Supplementary Note 1

Denaturation of cleaved hairpin tethers by SDS

To verify that the DNA was cleaved during the observed extension increases, we used 1% SDS to denature the protein. One percent SDS has been shown to have no discernable direct effect on DNA tethers in magnetic tweezers experiments, but to cause irreversible cleavage and loss of the tethered magnetic bead in the presence of topoisomerase[1]. For these experiments, the hairpin construct was unfolded under high force (~24 pN), as this allowed us to readily identify cleavage events and to trap the topoisomerase-DNA complex in the presumed cleaved state. When buffer containing 1% SDS was added to a sample with 500 pM topoisomerase, all tethers with evidence of topoisomerase-dependent extension increases disappeared within 20-40 seconds (n = 2 for topo III, n = 3 for topo I) (**Supplementary Fig. 1g**). The average lifetime of the cleaved DNA was 27.5 ± 0.7 s. In contrast, tethers without protein present did not respond to the addition of SDS. 8 out of 10 such tethers persisted under 24 pN of force for > 30 minutes in buffer containing 1% SDS. Data acquisition was stopped after 30 minutes if the tether had not been lost. The average lifetime of the DNA tethers in the absence of protein was 1486± 663 s. This lifetime represents a conservative lower bound since tethers that lasted 30 mins were assumed to have been lost at 30 min. The actual lifetime of the tethers in the absence of topoisomerases is likely considerably longer.

Free energy calculations from MD Simulations

To compare the simulation and experimental results, we calculated the free energy under applied force using the equation $\Delta G_F = \Delta G_0 - F\Delta x$, where F is the applied force and Δx is the distance from the origin of force along the gate opening. We modeled the effect of force on the free energy for two conditions: with the origin of the gate opening distance subject to force corresponding to the closed state (**Supplementary Fig. 6a**); and with the origin of the gate opening distance subject to force corresponding to the state C* (**Supplementary Fig. 6b**). In the first scenario the transition from C to O would be force dependent. In the second scenario the transition from C to C* would be force independent, whereas the transition from C* to O would be force dependent. This second condition is consistent with our experimental results where k_{open} is insensitive to force (**Fig. 4c, Supplementary Table 2**).

In addition to the force dependence, the simulation free energies provide insight into the differences between the gate dynamics of topo III and topo I. The two-dimensional free energy profile along both the restrained coordinate (the distance between domains III and IV) and x (the change in distance between the catalytic tyrosine and the DNA cleavage site) shows two alternate opening pathways (**Supplementary Fig. 6c**). In one of these pathways, the decatenation latch is formed while in the other it is not. In the second pathway, the free energy difference between C* and O is smaller. Because it lacks the decatenation and acidic loops, topo I is likely to follow a pathway similar to this one. In the absence of latch formation, the hinge region of domain II also adopts a more extended structure (**Supplementary Fig. 6d**).

Relaxation at high force

Our topoisomerase gate dynamics measurements were conducted at forces ranging from 8 to 18 pN. These forces are possibly higher than the enzymes are expected to encounter in the cell. Previous studies have shown an inhibitory effect of force on type IA topoisomerase relaxation activity.[2] To test whether such high forces would inhibit the relaxation activity of type IA topoisomerases, we conducted magnetic tweezers relaxation assays under high force (**Supplementary Fig. 8**). At forces above ~0.8 pN, negatively supercoiled DNA is fully melted and indistinguishable from relaxed DNA (**Supplementary Fig. 8a**). To measure relaxation at high forces, magnetic tweezers were first used to negatively supercoil an 11 kb DNA at low force (**Supplementary Fig. 8b**). The force was then raised to 12 pN for 5-15 minutes and lowered again to < 0.5 pN. Relaxation was observed as an increase in the baseline DNA extension upon returning to low force (**Supplementary Fig. 8b**). We observed relaxation for both topo III and topo I (**Supplementary Fig. 8c**). However, the relaxation appears to be inefficient at this force, possibly due to either the slower gate dynamics or strain applied to the passed strand.

1. Dekker, N. H. *et al.* Thermophilic Topoisomerase I on a Single DNA Molecule. *J. Mol. Biol.* **329,** 271–282 (2003).
2. Dekker, N. H. *et al.* The mechanism of type IA topoisomerases. *Proc. Natl. Acad. Sci.* **99,** 12126–12131 (2002).

Supplementary Note 2

SDS denaturation of cleaved DNA

Samples were prepared as described for the hairpin assay. Topoisomerase was diluted to 500 pM in 1 mM Mg reaction buffer. Hairpins were unfolded under a force of 24 pN. Once cleavage was observed, 1mM Mg reaction buffer containing 1% SDS was flowed into the sample cell. Cleavage was observed as subsequent loss of the tethered bead. For DNA only experiments, tethers were unfolded under 24 pN of force in 1mM Mg reaction buffer containing 1% SDS and followed for up to 30 minutes. After 30 minutes, the force was lowered to 9 pN to verify that hairpins were intact.

High force relaxation assay

An 11 kb coilable DNA was generated as described in Seol and Neuman (2011)[1]. Topoisomerase was diluted to 1 μM in 0 Mg reaction buffer and incubated briefly at 37 °C, then diluted to 500 pM in reaction buffer containing 3 mM Mg L-glutamic acid. -50 turns were introduced at a force of 0.05 pN. The force was then raised to 12 pN. After 5 -15 minutes, the force was lowered again to 0.1 pN. If relaxation had not occurred, the high-force, low-force cycle was repeated. Hat curves at 0.1 pN were taken before and after relaxation to determine how many turns were removed and to verify that the molecule was not nicked.

1. Seol, Y. & Neuman, K. Magnetic Tweezers for Single-Molecule Manipulation. in *Single Molecule Analysis* (eds. Peterman, E. J. G. & Wuite, G. J. L.) 265–293 (Humana Press, 2011). doi:10.1007/978-1-61779-282-3_15

Supplementary Tables

	Topo III		Topo I	
Force (pN)	**K_D**	**ΔExt (nm)**	**K_D**	**ΔExt (nm)**
8	0.043 ± 0.001	5.9 ± 0.2	-	-
10	0.236 ± 0.001	5.0 ± 0.1	-	-

12	0.143 ± 0.001	5.7 ± 0.1	0.327 ± 0.003	5.0 ± 0.6
14	0.432 ± 0.001	5.2 ± 0.1	2.28 ± 0.01	6.1 ± 0.2
16	0.1548 ± 0.0008	5.9 ± 0.1	3.84 ± 0.04	6.2 ± 0.2
18	-	-	24 ± 1	6.5 ± 0.6

Supplementary Table 1. Force dependence of K_D and ΔExtension values. ΔExtension is the experimentally measured increase in DNA extension associated with gate-opening. To obtain K_D and ΔExt values, the extension versus time data traces were smoothed (4^{th} order Savitzky-Golay, 51 points), multiple traces for each force were combined, and the data was histogrammed and fit to the sum of two Gaussians (**Supplementary Fig. 2**). K_D values were calculated by taking the area of the Gaussian fit to the higher extension (open state) divided by the area of the Gaussian fit to the lower extension (closed state). ΔExtension corresponds to the distance between the centers of the Gaussian fits. Data was taken at 3 mM Mg^{2+}. All errors are standard error.

Topo III

Force (pN)	k_{open} (s^{-1})	k_{close} (s^{-1})	$k_{cleavage}$ (s^{-1})	$k_{ligation}$ (s^{-1})
8	1.0 ± 0.3	0.21 ± 0.02	0.0011 ± 0.0006	0.76 ± 0.07
10	0.9 ± 0.1	0.1 ± 0.01	0.0012 ± 0.0007	2.6 ± 0.07
12	1.2 ± 0.2	0.2 ± 0.02	0.0012 ± 0.0007	2 ± 0.13
14	1.3 ± 0.7	0.03 ± 0.007	0.0014 ± 0.0008	0.8 ± 0.15
16	0.96 ± 0.08	0.022 ± 0.002	0.0011 ± 0.0002	1.5 ± 0.05

Topo I

Force (pN)	k_{open} (s^{-1})	k_{close} (s^{-1})	$k_{cleavage}$ (s^{-1})	$k_{ligation}$ (s^{-1})
12	2.7 ± 0.8	2.7 ± 0.1	0.72 ± 0.23	4.6 ± 2.3
14	4.3 ± 0.9	0.97 ± 0.04	0.98 ± 0.22	3.8 ± 1.8
16	4.6 ± 1.9	0.36 ± 0.02	1.2 ± 0.54	7.5 ± 5.3
18	5.1 ± 0.7	0.16 ± 0.01	0.75 ± 0.27	2.5 ± 2.7

Supplementary Table 2. Force dependence of topo III and topo I rates. For topo III, the opening rate was calculated by fitting an exponential to the histogram of closed state lifetimes below 20 s. The cleavage rate was determined from an exponential fit to closed state lifetimes above 20s. The probability of opening (P_{open}) was determined from

the number of short lived closing events relative to the total number of closing events and the ligation rate was calculated using the equation describing the kinetic competition between ligation and opening: $P_{open} = k_{open}/(k_{open} + k_{ligation})$. For topo I, the opening and cleavage rates were determined by fitting the closed state lifetimes to a double exponential. The higher rate was assigned to k_{open} and the lower rate to the convolution of k_{open} and $k_{cleavage}$. The cleavage rate was then calculated using the equation $t_{cleavage} = t_{open+cleavage} - t_{open}$, where t indicates the lifetime. P_{open} was calculated from the relative areas of the two exponents fit to the lifetime histogram. Closing rates for both proteins were determined from exponential fits to the open state lifetimes. Data for topo III was collected at 3 mM Mg ($n_{tethers} = 8$). Data for topo I was taken at 0.5 mM Mg and 3 mM Mg ($n_{tethers} = 7$).

Topo III	**Gate dynamics analysis**	**Terekhova 2012[2] (0.7 pN)**	**Zhang 2017[3]**	**Topo I**	**Gate dynamics analysis**	**Terekhova 2012[2] (0.7 pN)**	**Gunn 2017[4] (1 pN)**	**Dekker 2002[1] (0.53 pN)**
$k_{cleavage}$	0.0012 ± 0.0004	0.0077*	10.9	$k_{cleavage}$	0.9 ± 0.2	0.16*	0.92*	
$k_{ligation}$	1.5 ± 0.7		2.62	$k_{ligation}$	4.6 ± 1.0			
k_{open}	1.07 ± 0.17			k_{open}	4.1 ± 1.0			
k_{close}	1.8 ± 0.3			k_{close}	1642 ± 549			
$k_{relaxation}$	0.0011 ± 0.0004	0.2 ± 0.02		$k_{relaxation}$	0.64 ± 0.38	3.3 ± 0.6	0.17	1

Supplementary Table 3. Comparison of rates with previous measurements. Estimated rates from gate dynamics experiments at $F = 0$ compared to previously measured rates. Lag rates from Terekhova et al. are presented as analogous to $k_{cleavage}$, as is the rate of conformational change measured in Gunn et al. Rates from Zhang et al. are for *S. solfataricus* topo III at 75 °C. Relaxation rates from gate dynamics are calculated from

the total rate of one cleavage-religation cycle. Relaxation rates from Terekhova et al. are for negatively supercoiled DNA and include lag time. Rates are in s^{-1}. Relaxation rates are ΔLk/s.

1. Dekker, N. H. *et al.* The mechanism of type IA topoisomerases. *Proc. Natl. Acad. Sci.* **99,** 12126–12131 (2002).
2. Terekhova, K., Gunn, K. H., Marko, J. F. & Mondragón, A. Bacterial topoisomerase I and topoisomerase III relax supercoiled DNA via distinct pathways. *Nucleic Acids Res.* **40,** 10432–10440 (2012).
3. Zhang, J., Pan, B., Li, Z., Sheng Zhao, X. & Huang, L. Kinetic insights into the temperature dependence of DNA strand cleavage and religation by topoisomerase III from the hyperthermophile Sulfolobus solfataricus. *Sci. Rep.* **7,** 5494 (2017).
4. Gunn, K. H., Marko, J. F. & Mondragón, A. An orthogonal single-molecule experiment reveals multiple-attempt dynamics of type IA topoisomerases. *Nat. Struct. Mol. Biol.* **24,** 484–490 (2017).

Topo III

Mg^{2+} conc. (mM)	k_{open} (s^{-1})	k_{close} (s^{-1})	$k_{cleavage}$ (s^{-1})	$k_{ligation}$ (s^{-1})	$n_{tethers}$
1	8.4 ± 0.8	0.13 ± 0.01	0.0013 ± 0.0005	0.31 ± 0.03	3
3	0.9 ± 0.3	0.21 ± 0.01	0.0011 ± 0.0006	0.76 ± 0.07	8
5	0.9 ± 0.2	0.15 ± 0.01	0.0018 ± 0.0006	2.1 ± 0.3	3
10	-	0.12 ± 0.01	0.0012 ± 0.0004	12.4 ± 1.6	2

Topo I

Mg^{2+} conc. (mM)	k_{open} (s^{-1})	k_{close} (s^{-1})	$k_{cleavage}$ (s^{-1})	$k_{ligation}$ (s^{-1})	$n_{tethers}$
0+	2.8 ± 1.0	1.0 ± 0.1	0.37± 0.18	2 ± 2	6
0.1	4 ± 7.8	0.98 ± 0.18	1 ± 2	16 ± 54	4
0.3	5.5 ± 5	0.9 ± 0.0	0.94 ± 0.86	15 ± 29	3
0.5	4.5 ± 1.3	1.0 ± 0.1	0.71 ± 0.27	4.6 ± 3.1	5
3	3.1 ± 0.5	1.0 ± 0.0	0.63 ± 0.14	1.7 ± 1.2	2

Supplementary Table 4. Magnesium dependence of topo III and topo I rates. Rates were calculated as described in **Supplementary Table 1** with the exception of 0.1 mM and 0 added Mg^{2+} data for topo I. At these low Mg^{2+} concentrations, we observed a third closed state. The closed state histograms for 0.1 mM and 0 added magnesium were therefore fit with three exponentials. The first two rates were assigned as before, while the slowest rate we assumed to be limited by either Mg^{2+} or DNA binding. This slow rate was 0.007 ± 0.004 for 0 added Mg^{2+} and 0.04 ± 0.29 for 0.1 mM. Decreased binding was also observed for topo III below 1 mM Mg^{2+}, resulting in too few events for statistical analysis.