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Engineering of a super-helicase through conformational control

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Abstract

Conformational control of biomolecular activities can reveal functional insights and enable the engineering of novel activities. Here, we show that conformational control through intramolecular crosslinking of a helicase monomer with undetectable unwinding activity converts it into a super-helicase 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 how nature achieves the regulation of nucleic acid unwinding activity and introduces a monomeric super-helicase without nuclease activities which may be useful for biotechnological applications.

Protein functions can be regulated by controlling its conformation, binding of ligands and effector molecules, as well as interactions with other proteins. Many novel 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 crosslinking of an enzyme without detectable unwinding activity can convert it to a super-helicase 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). Since 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.

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Rep, PcrA and UvrD are structurally homologous 3' to 5' single-stranded DNA (ssDNA) translocases and helicases that can unwind double-stranded DNA (dsDNA) utilizing energy from 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 unwinding-capable (4, 9-11). Crystal structures revealed a flexible domain (2B) that can rotate 130-160° in a swiveling motion between two conformations, referred to as the *open* and *closed* forms (12-14) (Fig. 1A). It has been debated whether 2B is essential for unwinding or it plays a regulatory role, and 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 *E. coli* Rep mutants that are intramolecularly crosslinked to constrain the 2B domain in *closed* or *open* conformations, termed Rep-X and Rep-Y, respectively. Residues for the cysteine substitution mutagenesis and the length of the bis-maleimide crosslinkers were selected such that when crosslinked, 2B cannot rotate appreciably, effectively locking the protein in one conformation (Fig. 1A)(18). Mutagenesis, purification, crosslinking procedures, and validation that crosslinking was intramolecular rather than intermolecular are described in fig. S1 and Supplementary Text (18). Crosslinking 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 bp) with a 3' overhang at a much faster rate and higher reaction amplitude than the wild type Rep or the uncrosslinked double cysteine mutant (fig. S2A-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 if the large enhancement in unwinding activity results from the activation of a monomer or from enhanced oligomerization, we performed single molecule FRET (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 (8). 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_{FRET}) 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_{FRET} 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. S3A-B). 82% of the DNA molecules (661/809) that initially bound to Rep-X monomers were unwound (fig. S3C). 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 30-50 bp (5, 8, 20). We investigated the processivity of Rep-X using a dual optical tweezers assay (Fig. 2A) (18, 21). The two traps held sub-micron sized polystyrene beads. The first was coated with 6-kbp dsDNA with a 3' poly-dT ssDNA overhang ((dT)_{10,15,75}, as specified in the figures) via the blunt end. The other bead was coated with Rep-X molecules via the His₆-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 unwound and pulled the DNA (Fig. 2B). Unless otherwise stated, *E. coli* SSB was added to the second laminar stream in order 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/omission of SSB (Supplementary Text). Remarkably, 95% (38/40) of the Rep-X-DNA complexes resulted in the unwinding of the entire 6-kbp DNA in a processive manner and the average pause-free speed was 136 bp/s (Fig. 2B and 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, currently only limited by the length of the DNA used. We confirmed that Rep-X, but not Rep, can unwind 3.5 kb DNA in the absence of force as well indicating 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 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), approximately 60 pN (18). These results suggest that Rep-X is the strongest helicase known to date (22, 23).

In order to test if generation of a super active 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 crosslinking 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. S5A-B). smFRET experiments showed that PcrA-X monomers can unwind 39 % (228/578) compared to 4% (26/617) for PcrA (fig. S5C-D).

In the optical tweezers assay, PcrA-X monomers were capable of processively unwinding 1-6 kbp long DNA, albeit at a much lower rate (2-15 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_{FRET} in the *closed* form and low E_{FRET} in the *open* form (Fig. 3D schematics) (26). The E_{FRET} distributions of PcrA-DM bound to the *oriD* DNA-RepD adduct and the *oriD* DNA alone showed that RepD indeed biases PcrA toward the *closed* high E_{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 super helicase? 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 crosslinking to the *closed* form prevents this inhibitory mechanism. We prefer the latter for the following reasons. First, Rep-Y does unwind 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 about 10 bp it reverts to the *open* conformation and rewinds 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 (Rep 2B) makes it active in unwinding as a monomer (16), possibly by inhibiting strand switching. The poorer processivity of Rep 2B compared to Rep-X (16) may stem from 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. The resulting enzyme is a super-helicase with unprecedentedly high processivity for a single motor helicase. RecBCD has similarly high processivity but contains two motors and associated nucleases. Moreover it is known to backslide at opposing forces below 10 pN (22) whereas Rep-X can be active against forces as high as 60 pN. High processivity and high tolerance against load without nuclease activities may also be useful for biotechnological applications such as nanopore sequencing and isothermal DNA amplification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Crosslink-mediated conformational control of helicase activity

(A) *Open* and *closed* form Rep crystal structures (PDB entry 1UAA). Domains are colored and named. Cysteine pairs that were crosslinked to lock the protein into the *closed* (*open*) conformation are shown in red (orange). Distances between the pairs are noted. Close-ups show the pairs that were crosslinked. (B) Schematics of smFRET analysis. Brightness of 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. (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). Mean speed and standard deviation 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 Rep-X in the fixed trap assay. (F) Normalized unwinding velocities of 58 Rep-X molecules plotted vs force. Error bars = s.e.m.

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. **(C)** Fractions of enzyme-DNA binding that led to processive unwinding of 6-kbp DNA in the optical tweezers assay. **(D)** The effect of RepD on PcrA was measured using smFRET assay. E_{FRET} histograms show that the PcrA bound to RepD adduct is biased toward the *closed* form (high E_{FRET} population) compared to PcrA bound to the bare *oriD* DNA. In C and D, error bars represent the 95% confidence bounds.