 pN were applied. Data from multiple tethers (n=8 for topo III; n=15 for topo I) were collected. After each experiment, data were fit to the range of forces for each tether, because the data were only taken at multiple forces ranging from 8 to 18 pN, although we do not have data for the full range of forces for every tether, because tethers were lost over time. Below 12 pN, the closing rates for topo I were too fast to be sampled accurately with the time resolution of our experiments. The closing rates were too slow to provide adequate statistics at forces higher than 16 pN for topo III and 18 pN for topo I. For magnesium-titration experiments, measurements were conducted at 8 pN on 250 pM topo III in reaction buffer containing 1 mM, 3 mM, 5 mM, or 10 mM magnesium l-glutamate and at 14 pN for topo I at 0.1 mM, 0.3 mM, 0.5 mM, and 3 mM magnesium l-glutamate. Additional experiments were conducted for topo I in reaction buffer containing no magnesium l-glutamate and 1 mM EDTA.

Molecular dynamics simulations. Simulations were conducted in NAMD\(^7\) v. 2.12 with the Charmm 36 force field\(^8\). Topo III bound to ssDNA (PDB ID 17ID) was solvated with 148,705 TIP3P\(^9\) water molecules. Sodium and chloride ions were added to a concentration of 50 mM. The fully solvated system was minimized for 2,500 steps with the protein atoms restrained with a force constant of 5 kcal/mol/Å\(^2\) and an additional 5,000 steps without restraints. After minimization, the system was equilibrated at constant pressure and temperature for 500 ps. For the CPT equilibration step, pressure was held at 1.03125 bar with Berendsen's method, and the protein atoms were harmonically restrained with a force constant of 5 kcal/mol/Å\(^2\). The full systems were then further equilibrated without restraints for 10 ns at constant volume and temperature. A Langervin thermostat was used to maintain a temperature of 300 K. From the closed structure, the protein gate was manually opened by applying a harmonic restraint of 4–10 kcal/mol/Å\(^2\) on the distance between the center of mass of residues 290–415 and 492–620. The full system was gradually increased over a series of windows from 2.5 nm to 6.5 nm in 0.1 nm increments. Umbrella-sampling windows were run for 2 ns–20 ns, with a 200-ps equilibration period for each new window. Coordinates were saved every 1 ps.

Data analysis. Hairpin assays. For magnetic tweezers hairpin experiments, data analysis was performed with custom-written routines in Igor Pro 6.3 A. Binding events were defined as pauses in unfolding lasting >100 ms, and opening events were defined as discrete extension increases of ~6–7 nm from the fully unfolded state. To determine the sizes of the extension increases, the data were smoothed with a second-order Savitzky–Goalay algorithm over 51 points, then histogrammed and fit to a sum of Gaussian distributions with the multiplex function in Igor. Equilibrium increases on the basis of the fitted Gaussians centered on the centers of the individual Gaussian fits. Because of the fast nature of topo I opening, for some events, the number of points was too few to appear in the distribution, thus leading to apparent increases of approximately twice the average size. The data for each unfolding transition were combined and histogrammed with a bin size of 1 nm. The number of binding and opening events for each unfolding–refolding cycle was determined, and the average number of opening events for a given number of bound topoisomerases was plotted.

Gapped-DNA assays. To determine the size of gate opening, data were smoothed with a second-order Savitzky–Goalay algorithm over 51 points, then histogrammed and fit to a double-Gaussian distribution with the multiplex function in Igor Pro 6.3 A. Extension increases were calculated on the basis of the difference between the centers of the individual Gaussian fits. Kc values were determined from comparison of the area under the two Gaussians.

To determine kinetics, we analyzed traces in vbFRET\(^10\) to assign states and then further analyzed the data in Igor Pro. Default values of priors were used in the analysis of 11 kb DNA tethers. Force-calibration measurements over many beads indicated that relying on the average force calibration introduced an error in force of ~10%. The bead position in three dimensions was acquired at 60 Hz.
vbFRET analysis (upi = 1, mu = 0.5, beta = 0.25, W = 50, v = 5, ua = 1, uad = 0).

Lifetime analysis of the idealized traces from vbFRET were calculated with a thresholding algorithm in Igor Pro 6.3 A. The open- and closed-state lifetimes were histogrammed and fit to exponentials to determine the cleavage, opening, and closing rates. For topo III, closed-state lifetimes were treated as two separate datasets, with short time bins for short-lifetime states (t ≤ 20 s) and longer time bins for the long-lifetime states (t > 20 s). We assumed that the long-lifetime closed state corresponded to cleavage followed by an opening event (t_{long} = t_{cleavage} + t_{open}), whereas the short-lifetime closed states corresponded to opening events (t_{short} = t_{open}). Because the short lifetimes were orders of magnitude less than the longer lifetimes for topo III, we assumed that k_{cleavage} ~ k_{long}. For topo I, the closed states were fit to a double exponential. We assigned the faster rate to k_{short} and the slower rate to k_{long}, and calculated k_{cleavage} as 1/(t_{long} − t_{open}). Closing rates were calculated from single-exponential fits to histograms of the open-state lifetimes. In some traces, we observed two topo III molecules that were active simultaneously. Cleavage rates were adjusted accordingly. Closing rates for events in which two molecules were open simultaneously were excluded from analysis. Ligation rates were calculated with the kinetic-competition model described in the results section.

Molecular dynamics simulations. The reweighted free-energy profile of the distance between the catalytic tyrosine and the cleavage site was calculated with a modification of the weighted histogram analysis method (WHAM)47 that allows for calculation of the free energy of reaction coordinates orthogonal to the restrained coordinates49. Values for the experimental reaction coordinate, x, defined as the distance between the oxygen of the catalytic tyrosine (Y328) and the cleavage site on the DNA backbone (P7), were obtained from the coordinates saved from each simulation window with VMD v. 1.9.2 (ref. 68). To determine the free energy along our experimental reaction coordinate x, we calculated two-dimensional histograms of the experimental and the restrained reaction coordinates for each simulation window. These histograms were then unbiased, and the free-energy profiles were calculated with WHAM version 2.0.9.1 (http://membrane.urmc.rochester.edu/content/wham/). The two-dimensional free-energy profile was collapsed along the restrained coordinate to determine the free energy along x.

Additional methods. Additional methods can be found in Supplementary Note 2.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Source data for Fig. 1 are available with the paper online. The data that support the findings of this study are available from the corresponding author upon reasonable request.

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|                |                                                                 |
|----------------|-----------------------------------------------------------------|
| **Sample size**| Sample size was determined based on number of DNA tethers from which data was collected. |
| **Data exclusions** | Data was excluded if instrument drift made analysis impossible or if excessive noise made the results of numerical analysis questionable. Data at forces outside of the range presented in the manuscript was also excluded due to the low number of observed events leading to poor statistics. |
| **Replication** | Data was taken for multiple tethers and collected on different samples to ensure reproducibility. Comparisons between data sets at different applied forces and magnesium concentrations were used to verify that results were consistent. |
| **Randomization** | n/a |
| **Blinding**    | Blinding was not considered necessary for this study as all data was processed numerically. |

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
|     | ✓ Unique biological materials |
|     | ✓ Antibodies |
|     | ✓ Eukaryotic cell lines |
|     | ✓ Palaeontology |
|     | ✓ Animals and other organisms |
|     | ✓ Human research participants |

Methods

| n/a | Involved in the study |
|-----|-----------------------|
|     | ✓ ChIP-seq |
|     | ✓ Flow cytometry |
|     | ✓ MRI-based neuroimaging |