nature

SUPPLEMENTARY INFORMATION

https://doi.org/10.1038/s41586-019-1397-7

In the format provided by the authors and unedited.

Rotation tracking of genome-processing enzymes using DNA origami rotors

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Supplementary Information for

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Supplementary Discussion

Origami rotor design

The origami rotor was designed with rotor blades sufficiently long to amplify the motion of the DNA substantially, yet still have low hydrodynamic drag and torsional flexibility in order to minimize the obscuring effect of Brownian fluctuations, thereby allowing measurements with high spatiotemporal resolution. In our design, the origami rotor consisted of four rotor blades, each extending 80 nm perpendicular to the axis of rotation (Extended Data Fig. 1a-e, Supplementary Table 1). The rotor's planar structure was selected to generate low hydrodynamic drag (see Estimate of the hydrodynamic drag of the origami rotor below).

In addition, the use of DNA origami as rotors allowed us to create a direct connection between the rotor and the dsDNA substrate and to keep the linker between the rotor and the surface-anchored enzyme short, both of which helped minimize torsional flexibility. To create this connection, a short double-stranded DNA (dsDNA) segment with single-stranded overhang was extended from the center of the rotor along the axis of rotation (Extended Data Fig. 1a-c); this short DNA segment can be ligated to the DNA substrate of a DNA-interacting enzyme (Fig. 1a; Extended Data Fig. 1d). In the absence of an applied external force, there is an inherent trade-off between resolution and the length of this DNA linker. While the use of a short linker limits the distance over which we can study enzyme motion on DNA, the resulting increase in resolution would ideally allow us to resolve the fundamental mechanistic steps of the enzymatic reaction cycles, which in general occur on a short length scale.

Brownian dynamics characterization

The resolution of ORBIT is limited by the Brownian dynamics of the origami rotor. The extent and timescale of these Brownian motions is determined by the torsional stiffness of the connection between the origami rotor and the enzyme (or other attachment point), κ, and the drag of the origami rotor, γ. Due to the drag, the angular fluctuations are correlated in time, with a relaxation time constant, $\tau = \gamma / \kappa$. In addition, photon-limited localization uncertainty of fluorescence dye molecules can also contribute to the noise.

In order to characterize these parameters, origami rotor-anchor complexes (Extended Data Fig. 1h) were attached to cleaned glass coverslips using biotinylated BSA and streptavidin. These were imaged in RecBCD reaction buffer or a similar buffer without glycerol at 1500 Hz or 3000 Hz. Localization trajectories were determined in a similar fashion to the RecBCD analysis described in Supplementary Methods. Localization positions were fit to a circle and the center position was used to convert the (x,y) positions to polar coordinates From this trajectory, we extracted κ and γ by fitting the power spectrum of this trajectory. The power spectrum was generated from each angular trajectory by determining the squared magnitude of the Fourier transform of the rotor angle. The power spectrum, $P(f)$, where f is the frequency, was fit to a model of the observed Brownian noise that takes into account motion blur and aliasing[31] and the frequency-independent contribution of the photon-limited localization uncertainty[32,33],

$$P(f) = \frac{2k_B T\gamma}{\kappa^3}\left(\kappa + \frac{2\gamma f_s \sin^2\left(\frac{\pi f}{f_s}\right)\sinh\left(\frac{\kappa}{\gamma f_s}\right)}{\cos\left(\frac{2\pi f}{f_s}\right) - \cosh\left(\frac{\kappa}{\gamma f_s}\right)}\right) + \varepsilon \qquad \text{(S1)}$$

where k_B is Boltzmann's constant, T is the temperature, f_s is the camera frame rate, and ε is the contribution from the localzation uncertainty. The contribution of localization uncertainty ε was fixed for each trajectory and we determined this term from the trajectory's radial position variance as we found that the radial variance was dominated by localization uncertainty (Extended Data Fig. 2f). The radial position variance was converted to an angular equivalent σ_L^2 using the radius of the arc of localizations. To determine ε from the radial variance, the variance σ_L^2 was divided by the size of the frequency range of the power spectrum (f_s/2), giving

$$\varepsilon = \frac{2\sigma_L^2}{f_s} \qquad \text{(S2)}$$

Predicted angular precision

Based on the characterization of the Brownian dynamics obtained by fitting the power spectrum with Equation S1 as described above, the angular precision after integrating measured positions over an integration time t can be estimated as[33]

$$\sigma(t) = \sqrt{\frac{2k_BT}{\kappa}\left(\frac{\tau}{t} - \frac{\tau^2}{t^2}\left(1 - \exp\left(-\frac{t}{\tau}\right)\right)\right) + \frac{\sigma_L^2}{f_s t}} \qquad \text{(S3)}$$

Note that unlike the Brownian noise, the localization uncertainty σ_L^2 per camera frame is uncorrelated from one frame to the next and the variance due to localization uncertainty falls as the inverse of the square root of the integration time t, as expected. This estimate is shown in Extended Data Fig. 2e, with and without the contribution of localization uncertainty term σ_L^2 taken into account.

Estimate of the effective torsional rigidity of DNA

The torsional stiffness κ derived from the power spectrum fit (Equation S1) of the anchored origami complex can be used to estimate the effective torsional rigidity of dsDNA. The value of κ contains contributions from the dsDNA linker between the tripod anchor and the rotor as well as contributions from the flexibility of the attachment points to the origami and between the anchor and surface. These components contribute to the flexibility as springs in series, giving

$$\frac{1}{\kappa} = \frac{1}{\kappa_{DNA}} + \frac{1}{\kappa_{other}} = \frac{L}{C} + \frac{1}{\kappa_{other}} \qquad \text{(S4)}$$

where κ_{DNA} is the contribution of the dsDNA linker, κ_{other} is the other contributions from the attachment points, L is the length of the dsDNA linker, and $C = \kappa_{DNA} * L$ is the effective torsional rigidity constant of DNA. By measuring κ of the anchored origami complexes with several dsDNA linker lengths, but otherwise identical design (i.e. identical κ_{other}) (Extended Data Fig. 2b), we find that C = 200 ± 10 pN nm^2 rad^{-1}, which is within the range of previously measured results under zero force[12]. Note that due to the contribution of twist-bend coupling at zero force, this value is an effective torsional rigidity which is smaller than the torsional rigidity of DNA under force, where the bending modes are suppressed[12].

Estimate of the hydrodynamic drag of the origami rotor

We estimate the hydrodynamic drag of the origami rotor by treating it as a pair of cylinders of length 160 nm and radius 3 nm rotating about the middle point along the length of the cylinder. For a single cylinder rotating about its middle point along the long axis, the drag γ can be estimated as[34]

$$\gamma = \frac{\pi\eta L^3}{3\,(\ln(p) + \delta_T)} \qquad \text{(S5)}$$

where η is the solvent viscosity, L is the cylinder length, δ_T is an end-effect correction, and

$$p = \frac{L}{2R} \qquad \text{(S6)}$$

where R is the cylinder radius. For $p \approx 25$ we use $\delta_T = -0.616$ as an approximate end-effect correction. Using this approach, and taking into account both arms, we estimate the drag of the origami rotor in water to be ~3 fN·nm·s. The viscosity of 10% glycerol is 30% higher than water, giving an expected drag of ~4 fN·nm·s under conditions used for our RecBCD experiments. These values are comparable to our values of the drag determined from the power spectrum of the anchored origami complex (Extended Data Fig. 2d), 3.7 ± 0.1 fN·nm·s and 4.9 ± 0.1 fN·nm·s at 0% and 10% glycerol, respectively. For comparison, for a bead of diameter $D = L$ rotating about its center, which would give the same amplification of DNA rotation as our origami rotor, the hydrodynamic drag would be $\gamma = \pi\eta D^3$ (Ref. [5]), which is equal to 12 fN·nm·s at 0% glycerol and ~ 4 times larger than the drag of our origami rotor, which leads to a substantially lower time resolution in rotation measurements.

Estimate of torque and angular lag due to the hydrodynamic drag for the origami rotor

The motion of the DNA origami rotor through solvent causes a resisting torque due to the origami's hydrodynamic drag. This torque due to the drag is given by $\tau_d = -\gamma\omega$, where γ is the drag and ω the angular velocity. At 10% glycerol and the highest ATP value used in this work for RecBCD experiments, 300 μM, the average rotation rate was measured to be 215 bp/s, or 130 rad/s. Using the rotor's drag of 4.9 fN·nm·s, measured using the power spectrum fit of the anchored origami rotors, we estimate the typical torque under these conditions as -0.6 pN·nm·rad^{-1}, which is much smaller than the contribution from the thermal energy, ~4 pN·nm, and thus inconsequential in rotation measurements. The torque is smaller during RNAP experiments as the enzyme is slower.

The drag will also cause the origami rotor to lag behind the true angular change of DNA at the enzyme. Since this lag is resisted by the stiffness of the DNA, we can estimate the angular lag as

$$\theta_{lag} = \frac{\tau_d}{\kappa} = -\frac{\gamma\omega}{\kappa} \qquad \text{(S7)}$$

where κ is the torsional stiffness of the complex. At the beginning of RecBCD translocation when the dsDNA linker is 80 bp long, we use the relationship between the torsional stiffness and

dsDNA length in Extended Data Fig. 2b to find κ ~6 pN·nm·rad^{-1}. At this stiffness, we estimate θ_{lag} to be -0.10 rad (or -6°) at 10% glycerol using the average DNA rotation rate of 130 rad/s. Later during translocation, the DNA linker is shorter, which will further increase the stiffness and reduce the angular lag. Thus, the angular lag is small compared to our measured angular noise (~35°) due to Brownian motion and can be neglected.

Local pH shift at the coverslip surface

Silicate glass coverslip surfaces such as those used in this experiment are negatively charged[35], which leads to local accumulation of H^+ ions and thus induces a local pH shift experienced by the surface-bound enzyme. This change can be described by

$$pH_s = pH_b + \frac{F\Psi}{2.3RT} \qquad \text{(S8)}$$

where pH_s is the effective pH at the surface, pH_b is the bulk solution pH, F is Faraday's constant, Ψ is the electric potential due to the surface charge, and RT is the thermal energy[36]. An experimental measurement of this pH shift for an enzyme attached to a silicate surface previously gave the value of ~2 pH units (i.e. local pH at the surface is ~2 pH units lower than the bulk pH)[37].

This effect should be taken into account in the comparison because the initiation kinetics of RecBCD depended quantitatively on pH (Extended Data Fig. 9). Therefore, we considered this local pH shift in the comparison between the RecBCD unwinding kinetics measured using ensemble stopped-flow assay with those measured by the single-molecule experiments (Extended Data Fig. 8). As single-molecule experiments are often carried out using surface-anchored molecules, we note that it may be generally important to characterize the effect of surface-induced pH shift on enzyme behavior if the enzyme is near a charged surface.

We envision that alternate strategies that anchor the enzyme to the surface through specific linkage at multiple sites[38] could also be used to achieve torsionally constrained enzyme immobilization.

Supplementary Methods

DNA origami preparation and purification

DNA origami rotors (Extended Data Fig. 1a-e, Supplementary Table 1) and anchors (Extended Data Fig. 2f-i, Supplementary Table 2) were designed using CADNano[39] and prepared as described previously[11], with modifications. All DNA oligomers, including origami staple strands and additional DNA linkers, were ordered from Integrated DNA Technologies (IDT). All origami structures contained extension strands with single-stranded DNA (ssDNA) overhangs for ligation to additional DNA. Oligomers containing dye, phosphorylation for ligation, or biotinylation modifications and any strands being ligated were ordered with HPLC or PAGE purification. DNA modifications, namely Cy3 fluorescent dyes, biotinylation, and 5' phosphorylation, are indicated using their IDT codes (see Supplementary Tables 1-4). To attach the DNA origami base to the streptavidin-coated coverslip, it contains six strands (labeled 'TTHr21' in Supplementary Table 2) with binding sites for the biotinylated oligomer ('Hr21_5Bio').

DNA staple strands (Supplementary Tables 1, 2) and the M13mp18 viral DNA (single-stranded, New England Biolabs) used as the scaffold[10] were mixed in folding buffer: 10 mM Tris, pH 8.0, 1 mM EDTA, and 18 or 9 mM $MgCl_2$ for the origami rotor and for the anchor, respectively. The concentrations of DNAs were 10 nM for the scaffold strand and 100 nM for the unlabeled staple strands, and 0.5 - 1 μM for the Cy3-labeled staple strands. The origami mixtures were incubated and annealed using a thermocycler. For the origami rotor, these mixtures were held at 80°C for 5 minutes, and annealed by cooling, first to 65°C in 1°C steps every 5 minutes, then to 25°C in 1°C steps every 105 minutes. The origami anchor was folded by heating to 80°C for 5 minutes, and annealed by cooling, first to 65°C in 1°C steps every 1 minute, then to 25°C in 1°C steps every 20 minutes.

For RecBCD and RNAP experiments, the folded origami rotors were purified, ligated to double-stranded DNA (dsDNA) segments serving as RecBCD substrates, and then again purified. We first PEG precipitated the origami to remove most of the free staple strands[40]. The origami sample was mixed 1:1 with 2x PEG precipitation buffer (15% PEG-8000, 5mM Tris, pH 8.0, 1 mM EDTA, 500 mM NaCl), incubated 30 minutes at 4°C, and centrifuged at 8000g for 30 minutes. The pellet was washed with 1x PEG wash buffer (7.5% PEG, 10 mM Tris, pH 8.0, 1 mM EDTA, 18 mM $MgCl_2$). After being resuspended in T4 ligation buffer, the pre-annealed short extension strands were ligated to longer DNA oligomers (Supplementary Table 3; note the sequence descriptions in Supplementary Table 3 indicate final extension DNA length after ligation) using T4 DNA ligase (New England Biolabs) for 2 hours at room temperature. The reaction mixture was treated with Proteinase K (New England Biolabs) for 1 hour at room temperature to degrade the ligase. The origami sample was then purified by agarose gel electrophoresis. Electrophoresis was performed with a 2% agarose gel in an ice bath in running buffer containing 89 mM Tris, 89 mM borate, 2 mM EDTA, and 10 mM $MgCl_2$. The origami band was excised from the gel and the origami extracted using a freeze 'n' squeeze spin column (Bio-Rad) by spinning at 1000g for 60 minutes. The sample was concentrated by PEG precipitation as described above.

To characterize the Brownian-limited angular resolution of the rotor, we prepared a complex with a DNA origami rotor attached to an origami anchor via dsDNA linkers of various lengths (Extended Data Fig. 1h). To remove excess origami extension strands, the origami structures were gel purified before ligation. The two origami structures were ligated together as described above. The length of the linker DNA between the two origami structures included the

14 base pairs (bp) extending from the origami rotor (Extended Data Fig. 1c), 26 bp on the origami anchor (Extended Data Fig. 1f), 12 nucleotides (nt) of ssDNA overhang on both structures, and any additional DNA added between the two origami. For the shortest DNA linker length, 52 bp, the linker consisted entirely of DNA present on the rotor and anchor structures (Supplementary Tables 1-3). Note that for this sample, to create the direct connection, the DNA oligomer Anchor_Ext0_oh (Supplementary Table 2) was replaced with Anchor_Ext0_oh_direct (Supplementary Table 3). The two longer lengths used additional dsDNA linkers in the ligation reaction (Supplementary Table 3). These were either purchased from IDT (92 bp) and annealed prior to ligation or prepared by PCR and dU excision (163 bp). In the latter case, the DNA was prepared using PCR with PfuTurbo Cx Hotstart DNA Polymerase (Agilent) and primers with a dU base 12 nt from their 5' ends (Supplementary Table 3). Following purification on a column (Zymo DCC-100), the product's dU bases were excised with the USER enzyme system (New England Biolabs) to create 12 nt overhangs for ligation with the rotor and anchor structures. Finally, the ligation products were again purified using electrophoresis.

AFM imaging

AFM images were obtained using an Asylum MFP-3D system (Asylum Research) at the Center for Nanoscale Systems at Harvard University. A 2 μL droplet of purified sample (low nM concentration) and then a 20 μL drop of buffer containing 5 mM Tris, 0.5 mM EDTA, 10 mM $MgCl_2$, 10 mM $NiCl_2$ was applied to a freshly cleaved mica surface and left for approximately 2 minutes. The images were taken under liquid tapping mode, with C-type triangular tips (resonant frequency, f_0 = 40-75 kHz; spring constant, k = 0.24 N m^{-1}) from the SNL-10 silicon nitride cantilever chip (Bruker Corporation).

TEM imaging

For TEM imaging, sample was adsorbed onto glow discharged carbon-coated TEM grids for 2 minutes and then stained for a few seconds using a 2% aqueous uranyl formate solution containing 25 mM NaOH. Imaging was performed using a JEOL JEM-1400 TEM operated at 80 kV.

Single-molecule imaging of RecBCD-induced DNA unwinding using ORBIT

Single-molecule fluorescence imaging was conducted using a Nikon Eclipse Ti inverted microscopy body with a 60x 1.4 NA oil objective (Nikon) and a high speed scientific CMOS camera (Hamamatsu Orca-Flash 4.0 v2). The camera field of view was cropped as needed to achieve high frame rates. The sample was illuminated using objective-type total internal reflection with a 1 W 532 nm laser (CrystaLaser). Laser intensity was controlled using an acousto-optical tunable filter (Crystal Technologies). The microscope filter cube contained a dichroic mirror (Chroma Technology Corp ZT532/640rpc-UF3) and an emission filter (Chroma Technology Corp ZET532/640m-TRF). The focus was maintained with an IR laser reflection focus lock system. Each camera pixel corresponded to 160 nm in the sample plane. The microscope hardware was controlled with custom software written in Python.

Origami were imaged in a flow chamber consisting of a glass coverslip (VWR, No. 1.5) attached to a microscope slide with double sided tape. Two holes were drilled in the slides to facilitate buffer exchange. The coverslips were cleaned by sonication in 95% ethanol, rinsing in water, drying thoroughly with compressed nitrogen, and plasma cleaning under argon atmosphere (Harrick Plasma PDC-32G). Between uses, slides were soaked in acetone and water

to facilitate flow chamber disassembly, then scrubbed with alconox, sonicated in 1 M KOH, rinsed in water, briefly flamed with a propane torch, and plasma cleaned. After assembling the flow chambers with double sided tape and sealing the sides with epoxy, they were used the same day or vacuum sealed until use to prevent dust accumulation. Tubing was inserted into the slide holes and epoxied in place. A syringe pump (KD Scientific KDS210) was used to pull solution into the chamber.

Standard reaction buffer contained 50 mM Tris, 2 mM Trolox, 50 μM of the Trolox quinone (prepared using UV irradiation and quantified by UV-Vis absorbance spectroscopy before adding to the buffer[41]), 5 mM protocatechuic acid (PCA), 10% glycerol, and 10 mM $MgCl_2$, adjusted to pH 8.0 unless otherwise indicated. Before imaging, 0.25 U/mL protocatechuate-dioxygenase (PCD; sold by OYC Americas as rPCO) was added. The PCA/PCD system acts as an oxygen scavenger and Trolox suppresses dye blinking[42,43]. ATP (Affymetrix or ThermoFisher Scientific) was added when indicated in the text. PCD was added ~10 minutes before imaging to allow the PCA/PCD system to remove oxygen. All experiments were done at room temperature (~23° C).

Experiments were conducted by first flowing 300 units/mL RecBCD (New England Biolabs) in reaction buffer into the chamber to passively adsorb RecBCD molecules to the slide surface. After ~1 minute, unbound RecBCD was washed out with ~100 μL of reaction buffer twice. The second wash contained PCD and the desired ATP concentration. Finally, the origami-dsDNA substrate complex was added to the chamber in reaction buffer with PCD and the desired ATP concentration. Data were typically acquired for 3-4 minutes at 500 Hz – 1 kHz.

Single-molecule data analysis for RecBCD

Single-molecule imaging data were analyzed using custom Python code. Briefly, to identify RecBCD-bound rotor complexes, the movie was first divided into 100-frame segments, and a median image was generated for each such segment by computing the 100-frame median value for each pixel. We then identified local maxima in these median images and used the corresponding coordinates to select persistent fluorescent spots for further analysis. Using median images allowed us to ignore rotors that entered the evanescent field transiently without successful binding to RecBCD. However, these median images where not used for reconstructing single molecule trajectories, which were instead determined by fitting each selected fluorescent spot to a 2D Gaussian in each raw-data frame.

Single molecule trajectories were further analyzed using custom code in Igor Pro. Among the binding events of the rotor-dsDNA substrate to the surface (fluorescent spots appearing at the surface for at least 0.2 s; typically, ~250 spots per field of view), ~50% of these spots showed localizations moving along the full circumference of a circle and were selected for further analysis. The remaining 50% showed either no apparent motion (~30%), likely corresponding to rotors bound directly to the surface (i.e. not through the dsDNA linker which would have led to Brownian motion of rotors), or showed constrained movement along a partial circular arc (~20%). The latter fraction (~20%) could be due to rotors bound in a non-canonical manner with some degree of flexibility, for instance via the dsDNA linker to an incorrect part of an enzyme or directly to the coverslip surface, or from canonical attachment of dsDNA linker to inactive enzymes (which would be expected due to the fraction of inactive enzymes present in typical preparations of RecBCD solutions).

For the spots displaying motion along a complete circle, we fit each set of localizations to a circle, the center of which was used to convert (x,y) positions to polar coordinates (see Fig. 1c).

~50% of these circular trajectories showed processive rotation, due to processive RecBCD activity, while the remainder showed random fluctuations in the angular direction. This latter fraction, which is likely due to RecBCD bound to the surface in a torsionally unconstrained manner or due to mis-assembly of the rotor-dsDNA junction (leaving a portion being single stranded), was not considered for subsequent analysis. Furthermore, high localization precision is required for high-accuracy rotational tracking. We used the radial variance (localization variance in the radial direction, orthogonal to the circular path), which depended on the photon number detected from the fluorescent spot in each frame (Extended Data Fig. 2f), as an approximate measure of the localization precision and included only trajectories with a localization precision better than 16 nm (0.1 camera pixel width) in our analysis of DNA unwinding by RecBCD. We expect the Brownian dynamics in the radial direction to be small because (1) unlike the angular fluctuations, the radial motions due to lateral motions of the origami (resulting from DNA bending) are not amplified by a lever arm effect and (2) tilting motion of the arms relative to the surface, although amplified by the lever arm, has only a very small lateral projection on the imaging plane because the maximum tilting angle is small. Typically, among the trajectories that displayed processive RecBCD activity, ~30% of them were removed due to this localization precision cut. In the remaining trajectories, the angular noise was dominated by the Brownian dynamics, which was substantially larger than the localization precision measured by the radial variance. However, a small fraction of traces (~10% of the remaining trajectories) showed periods when the angular motion of the origami was comparable to the localization precision, likely due to interactions with the surface or steric hindrance by the surface if the rotor is tilted, and were excluded from further analysis. The surface sticking of origami rotor was minimal likely because the negative charge on the glass (silica) surface tends to repel the negatively charged DNA, and inclusion of this small fraction of trajectories showing surface interaction of rotors did not change our results appreciably. After the above three filters, a total of ~30% (~50% x 70% x 90%) of circular trajectories remained and were considered in our RecBCD activity analysis. Angular changes were converted to base pairs unwound using the average DNA angular twist of 34.6°/bp.

RecBCD pausing analysis

We used an automated pause-finding algorithm to identify pauses in the single-molecule unwinding trajectories. Briefly, the trajectories were subjected to a 20 Hz half-transmission frequency binomial smoothing filter, and time derivatives of the trajectory were than used to determine the instantaneous velocity. Frames showing a velocity below a threshold (1° per frame at 500 Hz), were identified as pause frames and frames moving backwards faster than this rate were identified as potential backtracking frames. Because long pauses tended to get broken up due to short fluctuations, another binomial smoothing (8.4 Hz) was applied and additional frames were called as pauses here using the same threshold. Since smoothing tends to blur the edges of the pausing phase, pauses were extended forward and backward until the angle in the raw data moved outside of a 1 bp window from the pause location. Adjacent pauses at the same angular location were merged. Because of signal fluctuations, we only consider pauses that lasted at least 100 ms as real pauses during forward unwinding to avoid false positive detection of pauses. Likewise, we only considered backtracks that lasted at least 100 ms (including pre-backtracking pause and recovery pause) and exhibited a minimum of 100° (3 bp) backward motion as real backtracks. For pauses that occurred between backtracking and recovered forward unwinding,

we did not set a threshold on pause duration because of the low probability of false positive detection of such events.

RecBCD initiation analysis

The RecBCD initiation phase was defined as the period of the trajectory between binding and the start of processive unwinding. Reversible unwinding transitions were detected during this initiation phase. To determine the transition rates between the wound (magenta) and unwound (green) states, the initiation phase was segmented according to the angle, and the dwell time in each state was determined. In the presence of ATP, exit from the green state is a competitive process, with transitions occurring either back to the magenta state or forward to processive unwinding. This reduces the dwell time in the green state, increasing the apparent transition rate between the green state and the magenta state. Assuming both processes are first order transitions, the apparent transition rate, measured as the average dwell time of individual green states, should be equal to the sum of the real transition rate from green to magenta state and the transition rate from green state to the processive unwinding state. Mathematically, the real transition rate from the green state to magenta state, $k_{g\text{-}m}$, is related to the average dwell time in the green state, $<t_g>$, and the probability of transitioning from the green state to the magenta state, $P_{g\text{-}m}$ by the following equation[44]

$$k_{g-m} = \frac{P_{g-m}}{< t_g >} \tag{S9}$$

$P_{g\text{-}m}$ is related to the average number of times each trajectory visits the green state, c_g, before unwinding by considering all possible number of visits and their probability[45],

$$< c_g > = \sum_{n=1}^{\infty} \left(1 - P_{g-m}\right) * (P_{g-m})^{n-1} * n = \frac{1}{1 - P_{g-m}} \tag{S10}$$

Based on our measured distribution of the number of times each trajectory visited the green state before processive unwinding, we obtained $P_{g\text{-}m}$ = 0.52 ± 0.06 (mean ± s.e.m.; n = 104 trajectories; 50 μM ATP) and $P_{g\text{-}m}$ = 0.29 ± 0.03 (mean ± s.e.m.; n = 80 trajectories; 300 μM ATP). We then used this probability number and the average dwell time in the green state to determine $k_{g\text{-}m}$, based on Equation S9. For the 50 μM ATP condition, we excluded a single outlier with >6x more transitions than the trajectory with the next largest number of transitions in calculating $P_{g\text{-}m}$; including this point does not significantly affect the result ($P_{g\text{-}m}$ = 0.65 ± 0.10; mean ± s.e.m.; n = 105 trajectories). Error bars for the $k_{g\text{-}m}$ values reported in Fig. 3c were calculated by propagating the individual s.e.m. values of both $P_{g\text{-}m}$ (as given above) and $< t_g >$ (n = 441, 302, 106 dwells at 0, 50, 300 μM ATP). Since the transition rate from the magenta to green state, $k_{m\text{-}g}$, is not affected by the transition from the green to processive unwinding state, $k_{m\text{-}g}$ was simply determined from the average dwell time in the magenta state. To determine these transition rates in the absence of ATP, experiments in which ATP was initially absent and then added after 6 seconds were similarly segmented into states. In these experiments, dwell times beginning after the addition of ATP were excluded. Only trajectories which showed processive unwinding after adding ATP were considered.

Stopped-Flow

DNA unwinding by RecBCD was measured in a pre-steady state stopped-flow assay similar to that of Ref. [21] using a stopped-flow spectrophotometer (KinTek SF-2004) to measure the unwinding of fluorescently labeled DNA substrates (Extended Data Figs. 5a and 8a; Supplementary Table 4). In this substrate design, each sample forms a hairpin consisting of a "C" strand annealed to a pair of "A" and "B" strands, as shown in Extended Data Fig. 5a. The emission intensity from a Cy3 dye was initially quenched by energy transfer to a Cy5 dye in each substrate. RecBCD-catalyzed DNA substrate unwinding dissociated the Cy3 labeled DNA strand, resulting in reduced quenching of Cy3, which was detected as an increase in Cy3 fluorescence.

We used a 2-stage mixing protocol in order to measure single-turnover unwinding reactions shortly after enzyme-substrate binding (Extended Data Figs. 5b and 8a). In the first stage, we mixed RecBCD with DNA for 200 ms, allowing the enzyme-substrate complex to form. In the second stage, we mixed the enzyme-substrate complexes with a solution containing ATP and heparin while recording the resulting fluorescence. Heparin inhibits RecBCD-DNA binding and thus prevents enzymes from engaging new DNA substrates after the first turnover[21]. The use of heparin to achieve single-turnover conditions requires that the RecBCD-DNA complex is formed before the ATP and heparin are added. Although this can be achieved by pre-mixing the RecBCD and DNA prior to loading the mixture into the stopped-flow instrument, we chose to instead briefly mix RecBCD and DNA for 200 ms in the stopped-flow apparatus before the addition of ATP to trigger unwinding in order to prevent long equilibration of the RecBCD-DNA complex in the absence of ATP which could potentially change initiation phase characteristics and to more closely match our single molecule experimental conditions, where the substrate and ATP were simultaneously added to the surface bound enzyme. The 200 ms mixing time was the shortest time that allowed enough RecBCD-DNA complexes to form to create a reliably detectable fluorescence signal after adding ATP. Each experimental fluorescence time course shown in the figures represents an average of $n = 10$ individual experiments.

Single-molecule experiments of RNAP-induced DNA rotation during transcription

RNAP experiments were conducted similarly to the RecBCD experiments described above. The origami complexes were ligated to dsDNA extensions (see Supplementary Table 3) which contained the T7A1 promoter. The reaction buffer for these experiments contained 50 mM Tris, 2 mM Trolox, 50 μM of the Trolox quinone, 5 mM protocatechuic acid (PCA), 20 mM NaCl, and 10 mM $MgCl_2$, adjusted to pH 8.0. Stalled ternary (RNAP-DNA-RNA) complexes were formed by adding RNAP and origami complexes to 10 μM ATP, GTP, and CTP, 250 μM of the dinucleotide ApU, and 5 mM DTT in reaction buffer. The dsDNA extensions (see Supplementary Table 3) contained a 20 bp sequence without T's after the T7A1 promoter, causing the complexes to become stalled in the absence of UTP[6]. After ~20 minutes at room temperature, 200 μM sheared salmon sperm DNA (Invitrogen) was added to the solution containing stalled ternary complexes. The complex was then incubated at least 5 minutes at room temperature and added to microscope flow chambers. After washing out excess complexes, reaction buffer with all four NTPs, 100 μM salmon sperm DNA, and 0.25 U/mL PCD was added to start the reaction while imaging. RNAP experiments were recorded at 200 Hz, except for those at low [NTP] for single-base-pair stepping detection, which were recorded at 50 Hz or 100 Hz and down-sampled to 50 Hz. Following previous work[27], experiments for base-pair stepping

analysis were conducted with a 4:4:2:1 ratio of GTP:UTP:ATP:CTP (5, 5, 2.5, and 1.25 μM, respectively). All other experiments were done with equimolar concentrations of NTPs.

The RNAP data was processed similarly to the RecBCD data. Step sizes were analyzed using a previously described hidden Markov model (HMM)[46] on the elongation phase of each trajectory with high localization precision. We used a 5° cutoff to reject spurious steps smaller than the noise.

Statistics

Recovery pause durations were compared using the two-sided Kolmogorov-Smirnov (K-S) test. The RecBCD initiation times were compared using two-sided t-tests.

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Supplementary Videos

Supplementary Video 1 | A single-molecule ORBIT trajectory of RecBCD-induced processive DNA unwinding. The origami-dsDNA complex diffuses from solution and binds to RecBCD. After a relatively brief initiation period, the origami rotates processively due to the unwinding of the dsDNA substrate by RecBCD at 50 μM ATP. The localizations of the dyes at the tip of the origami rotor are shown. Trajectory is representative of at least three independent experiments.

Supplementary Video 2 | A single-molecule ORBIT trajectory of RecBCD-induced processive DNA unwinding with a long initiation phase. The origami-double stranded DNA complex diffuses from solution and binds to RecBCD. After an initiation phase showing several reversible transitions between two angular positions ~170° apart due to reversible, ATP-independent unwinding transitions of the first 5 bp, the origami begins rotating processively due to the unwinding of the DNA substrate by RecBCD at 50 μM ATP. The localizations of the dyes at the tip of the origami rotor are shown. Trajectory is representative of at least three independent experiments.

Supplementary Tables

Supplementary Table 1 | DNA oligomers used for the origami rotor (Table contained in a separate Excel file). DNA modifications (Cy3 dye labels and phosphorylation) are included using their IDT codes. The final two strands extend outside of the origami. They have 14 nt of complementarity (green) followed by a 12 nt single stranded overhang (purple) for ligation to additional DNA (Supplementary Table 3).

Supplementary Table 2 | DNA oligomers used for the origami anchor (Table contained in a separate Excel file). DNA modifications (biotinylation and phosphorylation) are included using their IDT codes. The six strands ending in 'TTHr21' contain a 21 nt binding site (red) for the Hr21_5Bio biotinylated secondary strand. The final three strands form the adaptor (Extended Data Fig. 1f-g) for ligating additional DNA to the anchor using the 12 nt overhang (purple). Two regions of complementarity between these adaptor strands are indicated in green and orange. Note Anchor_Ext0_oh by is replaced by Anchor_Ext0_oh_direct (Supplementary Table 3) for the anchor-rotor complex linked by a 52-bp dsDNA.

Supplementary Table 3 | Additional DNA oligomers. DNA modifications (phosphorylation) are included using their IDT codes. Sample descriptions indicate the final linker duplex DNA length after ligation. This length includes contributions from one or both origami structures (14 bp for the origami rotor, 26 bp for the origami anchor, and a 12 nt overhang on each). Substrates with 3' or 5' overhang for RecBCD experiments are designed based on the 80 bp blunt-end DNA with additional 5' or 3' dT nucleotides as indicated in the main text. The strands for 80- and 92-bp duplexes are annealed prior to ligation to the origami structure(s). The primers used to generate the 163-bp DNA linker between the origami rotor and anchor contained dU bases. One strand of the PCR product for the 163-bp linker is shown prior to dU excision of the nucleotides shown in red, which created the 12-nt ssDNA overhang required for ligation to the origami structures. The other strand also contains a dU base to create ssDNA overhangs on each end. To generate the origami anchor-rotor complex with a 52-bp linker, strand Anchor_Ext0_oh (Supplementary Table 2) in the anchor folding reaction was replaced with Anchor_Ext0_oh_direct. For this sample, no additional DNA was required to connect the rotor and

anchor. Overhangs for ligation to the origami rotor or anchor are shown in purple and bases complementary to Anchor_Ext1 (Supplementary Table 2) are in green. The RNAP kinetic series data was collected using substrates with the "Extension for variable [NTP] RNAP experiments" sequences and the single base pair stepping data collected with the "Extension for low [NTP] RNAP experiments" sequences. In both cases, the extensions contained a 3' overhang (purple) for ligation to the origami rotor.

Sample	DNA sequences
80-bp blunt-end DNA for RecBCD experiments	TCGAATTCGCCCTATAGTGAGTCGTATTACAATTC ACTGGCCGTCGTTTTACAAACTAGTC CGAGA
	/5Phos/TTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGA
80-bp 10nt 5' overhang DNA without GC in initial 5 bp ("-GC")	TTTTTTTTTTATTATATCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACA AACTAGTCCGAGA
	/5Phos/TTGTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGATATAAT
Adaptor for 92-bp linker between rotor and anchor	/5Phos/CCTCTAGAGTGGGAGCTCGGAACACTATACGGAGTTCGCA
	/5Phos/ATAGTGTTCCGAGCTCCCACTCTAGAGGACTAGTCCGAGA
Primers used for 163-bp linker production	TCTCGGACTAGUTTGTAAAACGACGGCCAGTGAATTG
	TGCGAACTCCGUATAGTGTTCCGAGCTCCCACTC
Adaptor for 163-bp linker prior to dU excision	TGCGAACTCCGUATAGTGTTCCGAGCTCCCACTCTAGAGGATCCCCGGGTACCGAGCTCG AATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACTAGTCCGA GA
Replacement strand for 52-bp linker: Anchor_Ext0_oh_direct	TTAGTTAATTTCATCTTTATGTTTATTGCTCACTATCATTGCACTAGTCCGAGA
Extension for variable [NTP] RNAP experiments	TTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCATCGAGAGGG ACACGGGGGATCCTCTAGACTGCAGAAAGGTTGGTGGCTACAATTCACTGGCCGTCGTTT TACAACCAGTCACGCAGTAACGTTCATCAGCTAACGTAACAGTTAGAGGCTCGCTAAATCG CACTGTACTAGTCCGAGA
	/5Phos/ACAGTGCGATTTAGCGAGCCTCTAACTGTTACGTTAGCTGATGAACGTTACTGCGT GACTGGTTGTAAAACGACGGCCAGTGAATTGTAGCCACCAACCTTTCTGCAGTCTAGAGG ATCCCCCGTGTCCCTCTCGATGGCTGTAAGTATCCTATAGGTTAGACTTTAAGTCAATACT CTTTTTGATAA
Extension for low [NTP] RNAP experiments	TTATCAAAAAGAGTATTGACTTAAAGTCTAACCTATAGGATACTTACAGCCATCGAGAGGG ACACGGGGGATCCTCTAGACTGCAGAAAGGTTGGTGGCTACAATTCACTGGCCGTCGTTT TACAAACTAGTCCGAGA
	/5Phos/TTGTAAAACGACGGCCAGTGAATTGTAGCCACCAACCTTTCTGCAGTCTAGAGGAT CCCCCGTGTCCCTCTCGATGGCTGTAAGTATCCTATAGGTTAGACTTTAAGTCAATACTCT TTTTGATAA

Supplementary Table 4 | DNA oligomers for hairpin substrates used in stopped-flow fluorescence experiments. Each hairpin consists of a "C" strand annealed to a pair of "A" and "B" strands, as shown in Extended Data Fig. 5a. DNA modifications are included using their IDT codes.

HBE26_C	AGATCCTAGTGCAGGTTTTCCTGCACTAGGATCTTTCCTCAGTTGTGTTCTCGGACTAGTT TGTAAAACGACGGCCAGTGAATTGTAATTCGACTCACTATAGGGCGAATTCGA
HBE26_A	TCGAATTCGCCCTATAGTGAGTCGA/iCy3/A
HBE26_B	/5Cy5/TTACAATTCACTGGCCGTCGTTTTACAAACTAGTCCGAGAACACAACTGAGGAA
HBE26_A_5oh10	TTTTTTTTTT TCGAATTCGCCCTATAGTGAGTCGA/iCy3/A
HBE26_C_3oh6	AGATCCTAGTGCAGGTTTTCCTGCACTAGGATCTTTCCTCAGTTGTGTTCTCGGACTAGTT TGTAAAACGACGGCCAGTGAATTGTAATTCGACTCACTATAGGGCGAATTCGA TTTTTT
HBE52_C	AGATCCTAGTGCAGGTTTTCCTGCACTAGGATCTTTCCTCAGTTGTGTTCTCGGACTAGTT ATTAAAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGGCGAATTCGA
HBE52_A	TCGAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTA/iCy3/A
HBE52_B	/5Cy5/TAACTAGTCCGAGAACACAACTGAGGAA