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The helicase-like domains of Type III restriction enzymes trigger long-range diffusion along DNA*

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Abstract

Helicases are ubiquitous ATPases with widespread roles in genome metabolism. Here we report a new functionality for ATPases with helicase-like domains, namely that ATP hydrolysis can trigger ATP-independent long-range protein diffusion on DNA in one dimension (1D). Specifically, using single-molecule fluorescence microscopy we show that the Type III restriction enzyme EcoP15I uses its ATPase to switch into a distinct structural state that diffuses on DNA over long distances and long times. The switching occurs only upon binding to the target site and requires hydrolysis of ~30 ATPs. This study defines the mechanism for these enzymes and shows how ATPase activity is involved in DNA target site verification and 1D signaling, roles that are common in DNA metabolism, for example, in nucleotide excision and mismatch repair.

Helicases were defined classically as ATPases that use directional translocation to unwind duplex nucleic acids. More recently however, many “pseudo-helicases” have been described that possess amino acid motifs characteristic of helicases yet fulfill their cellular functions without obligate DNA unwinding (1) and instead are often translocases that move directionally on single- (2) or double-stranded (3,4) nucleic acids. Pseudo-helicases include the ATP-dependent bacterial Type I and III restriction enzymes (REs) which use ATP hydrolysis to communicate between two distant restriction sites on the same DNA (5,6). Only if both sites are unmethylated is the DNA considered “foreign” and destroyed by the endonuclease. Type III REs form a heterooligomeric complex of two subunits: Mod (for target site methylation) and Res (for ATPase and endonuclease activities). Because they only hemimethylate their asymmetric recognition sequences, these enzymes must signal the relative site orientation during communication (7): cleavage occurs *only if* the sites are in an inverted-repeat orientation, arranged either “head-to-head” (HtH) (7) or “tail-to-tail” (TtT) (8). Type III REs hydrolyze only a few tens of ATP per dsDNA break (9,10), suggesting either passive three-dimensional looping between sites with limited translocation (11) or diffusion in 1D following an ATP-dependent switch (Fig. S1A) (8,10).

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To distinguish between the different communication models, we developed a real-time single-molecule assay using magnetic tweezers combined with TIRF microscopy (12) that can localize fluorescently-labeled enzymes on stretched 26 kbp DNA molecules containing two centrally-located HtH EcoP15I sites with 6 kbp intersite distance (Fig. 1A). EcoP15I was labeled with quantum dots on the C-terminus of the Res subunit (QR-EcoP15I), without compromising the biochemical activity (Fig. S2). While DNA binding by lone quantum dots was not detected, addition of QR-EcoP15I and ATP resulted in enzyme attachment at one (Fig. 1B and movie S1) or two (Fig. S3) specific recognition sites. Enzymes always arrived instantaneously at the sites, with no evidence for long-lived non-specific binding en route ($N = 61$). Following a delay of, on average, 17 ± 2 s (Fig. S4), bound enzymes started to move rapidly in both directions along the DNA (Fig. 1B), consistent with random walk simulations using measured parameters (13) (Fig. S5). All bound enzyme complexes ($N = 91$) could be assigned to one of the two target sites (Fig. S6A) and 90% exhibited fast bidirectional movement. The mean-square displacement determined from single particle tracking time trajectories (14) was found to increase linearly with time confirming a 1D diffusion with a diffusion constant, $D = 0.92 \pm 0.06 \mu\text{m}^2 \text{s}^{-1}$ (Fig. 1C). This is one of the highest values reported, suggesting that EcoP15I is optimized for rapid, long-distance DNA scanning (supplementary text). Rebinding to the original site or a second distant specific site was often observed, after which additional sliding events could restart with a similar initiation rate (Fig. S4) until final dissociation occurred. Rebinding occurred preferentially at sites with the same orientation as the start site (Figs. 1D, S4). The average lifetime of a diffusion event was 9 ± 1 s, whilst the average total DNA interaction time involving multiple sliding and rebinding events was 180 ± 10 s (Fig. S4). Thus an enzyme can explore thousands of bps up- and downstream of the original site before dissociation. On a substrate with a sufficient density of EcoP15I sites, DNA cleavage was observed following collisions between a mobile enzyme and a site-bound enzyme (Fig. S6B). Given the diffusion constant and the viscous drag of the quantum dot, QR-EcoP15I cannot follow a helical path along the DNA (15) (supplementary text). We interpret this to mean that EcoP15I can move on DNA independent of the helical structure, as might be explained by a proposed toroidal clamp configuration (16). Supporting such a structure is the observation that protein road-blocks at DNA ends increase endonuclease efficiency as they prevent dissociation by sliding (10).

The period of stable target site-binding prior to initiation of diffusion suggested the existence of a conformational switch, since enzymes binding the DNA from an ATP solution did not directly enter the diffusive state. To test whether ATP hydrolysis drives the switching we repeated our measurements in the absence (apo) or presence of different nucleotide analogues: non-hydrolyzable AMP-PNP; the transition state mimic ADP-Va²⁺; and ADP. In contrast to ATP, in all cases we observed numerous non-specific association events (Fig. 2A). For apo, AMP-PNP and ADP-Va²⁺ conditions, QR-EcoP15I was almost immobile. For ADP, non-specific binding was combined with occasional excursions into long-lived diffusion states, albeit with a diffusion constant 30-fold smaller than observed in ATP (Fig. S7). To examine the effect of ATP on non-specific complexes, we pre-bound QR-EcoP15I in the apo conditions for a fixed period. After flushing in ATP, most prebound enzymes dissociated from the DNA. The remaining enzymes slowly diffused to a target site and, once specifically bound, started to undergo cycles of fast diffusive motion and target site rebinding as before (Fig. 2B). Thus, ATP hydrolysis not only initiates fast diffusion from specific sites but also reduces non-specific binding, so increasing specificity.

We measured DNA-binding and ATP consumption by EcoP15I using stopped-flow fluorimetry (17,18). Prebinding EcoP15I to a short specific oligonucleotide, and then rapidly mixing with an enzyme trap, allowed measurement of enzyme-DNA dissociation. The kinetics were most rapid with ATP (6.6 ± 0.1 s lifetime on DNA, Fig. S8), of intermediate velocity with ADP and ADP+P_i and significantly slower with AMP-PNP, P_i or apo

conditions (Fig. 2C), in qualitative agreement with the single-molecule measurements. ATP hydrolysis was measured under the same reaction configuration (Fig. 2D). On specific DNA only, hydrolysis of ~30 ATP molecules was coupled to each release event with ~10 ATPs consumed within an initial 1 s burst followed by ~20 ATPs at a lower rate similar to the accompanying dissociation kinetics (Fig. 2D and Fig. S8). Tryptophan fluorescence changes during dissociation point to a single-step nucleoprotein remodeling event coupled to the initial 10 ATPs (Fig. S9), whilst short-range translocation events (i.e. “DNA scrunching” (19)) were not observed (Fig. S10). ADP did not produce the same changes in tryptophan fluorescence, consistent with a different sliding conformation.

To address if ATP is required during sliding, ATP hydrolysis was measured using DNA of different lengths, with and without streptavidin roadblocks at the ends (Figs. 3A, S11). End-capping prevents EcoP15I sliding off the DNA and increases the DNA-bound lifetime (~200 s) compared to uncapped DNA ($\ll 1$ s) (20). There are two distinct stages in the ATPase profiles: a burst phase, due to site-specific ATP hydrolysis and initiation of sliding; and a steady-state phase, due to enzymes binding from solution and initiating sliding (consuming ~30 ATPs) and any other ATPase activity during sliding. If significant levels of ATP were consumed during sliding or upon site rebinding, we might expect a lower steady-state rate on uncapped DNA than on capped DNA due to the longer sliding lifetime on the latter substrate. Instead we observed little difference in the steady-state rates and little dependence on DNA length, suggesting that negligible ATP is hydrolyzed during diffusion or following site rebinding.

We also measured the nucleotide-dependence of site-specific binding by EcoP15I (Fig. 3B). While saturated binding was observed with apo, AMP-PNP and ADP conditions, binding in ATP was characterized by a transient maximum followed by a lower steady-state. A two state model (free and bound) cannot describe this profile. However, satisfactory fits were obtained by introducing a third state (in agreement with the tryptophan fluorescence measurements) following ATP-dependent DNA dissociation, where an activated EcoP15I must slowly re-isomerise before it can rebind DNA (Figs. 3C, S12). The target site-binding lifetime of 5.9 ± 0.7 s matches that observed in Fig. 2D and is similar to the value from the single-molecule experiments. The activated state has a shorter lifetime (~25 s) than the total interaction time measured in the tweezers, but nonetheless we interpret this as representing the sliding conformation – the shorter observed lifetime may reflect the absence of bound DNA.

In this work we provide direct evidence for the 1D diffusion hypothesis for intersite communication and cleavage by Type III REs (Fig. S1B). 1D diffusion on DNA is a general model for target site localization by proteins (21), but sliding events in this situation are short-lived and can be interspersed by microscopic dissociation-reassociation events (“hopping”). Hopping can be ruled out for Type III REs as this would lose the original asymmetric binding orientation and would negate the site-orientation selectivity of these enzymes. The EcoP15I mechanism highlights that long-lived sliding is only achieved by a conformational switch catalyzed by site-dependent ATP hydrolysis. The necessity for an ATP hydrolysis step to establish long-lived sliding has precedent in the loading of processivity clamps at replication forks by the AAA+ family clamp loader complexes (22). There are also close functional parallels to mismatch repair: After mismatch targeting through helix-correlated sliding and 3D steps, the ABC transporter family MutS enzymes use a two-step conformational change to adopt a sliding clamp configuration that displays accelerated, helix-uncorrelated 1D diffusion (23–25). This transition requires a sole ADP to ATP exchange, contrasting with EcoP15I that hydrolyses many ATPs upon initiation (Fig. S1). Together this shows that ATP-triggered sliding is a general function that has evolved in different ATPase kingdoms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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One Sentence Summary

A bacterial helicase hydrolyses a few ATPs to produce a structural switch allowing diffusion over thousands of base pairs.

Fig. 1.

Direct observation of long-range diffusion on DNA by the Type III restriction enzyme EcoP15I in the presence of ATP (A) Detection scheme based on magnetic tweezers that allows simultaneous DNA stretching and TIRF microscopy of quantum dot-labeled enzymes. (B) Kymographs of the interaction of 0.7 nM QR-EcoP15I in the presence of ATP with a DNA substrate that contains two target sites in an inverse HtH orientation, spaced 6 kbp apart (see sketch on the left). The snapshot fluorescence image on the far left shows the magnetic particle and the bound enzyme after 60 s. The colored bar on the bottom of the kymograph highlights periods of enzyme diffusion (green) and target site binding (blue). (C) Cumulative mean-square displacement over time of different diffusion events of EcoP15I on DNA ($N = 32$). A weighted linear fit to the data provides a diffusion constant of $0.92 \pm 0.06 \mu\text{m}^2 \text{s}^{-1}$ (SEM). (D) Probability of EcoP15I to rebind during the diffusion to its original start site or to the distant target site normalized by the number of attempts ($N = 201$). (Error bars in C and D given as SEM).

Fig. 2.

ATP hydrolysis triggered release of the enzyme from its target site. **(A)** Interaction of 0.13 nM QR-EcoP15I with DNA in the presence of different nucleotides. Apo indicates the absence of any added nucleotide. Images and kymographs have identical scales. Snapshots were taken after 120 s. **(B)** ATP-triggered release of prebound enzyme from DNA. Period 1: Addition of 0.7 nM enzyme in the absence of nucleotide. Period 2: Flush with buffer only. Period 3: Flush with buffer containing ATP. Snapshots shown were taken at 200 s (before ATP addition) and 400 s (after ATP addition). The immobile quantum dot following ATP addition is stuck to the capillary surface in proximity to the DNA. **(C)** Stopped-flow measurements (see sketch) showing the release of prebound enzyme from its target site with different nucleotides **(D)** Stopped-flow measurements of DNA-dissociation by EcoP15I and the amount of ATP hydrolyzed upon adding ATP and heparin. The *inset* shows the DNA dissociation kinetics and the instantaneous ATPase rate obtained from the derivative of the ATP hydrolysis kinetics (both data from main figure, see Fig. S8).

Fig. 3. EcoP15I adopts a new conformation upon ATP hydrolysis driven target site release. **(A)** ATP hydrolysis during target site binding of EcoP15I in the absence of a trap using a short 0.5 kbp DNA with free ends and a longer 1.5 kbp DNA with streptavidin roadblocks at its ends. Stopped-flow measurements were initiated by mixing enzyme prebound to DNA with ATP (see sketch) with both solutions containing a phosphate sensor. **(B)** Binding kinetics of EcoP15I to its target site in the presence or absence of different nucleotides. Stopped-flow anisotropy measurements were initiated by mixing enzyme and DNA supplemented with nucleotide (see sketch) **(C)** Binding kinetics of EcoP15I to its target site in the presence of ATP as measured in **B** for different enzyme concentrations (gray curves, increasing grayscale values correspond to 0, 20, 26, 40, 53, 66, 99, 132, 265 nM EcoP15I). The experimental data was fitted according to the scheme sketched above based on four different rate constants (light to dark red curves). Curves for concentrations of 20 to 132 nM were obtained from fits to the experimental data, whereas mean rate constants were used to simulate the curve for 265 nM (Fig. S12).