Protein functions can be regulated by controlling protein conformation, binding of ligands and effector molecules, and interactions with other proteins. Many techniques have been developed for conformational control of protein function, mainly based on inhibition of activity and removal of that inhibition in a controlled manner (1–3). Here we demonstrate that intramolecular cross-linking of an enzyme without detectable unwinding activity can convert it to a superhelicase by stabilizing its active conformation.

Although studies have shown how various helicases can translocate on single-stranded nucleic acids directionally, the mechanism of nucleic acid unwinding and how the unwinding activity is regulated remain unclear (4). Because helicases that are free to unwind all nucleic acids encountered can be detrimental to genome integrity, it is critical to understand how their unwinding activities are regulated.

Rep, PcrA, and UvrD are structurally homologous 3′→5′ single-stranded DNA (ssDNA) translocases and helicases that can unwind double-stranded DNA (dsDNA) using energy from adenosine triphosphate (ATP) binding and hydrolysis. However, in vitro studies showed that monomers of these helicases have a very poor activity and cannot processively unwind DNA (5–8). They require oligomerization or association with cellular partner proteins to become capable of unwinding (4, 9–11). Crystal structures revealed a flexible domain (2B) that can rotate 130° to 160° in a swiveling motion between two conformations, referred to as the “open” and “closed” forms (12–15) (Fig. 1A). Whether 2B is essential for unwinding or whether it plays a regulatory role has been debated, as well as which of the two conformations is required for DNA unwinding (4, 12–17), but no direct evidence has been found linking these conformations to the unwinding function.

To determine which conformation is assumed during unwinding, we engineered *Escherichia coli* Rep mutants that are intramolecularly cross-linked to constrain the 2B domain in closed or open conformations, respectively, termed “Rep-X” and “Rep-Y.” Residues for the cysteine substitution mutagenesis and the length of the bis-maleimide cross-linkers were selected such that when cross-linked, 2B cannot rotate appreciably, effectively locking the protein in one conformation (Fig. 1A) (18). Mutagenesis, purification, cross-linking procedures, and validation that cross-linking was intramolecular rather than intermolecular are described in fig. S1 and the supplementary text (18). Cross-linking had only modest effects on ATPase activities of Rep-X and Rep-Y (fig. S2).

In multiple-turnover ensemble unwinding reactions using fluorescently labeled DNA, Rep-X unwound dsDNA [18 or 50 base pairs (bp)] with a 3′ overhang at a much faster rate and a higher reaction amplitude than the wild-type Rep or the un–cross-linked double-cysteine mutant (fig. S2, A to C). In contrast, Rep-Y unwinding rates were similar to that of Rep (fig. S2D), indicating that the dramatic unwinding enhancement is specifically achieved in the closed conformation. To determine whether the large enhancement in unwinding activity results from the activation of a monomer or from enhanced oligomerization, we performed single-molecule fluorescence resonance energy transfer (smFRET) experiments. Proteins were immobilized to a surface through the N-terminal His6-tag (Fig. 1B) (18) to ensure that the observed activity belonged to monomers (5). We used a 18-bp duplex DNA with a 3′-(dT)20 overhang labeled with a donor (Cy3) and an acceptor (Cy5) at opposite ends of the duplex (Fig. 1B). When the DNA and ATP were added to the reaction chamber, we could observe the capture of a single DNA molecule by a single protein as the sudden appearance of fluorescence signal (Fig. 1C). Unwound ssDNA coiled up due to high flexibility [FRET efficiency (E<sub>FRET</sub>) increase] (19), full unwinding released the acceptor strand (acceptor signal disappearance and donor signal increase), and then the donor strand dissociated (loss of fluorescence). The mean duration of unwinding measured from the E<sub>FRET</sub> increase to acceptor strand release was ~0.6 s, giving a lower limit on the unwinding speed of 30 bp/s for the 18-bp substrate (fig. S3, A and B). Most (82%) of the DNA molecules [661 of 809 (661/809)] that initially bound to Rep-X monomers were unwound (fig. S3, C and D).

In contrast, Rep and Rep-Y showed 2% (13/847) and 16% (357/2212) unwinding yields, respectively, suggesting that constraining Rep into the closed form selectively activates the unwinding activity of a monomer. The residual activities for Rep and Rep-Y may be due to conformational constraints caused by surface tethering in a small fraction of molecules or passive helicase activity of trapping thermally melted DNA.

The unwinding processivity of Rep and related helicases is limited, even in their oligomeric forms, ranging from 30 to 50 bp (5, 8, 20). We used a dual optical tweezers assay to investigate the processivity of Rep-X (Fig. 2A) (18, 21). The two traps held submicrometer-sized polystyrene beads. The first was coated with 6-kbp dsDNA (kb, thousand base pairs) with a 3′ poly-dT ssDNA overhang ([dT]<sub>10,15,20</sub>, as specified in the figures) via the blunt end. The other bead was coated with Rep-X molecules via the His<sub>6</sub>-tag. A laminar flow cell with two streams of buffer was used to control unwinding initiation (Fig. 2B, inset). When we brought the two beads in close proximity in the first stream (buffer C with 100 μM ATP and 100 μM ATP-γS), a single Rep-X binding to the 3′ overhang of the DNA formed a tether between the two beads without initiating unwinding. When we moved the tethered beads to the second stream (buffer C and 1 mM ATP), the DNA tether progressively shortened as the Rep-X monomer unwind and pulled the DNA (Fig. 2B). Unless otherwise stated, *E. coli* ssDNA binding protein (SSB) was added to the second laminar stream to prevent any subsequent interaction of unwound ssDNA with other Rep-X on the bead surface, but we did not find any difference in behavior with or without SSB (18). The DNA was held under constant force, ranging from 4 to 22 pN, as indicated. Additional controls and considerations ascertained that the observed activity stemmed from a single Rep-X, regardless of the 3′-tail length and inclusion or omission of SSB (supplementary text). Notably, 95% (38/40) of the Rep-X–DNA complexes resulted in the unwinding of the entire observable ~4-kbp region of the 6-kbp DNA in a processive manner, and the average pause-free speed was 136 bp/s (Fig. 2, B to D). In comparison, only 3% (2/61 at 4 pN, none at higher forces) of wild-type Rep and 7% (5/70) of Rep-Y complexes displayed such processive unwinding. Rep-X probably has even greater processivity than 6 kbp and is currently limited only by the length of the DNA used, because all of the initiated unwinding events proceeded to the end of the measureable range of DNA.

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**Protein Structure**

**Engineering of a superhelicase through conformational control**

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Conformational control of biomolecular activities can reveal functional insights and enable the engineering of novel activities. Here we show that conformational control through intramolecular cross-linking of a helicase monomer with undetectable unwinding activity converts it into a superhelicase that can unwind thousands of base pairs processively, even against a large opposing force. A natural partner that enhances the helicase activity is shown to achieve its stimulating role also by selectively stabilizing the active conformation. Our work provides insight into the regulation of nucleic acid unwinding activity and introduces a monomeric superhelicase without nucleasen activities, which may be useful for biotechnological applications.
our assay where we manually stopped the experiment. We confirmed that Rep-X, but not Rep, can unwind 3.5-kb DNA in the absence of force, which indicates that the tension is not needed for high processivity (fig. S4).

We determined how much force Rep-X can generate by performing measurements without maintaining a constant force. Fixing trap positions led to a rapid build-up of force in the direction opposing unwinding until the measurement was terminated due to the breakage of the connection between the two beads (Fig. 2E). The highest loads achieved in this experiment were not enough to stall the helicase permanently. More detailed analysis showed that the pause-free unwinding rate of Rep-X was not impeded by increasing loads up to the limits of the linear regime of our trap (Fig. 2F): ~60 pN (18).

These results suggest that Rep-X is the strongest helicase known to date (22, 23). To test whether the generation of a superactive helicase via conformational control can be reproduced for other helicases, we engineered PcrA-X from Bacillus stearothermophilus PcrA.

Mutations involved replacing two highly conserved cysteines (tables S1 and S2), reducing the apparent ATPase activity from ~40 ATP/s (wild type) to 5 ATP/s. Upon cross-linking in the closed form, PcrA-X retained the low-ATPase activity (4.3 ATP/s) but exhibited an enhanced helicase activity in comparison to PcrA in ensemble reactions (Fig. 3A and fig. S5, A and B). Our smFRET experiments showed that PcrA-X monomers can unwind 39% (23/578) of DNA molecules, compared with 4% (26/617) for PcrA (fig. S5, C and D). In the optical tweezers assay, PcrA-X monomers were capable of processively unwinding 1- to 6-kbp-long DNA, albeit at a much lower rate (2 to 15 bp/s) (Fig. 3B), whereas no PcrA molecule (0/51) could do the same (Fig. 3C). Despite the impaired activity levels of the PcrA mutant, conversion to PcrA-X made its monomers into highly processive helicases.

Strong helicase activity of Rep-X and PcrA-X raises the possibility that their cellular partners may switch on their unwinding activity by constraining them in the closed conformation. One such partner of PcrA is RepD, a plasmid replication initiator protein that recognizes and forms a covalent adduct with the oriD sequence of the plasmid and then recruits PcrA for processive unwinding (24, 25). We prepared an oriD DNA-RepD adduct and measured the intramolecular conformation of PcrA bound to this adduct (18). We used a double-cysteine mutant of PcrA (PcrA-DM) stochastically labeled with a mixture of donor and acceptor fluorophores that would be expected to generate high $E_{\text{FRET}}$ in the closed form and low $E_{\text{FRET}}$ in the open form (Fig. 3D, schematics) (26). The $E_{\text{FRET}}$ distributions of PcrA-DM bound to the oriD DNA-RepD adduct and the oriD DNA alone showed that RepD biases PcrA toward the closed high-$E_{\text{FRET}}$ conformation (Fig. 3D), which may be the basis for unwinding activation in vivo.

Why does constraining Rep and PcrA into the closed form convert an enzyme with undetectable unwinding activity to a superhelicase? The intrinsic unwinding activity itself may require the closed form; for example, via the torque-wrench mechanism proposed for UvrD (14). Alternatively, 2B may play a regulatory role (4); more specifically, the open form may inhibit helicase function, and

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**Fig. 1. Cross-link-mediated conformational control of helicase activity.** (A) Open and closed forms Rep crystal structures (Protein Data Bank entry 1UAA). Domains are colored and named. Cysteine pairs that were cross-linked to lock the protein into the closed or open conformation are shown in red or orange, respectively. Distances between the pairs are noted. Close-up views show the pairs that were cross-linked. C, Cys; S, Ser; D, Asp. (B) Schemes of smFRET analysis. The brightness of the donor (green) and acceptor (red) changes as unwinding progresses. (C) Representative single-molecule time traces for Rep-X, Rep, and Rep-Y.
Fig. 2. Rep-X processivity and force generation. (A) Schematics of optical tweezers assay for Rep-X DNA unwinding. nt, nucleotide. (B) 6-kbp DNA unwinding traces (colored according to overhang length, SSB, and force; offset for clarity). Background colors denote two laminar flows (see inset). (C) Distribution of unwinding speed (N = 38 molecules). Mean speed and SD for each molecule are plotted above [colors as in (B)]. (D) Fraction of complete DNA unwinding events. Error bars represent 95% confidence bounds. (E) Unwinding traces by five Rep-X molecules in the fixed trap assay (colored and offset for clarity). (F) Normalized unwinding velocities ($V_{\text{norm}}$) of 58 Rep-X molecules plotted versus force. Error bars denote SEM.

Fig. 3. Conformational control of PcrA helicase. (A) Representative smFRET time traces for a PcrA-X monomer. (B) Representative processive unwinding traces by PcrA-X in the optical tweezers assay. Each color represents a single PcrA-X molecule. (C) Fractions of enzyme-DNA binding that led to processive unwinding of 6-kbp DNA in the optical tweezers assay. Error bars represent the 95% confidence bounds. (D) The conformational effect of RepD on PcrA was measured using a smFRET assay. $E_{\text{FRET}}$ histograms show that the PcrA bound to the RepD adduct is biased toward the closed form (high $E_{\text{FRET}}$ population) compared with PcrA bound to the bare oriD DNA.
cross-linking to the closed form prevents this inhibitory mechanism. We prefer the latter for the following reasons: First, Rep-Y unwinds DNA as well as Rep when functioning as oligomers (fig. S2D), suggesting that the closed form per se is not absolutely required. Second, simultaneous measurement of unwinding and UvrD conformation showed that UvrD assumes the closed conformation during unwinding, but after it unwinds ~10 bp, it reverts to the open conformation and reunds the DNA after strand switching (27). Therefore, we suggest that Rep-X is highly processive because the open conformation, which is required for strand switching and rewinding, is disallowed (27, 28). The deletion of 2B in Rep (Rep2A2B) makes it active in unwinding as a monomer (16), possibly by inhibiting strand switching. The poorer processivity of Rep2A2B compared with Rep-X (16) may stem from a lack of 2B, which carries its own dsDNA binding capacity. Topological enclosure of DNA in Rep-X and Rep-Y is unlikely to be the reason, because Rep-X showed at least 10-fold higher yield of highly processive unwinding than Rep-Y or Rep (Fig. 2D).

We demonstrated a conformational control that activates a naturally inhibited unwinding function to create a superhelicase with high processivity and high tolerance against load without nuclease activities (9, 13). Inhibitory mechanisms against load without nuclease activities may also be useful for biotechnological applications such as nanopore sequencing and isothermal DNA amplification.

REFERENCES AND NOTES

18. Materials and methods are available as supplementary materials on Science Online.

ACKNOWLEDGMENTS

We thank J. Park and M. Schlierf for experimental help and R. Zhou, H. Balcì, K. Lee, J. Yodh, T. Ngo, B. Leslie, M. Comstock, and P. Jena, and A. Jain for helpful discussions. This work was supported by the NIH (grant GM056367) and the NSF (Physics Frontier Center grant PHY 0822631) and CAREER award MCB 09-52442 to Y.R.C. T.H. and S.A. have filed a patent application (62/107,183) that relates to superhelicases.

SUPPLEMENTARY MATERIALS

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Materials and Methods

Supplementary Text

Figures S1 to S5

Tables S1 to S2

References (29–41)

9 October 2014; accepted 11 March 2015

10.1126/science.aaa0445

AXONAL REGENERATION

Systemic administration of epothilone B promotes axon regeneration after spinal cord injury

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After central nervous system (CNS) injury, inhibitory factors in the lesion scar and poor axon growth potential prevent axon regeneration. Microtubule stabilization reduces scarring and promotes axon growth. However, the cellular mechanisms of this dual effect remain unclear. Here, delayed systemic administration of a blood-brain barrier–permeable microtubule-stabilizing drug, epothilone B (epoB), decreased scarring after rodent spinal cord injury (SCI) by abrogating polarization and directed migration of scar-forming fibroblasts. Conversely, epothilone B reactivated neuronal polarization by inducing concerted microtubule polymerization into the axon tip, which propelled axon growth through an inhibitory environment. Together, these drug-elicited effects promoted axon regeneration and improved motor function after SCI. With recent clinical approval, epothilones hold promise for clinical use after CNS injury.

A n ideal treatment to induce axon regeneration in the injured central nervous system (CNS) should reduce scarring (1) and growth-inhibitory factors at the lesion site (2–4), reactivate the axon growth potential (5), and be administrable as a medication after injury. Recently, a number of combinatorial approaches have led to axon regeneration (6, 7). These approaches, however, involve multiple drugs, enzymes, and interventions, and rendering clinical translation difficult. Moderate microtubule stabilization by the anticancer drug Taxol promotes axon regeneration by reducing fibrillar scarring and increasing axon growth (8, 9). However, it remains elusive how microtubule stabilization induces such divergent effects. Moreover, Taxol cannot be used for clinical CNS intervention because it does not cross the blood-brain barrier (10).

We aimed to target microtubule stabilization in the injured CNS in a clinically feasible way and to decipher its distinct cellular actions. We used epothilones, a class of U.S. Food and Drug Administration (FDA)–approved blood-brain barrier–permeable microtubule-stabilizing drugs (11). Mass spectrometry confirmed that

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17 APRIL 2015 • VOL 348 ISSUE 6232 347
Engineering of a superhelicase through conformational control
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Science 348 (6232), 344-347.
DOI: 10.1126/science.aaa0445

Engineering superenzyme function
Understanding how protein domains and subunits operate is critical for engineering novel functions into proteins. Arslan et al. introduced intramolecular crosslinks between two domains of the Escherichia coli helicase Rep, which unwinds DNA. By inserting linkers of different lengths, the domains can be held either "open" or "closed." The closed conformation activates the helicase, but it can also generate super-helicases capable of unzipping long stretches of DNA at high speed and with considerable force. Comstock et al. used optical tweezers and fluorescence microscopy to simultaneously measure the structure and function of the bacterial helicase UvrD. They monitored its DNA winding and unwinding activity and its shape during these activities. The motor domain also has a "closed" conformation during DNA unwinding and switches to a reversed "open" conformation during the zipping-up interaction.

Science, this issue p. 344 and p. 352

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