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DNA Unwinding Heterogeneity by RecBCD Results from Static Molecules Able to Equilibrate

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Single-molecule studies can overcome the complications of asynchrony and ensemble-averaging in bulk-phase measurements, provide mechanistic insights into molecular activities, and reveal interesting variations between individual molecules^{1–3}. The application of these techniques to the RecBCD helicase of *Escherichia coli* has resolved some long-standing discrepancies, and has provided otherwise unattainable mechanistic insights into its enzymatic behavior^{4–6}. Enigmatically, the DNA unwinding rates of individual enzyme molecules are seen to vary significantly^{6–8}, yet the origin of this heterogeneity remains unknown. Here we investigated the physical basis for this behavior. Although any individual RecBCD molecule unwound DNA at a constant rate for an average of ~30,000 steps, we discovered that transiently halting a single enzyme-DNA complex by depleting Mg²⁺-ATP could change the subsequent rates of DNA unwinding by that enzyme after reintroduction to ligand. The proportion of molecules that changed rate increased exponentially with the duration of the interruption, with a half-life of approximately 1 second, suggesting a conformational change occurred during the time that the molecule was arrested. The velocity after pausing an individual molecule was any velocity found in the starting distribution of the ensemble. We suggest that substrate binding stabilizes the enzyme in one of many equilibrium conformational sub-states that determine the rate-limiting translocation behavior of each RecBCD molecule. Each stabilized sub-state can persist for the duration (~1 minute) of processive unwinding of a DNA molecule, comprising tens of thousands of catalytic steps, each of which is much faster than the time needed for the conformational change required to alter kinetic behavior. This ligand-dependent stabilization of rate-defining conformational sub-states results in seemingly static molecule-to-molecule variation in

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RecBCD helicase activity but in fact reflects one microstate from the equilibrium ensemble that a single molecule manifests during an individual processive translocation event.

The RecBCD enzyme is an important helicase/nuclease in the repair of double-stranded DNA (dsDNA) breaks *via* homologous recombination⁸. RecBCD initiates homologous recombination by processing dsDNA to generate 3'-ended single-stranded DNA (ssDNA) upon recognition of the recombination hotspot sequence, χ (Crossover Hotspot Instigator, Chi) sequence 5'-GCTGGTGG-3'⁹. The RecB and RecD subunits are SF1 helicases with 3'→5' and 5'→3' translocation polarities, respectively^{10,11}. RecC holds the complex together and recognizes χ ¹². RecB and RecD drive dsDNA unwinding by acting as ssDNA motors, pulling the two antiparallel strands of the DNA across a pin in the RecC subunit and thus splitting the duplex DNA¹³.

Earlier single-molecule studies of DNA unwinding by RecBCD revealed significant variation in the unwinding rates of each molecule⁴⁻⁷. To understand the molecular origin of this intrinsic heterogeneity, we analyzed the unwinding behavior of a larger set of individual RecBCD molecules on bacteriophage λ DNA lacking χ (Fig. 1a, b). A total of 251 molecules were initially analyzed (Fig. 1c). The majority (96%) of the molecules did not change their speeds during unwinding (Fig. 1b). Individual RecBCD molecules were observed to unwind and degrade DNA at constant velocities for 30–60 s, for over tens of thousands of catalytic turnovers. Although the rate distribution in earlier studies could be fit to a single Gaussian function^{4,5}, the sizes of those datasets were limited; the comparatively large number of single-molecule unwinding rates obtained here provide clear evidence of a non-unimodal distribution (Fig. 1c). The distribution was fit to the sum of two Gaussian functions; the major population of molecules (71%) has a mean fitted rate of $1,584 \pm 95$ bp/s (\pm standard deviation, SD) while the minor population (29%) has a mean rate of 907 ± 500 bp/s (\pm SD). The difference in unwinding rates between the fast and slow populations is considerably beyond the experimental uncertainty. The slow population is not due to the recognition of χ -like sequences, because such events are readily discerned as pauses followed by a velocity change (Supplementary Fig. 1). Interestingly, the fast molecules are more processive than slow ones (Supplementary Fig. 2). Both the rate and processivity of the slow species are comparable to the behavior of RecBCD mutants with a defective motor subunit¹⁴, leading us to examine the single-molecule behavior of two such single-motor mutant enzymes. DNA unwinding by RecBCD^{K177Q} (RecBCD* in Fig. 1c) is manifest as a single Gaussian distribution with a mean rate of 729 ± 290 bp/s, and for RecB^{K29Q}CD (RecB*CD in Fig. 1c), it is 432 ± 227 bp/s (also Supplementary Fig. 2). These findings suggest that, for the wild-type enzyme, the slow species represents enzymes wherein one motor subunit is initially not engaged, but can be reversibly re-engaged when halted (see below).

The origin of heterogeneity can be dynamic or static¹⁵⁻¹⁷. Whereas dynamic heterogeneity was suggested to arise from conformational fluctuations of a protein, static heterogeneity can have different sources. It can arise from chemical heterogeneity due to the presence of multiple related genes, or from post-translational modifications¹⁸. It can also result from enzyme molecules with identical chemical composition that have different stable conformational sub-states in equilibrium^{15,17,19} or that are kinetically trapped in non-equilibrium states capable of multiple turnovers²⁰⁻²². We initiated experiments designed to

distinguish between these possible origins. Although the protein preparation contains no detectable heterogeneity in polypeptide composition (Supplementary Fig. 3), the distributions of unwinding rates for RecBCD eluted from different fractions of a chromatographic elution peak were examined as the first trivial source of heterogeneity; no experimentally significant differences in the distribution profiles were found (Supplementary Fig. 4). We next considered the possibility that the heterogeneity arose from RecBCD species that were not at equilibrium, but rather were trapped in different kinetic conformations. In an attempt to permit such hypothetically trapped conformations to relax to the equilibrium distribution, we subjected the enzyme population to experimental conditions that could potentially allow redistribution. Partial destabilization of protein structure, followed by refolding, can allow protein molecules to relax to their global minimum on the folding energy landscape, resulting in an equilibrium distribution of enzymes. We first utilized thermal annealing²³. Ensemble assays showed that RecBCD could be heated to a maximum of 45 °C for 10 min, with no loss of activity (Supplementary Fig. 5a,b). Therefore, an enzyme population that was treated at 45 °C, and slowly cooled at a rate of 1 °C/min, was analyzed using single-molecule methods. The distribution of the rates for the thermally-treated enzymes was not statistically different from the original distribution ($P = 0.45$; Supplementary Fig. 5c).

An alternative to thermal annealing is to use a chemical denaturant to unfold a protein, followed by slow removal, to permit refolding to the equilibrium distribution^{24,25}. Thus, we next investigated the effect of partial unfolding of RecBCD by the classical denaturant, guanidine hydrochloride (GuHCl). The enzyme could be reversibly renatured after treatment with up to 0.5 M GuHCl (Supplementary Fig. 6a). The velocity distribution of the resultant individual enzymes had a mean of 1736 ± 133 bp/s for the fast population *versus* 1773 ± 104 bp/s for the control (Supplementary Fig. 6b), which is the same within experimental uncertainty. The mean of the treated slow population is 556 ± 451 bp/s *versus* 793 ± 307 bp/s for the control population; although the mean for the slower group seems to be reduced, the difference is not significant ($P = 0.24$). In conclusion, neither thermal annealing nor chemical refolding produced a more homogeneous distribution, arguing that either these treatments are insufficient to permit redistribution, or that the population of RecBCD enzymes is intrinsically heterogeneous.

It remained possible that the conformational distribution of RecBCD enzyme was, in fact, at equilibrium due to the presence of multiple conformations of similar free energy²⁶, but the binding of substrates could lock an enzyme in a given conformation^{27,28}. For RecBCD, each DNA binding event allows unwinding of tens of thousands of base pairs, perhaps suggesting that the initial binding locks the enzyme in a conformation that lasts the duration of the unwinding process – a form of conformational selection²⁷. Given that we had been unable to alter the distribution of RecBCD enzyme rates by more traditional means, we next examined whether depletion of a ligand, ATP, permitted a change to an altered conformation while bound to the DNA. Consequently, we stopped individual RecBCD molecules during the course of unwinding by depleting this essential cofactor, and then measured the rate upon reintroduction of the ligand and restarting the same enzyme. This was achieved by first moving a single, optically-trapped enzyme-DNA complex into the reaction channel

containing ATP to initiate unwinding. After a length of time sufficient to accurately determine the rate of DNA unwinding (~10 s), the complex was moved to a third channel that contained 10 mM EDTA, but neither Mg^{2+} nor ATP, to stop unwinding. After a defined length of time, the arrested RecBCD-DNA complex was moved back to the reaction channel to resume unwinding. By halting RecBCD in this manner for 20 s, we found that about 50% (173 out of 354) of complexes restarted unwinding when moved back to the reaction channel; we presume that RecBCD dissociated from the remainder. Fig. 2a shows the time courses for three characteristic RecBCD molecules. For molecule 1, the unwinding rate decreased from 1443 bp/s to 507 bp/s; for molecule 2, it was the same upon resumption; and for molecule 3, it increased from 1447 bp/s to 1648 bp/s. After the 20-s incubation in EDTA, 53% (91 out of 173) of the molecules continued unwinding with the same rate (within a 20% difference), while 35% ($N = 61$) of molecules slowed, and 12% ($N = 21$) of molecules increased, speed (Fig. 2b). This finding shows that the rate of individual RecBCD molecules is not static and the heterogeneity in rates cannot be, at least not solely, due to variation in covalent or irreversibly trapped structures. Note that when DNA unwinding was observed in the continuous presence of ATP (Fig. 1b), spontaneous rate-change events (Supplementary Fig. 1) were rare (4%) and attributable to Chi-like recognition events. In contrast, when unwinding was interrupted by transiently removing ATP, at least 47% of the enzyme molecules resumed unwinding at a different rate upon re-introduction of ATP (Fig. 2a, b), suggesting that omission of the ATP ligand permitted a conformational switch that affects the rate-limiting translocation behavior of RecBCD. These results support the notion that ligand binding locks the enzyme into a conformational state that typically persists for the duration of a single processive DNA unwinding transaction, whereas the absence of ATP allows the enzyme molecule to change its conformation state within the time it was halted.

The blue box in Fig. 2b highlights a binned region of the single-molecule velocity distribution containing a relatively well-populated group of molecules ($N = 36$) that translocated at rates between 1450 and 1550 bp/s before pausing. After incubation in EDTA, the velocities became broadly redistributed, ranging from 300 bp/s to 1900 bp/s. The new distribution of velocities for this group is similar to the starting distribution for all the molecules (Fig. 2c and Supplementary Fig. 7), although the new distribution is overrepresented by molecules that switched to the slow macrostate (*i.e.*, with one motor disengaged). This finding demonstrates that an enzyme molecule with a fixed velocity can switch to any other velocity that was initially displayed by other enzymes in the original ensemble; similar redistribution was seen for other well populated bins of molecules (Supplementary Fig. 7b,c). These findings imply that all of the conformational sub-states of the ensemble are accessible to an enzyme after pausing. The velocity after ligand-depletion is not related to the starting velocity of the enzyme, but rather, each enzyme equilibrated to a new velocity that was represented in the initial ensemble. The velocity distributions for enzymes, both before and after arrest, are not unimodal although, after being halted, the percentage of molecules in the slow group is increases (Supplementary Fig. 7a). These results indicate that a RecBCD molecule can adopt any conformation on the free energy landscape, after being subjected to transient depletion of ATP. To ensure that the rate changes were not specific to the pausing by EDTA, experiments were conducted by

stopping the RecBCD-DNA complex in a channel devoid of ATP but containing Mg^{2+} . Similar results were obtained (Supplementary Fig. 8).

When the duration of the time arrested without ATP was decreased to 2 s, the percentage of complexes that resumed unwinding increased to 78%, although fewer (33%) switched velocity (Supplementary Fig. 9). Upon increasing the incubation time in the EDTA channel, the proportion of molecules that changed rate increased exponentially with a half-life of 1.3 ± 0.4 s (Fig. 2d), suggesting that a conformational change responsible for the change in velocity in the absence of ATP requires ~ 1 s. The combined data set for all pauses (Supplemental Fig. 9d; $N = 445$) shows that, with some underrepresentation of the slow starting velocities, there is switching from any one microstate to any other microstate. Given the existence of two macrostates (the fast population with two motors attached, and the slow population with one motor attached) when velocity switches that occur only within a macrostate are considered, the velocity redistributions are completely random (Supplementary Fig. 9e,f). Because the rate of ATP hydrolysis is rapid (ranging from a few hundred to a few thousand per s) relative to the half-life for the conformational change (1.3 s), the time between two adjacent ATP binding events would be too short (on the order of ms) for the unliganded apo-form of RecBCD to adopt a different conformation during the time that ADP has dissociated and before ATP has re-bound. For this reason, we presume spontaneous switching is rare. Our interpretation is in accord with an earlier study that found that a few individual RecBCD enzymes can spontaneously change velocity when examined at low (15 μ M) ATP²⁹. At such a low ATP concentration, the apo-form of the enzyme is longer lived and the time between adjacent ATP binding events would be ~ 67 -fold longer than for the studies in this report, making it more likely that RecBCD could switch to a new conformational sub-state. Therefore, we conclude that the binding of ATP and DNA to RecBCD fixes the conformational state, which in turn defines the unwinding rate for the duration of a single processive unwinding event, contributing to the observed heterogeneity in rates of (and between) individual enzymes.

To determine whether the conformational changes are stochastic for any individual molecules, we halted some enzyme molecules twice using the same procedure, and asked whether the rate changes after each interruption are correlated. The individual molecules ($N = 34$) exhibited both decreased and increased rates after each pause, as seen above (Supplementary Fig. 10), and we found no correlation between the relative changes in rate as the results of the two consecutive pauses (Fig. 2e).

Earlier studies on the behavior of other single enzymes have reported static heterogeneity in catalytic rates due to variation in the covalent structures¹⁸, the presence of metastable conformations^{15,17,19}, or dynamic heterogeneity caused by conformational fluctuation¹⁶. In this work, we found that the heterogeneity in the DNA unwinding rates by RecBCD is static on the experimental time scale of DNA unwinding for tens of thousands of base pairs. However, the rates are not intrinsic to individual molecules; thus, the heterogeneity cannot be explained by possible variations in the covalent structures of the enzyme. Instead, any individual molecule can adopt any conformation within the initially accessible free energy landscape after depletion of a ligand for a few seconds. The ergodic hypothesis posits that the (infinite) time-averaged behavior of a molecule at equilibrium is equal to the ensemble-

average of an infinite collection of those molecules. Thus, if a single enzyme molecule could be repeatedly stopped and observed, it should adopt all the possible conformations that are accessible for those conditions of thermodynamic state. Clearly, we cannot examine a single molecule for an infinite number of times, but a corollary of the ergodic hypothesis is that if one could watch any single-molecule in an equilibrium distribution that could randomly switch at least once to a new conformation, then the distribution of those new states should recapitulate the original distribution, if indeed the first distribution was at equilibrium. By watching a collection of individual enzymes switching a limited number of times, here we show that they can switch to velocities found in the original distribution. Therefore, we conclude that these seemingly static RecBCD molecules can switch into microstates existing within the original ensemble. Also, when transitions remain within each macrostate, the new distribution of velocities is completely random, manifesting an expectation of ergodic behavior. Unexpectedly, the lifetimes of these kinetic states are atypically long, and are dictated by ligand occupancy. We imagine that the conformation of the enzyme is dynamic in the absence of ligands and that a single conformation is selected and stabilized, *i.e.*, made seemingly static, upon ligand binding²⁷. These findings help to understand the influence of ligand binding on protein conformations, conformational selection, and enzymatic reactions, and they now raise the intriguing structural question of how sub-states that vary in speeds by hundreds of bp/s can be maintained by these quasi-stable enzymatic conformations. Finally, the possible biological function of heterogeneity in a population of individual molecules is unknown and is difficult to define. However, we offer the plausible speculation that the variation seen for populations of individual molecules is akin the epigenetic variation in the populations of organisms. Given the stochastic nature of life, a population of cells – bacteria in this specific case – needs both diversity and flexibility to respond to the random nature of natural challenges. We suggest that the variation in individual molecule behavior affords a molecular plasticity in the cellular functions of RecBCD to respond to unpredictable needs. RecBCD has two seemingly contradictory functions: one is degradation of foreign duplex DNA (*e.g.*, DNA viruses); the other is the repair of broken chromosomal DNA. The regulation of these activities is controlled by recognition of the DNA regulatory sequence χ^8 . Each *E. coli* cell contains only 10 RecBCD enzyme molecules, and each cell suffers ~0.5 DNA breaks per cell cycle and is exposed to an unpredictable amount of phage or foreign DNA. If RecBCD were limited to one conformation, or if it could adopt multiple conformations but these conformations rapidly equilibrated after each step of processive unwinding, then all DNA would be processed at the same rate. Given the probabilistic nature of DNA breaks and the appearance of foreign DNA, conformational heterogeneity coupled with conformation selection of a kinetically stable functional form of RecBCD can ensure a stochastic but broad cellular response. Consequently, if the few RecBCD molecules present can adopt a wide range of conformational states, then survival through random selection is more likely, and the surviving cells, within a population of cells, are not constrained genetically. By coupling dynamic disorder in the ensemble, with subsequent random selection of conformations that remain static during processive DNA unwinding, both molecules and cells can respond probabilistically to unpredictable situation with just a handful of molecules. From the perspective of a population of cells, although some will perish, a random fraction will have survived by throwing the dice productively.

METHODS

Proteins and DNA substrates

Escherichia coli RecBCD enzyme was expressed and purified as described previously^{31,32}. To check purity, protein was analyzed using a 12% denaturing polyacrylamide gel (1:29 bis:acrylamide in TBE buffer (89 mM Tris base, 2 mM EDTA, 89 mM boric acid), containing 10% SDS) stained with Coomassie blue dye. After electrophoresis, the gel was imaged using an AlphaInotech gel documentation system. The two mutant enzymes, RecBCD^{K177Q} (RecBCD*) and RecB^{K29Q}CD (RecB*CD), were purified as described³³. Bacteriophage λ DNA (*N*⁶-methyladenine-free lambda DNA, New England Biolabs) was biotinylated by ligation to a 3'-biotinylated 12-mer oligonucleotide (*cosA*: 5'-GGGCGGCGACCT-3' or *cosB*: 5'-AGGTCGCCGCC-3', Operon Technologies) that is complementary to one of the cohesive ends of λ DNA³⁴; except for the thermal reannealing and control experiments, where *cosA* was used, all other experiments used the *cosB* oligonucleotide.

The pUC19 plasmid DNA was purified by cesium chloride gradient centrifugation. The circular DNA was linearized with *NdeI* restriction endonuclease (New England Biolabs) followed by heat inactivation and phenol/chloroform/isoamyl alcohol extraction. The DNA concentration was determined by absorbance at 260 nm using an extinction coefficient of 6330 M⁻¹ (nucleotides) cm⁻¹.

ATP hydrolysis assays

The ATP hydrolysis activity of the enzyme was measured spectrophotometrically as reported³⁵ by coupling ATP hydrolysis to NADH oxidation³⁶ using an Agilent Technologies Model 8452A diode array spectrophotometer. The assay mixtures contained 25 mM Tris acetate (pH 7.5), 1 mM dithiothreitol (DTT), 2 mM ATP, 5 mM magnesium acetate, 1.5 mM phosphoenolpyruvate, 0.2 mg/ml NADH, 30 U/ml pyruvate kinase, 30 U/ml lactate dehydrogenase, and 50 μ M (nucleotides) poly(dT). Reactions were initiated by the addition of 0.5 nM RecBCD enzyme after pre-incubation of all other components at 37 °C for 5 min. The rate of ATP hydrolysis was calculated from the rate of change in absorbance at 340 nm due to oxidation of NADH using the following conversion: rate of A₃₄₀ decrease (s⁻¹) \times 9820 \div 0.0005 (μ M RecBCD) \div 60 = rate of ATP hydrolysis (s⁻¹)³⁵.

Re-purification of RecBCD

RecBCD enzyme (0.1 mg) from -80 °C freezer stock was thawed on ice, diluted 5-fold using cold B100 buffer (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, and 100 mM NaCl), and loaded onto a 1-ml MonoQ column (Amersham Biosciences). The enzyme was eluted using a gradient from 300 mM to 450 mM NaCl in 30 column volumes. Three fractions (100 μ l each) on one side of the peak in UV absorbance were used immediately for single-molecule helicase assays.

Stopped-flow dye-displacement helicase assay

Basically, the protocols used previously³⁷ were followed. Experiments were performed in an Applied Photophysics SX.18MV-R stopped-flow apparatus with excitation at 355 nm

(bandwidth 9.3 nm) and emission was measured using a 450 nm long pass filter. Unless stated otherwise, all reported concentrations are final after mixing of equal volumes in the stopped-flow apparatus. Reactions were performed at 25 °C in a buffer containing 25 mM Tris acetate (pH 7.5), 6 mM magnesium acetate, 1 mM DTT, 200 nM Hoechst 33258 dye (Molecular Probes), and 300 nM single-stranded DNA binding protein (SSB). The RecBCD enzyme, at the final concentration indicated, was incubated with 0.05 nM (molecules) *Nde*I-cut pUC19 DNA (equivalent to 0.1 nM RecBCD binding sites) for 5 min, and this was then mixed with 2 mM ATP to initiate the reaction. Data were analyzed using GraphPad Prism 5.02 (GraphPad Software, Inc.). Unwinding rates were determined by a linear fit to the first 2 s of each trace.

Thermal treatment of RecBCD

Aliquots of the RecBCD enzyme in storage buffer (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, 100 mM NaCl, 50% (v/v) glycerol) were thawed on ice and then heated to 45 °C for 10 min followed by slowly cooling by 1 °C/min down to 4 °C using GeneMate PCR machine. The untreated controls were kept on ice until use.

Chemical unfolding of RecBCD

Aliquots of the RecBCD enzyme were thawed on ice. Various concentrations of GuHCl were mixed in 1:1 volume ratio with the enzyme. After incubation at room temperature for one hour, the sample was dialyzed against B100 buffer (20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1 mM DTT, and 100 mM NaCl) at 4 °C for 24 hr and the dialysis buffer was changed once. The next day, samples were collected and the concentrations were measured after centrifugation. Samples were taken for ATPase assays, and the rest were used for single-molecule assays.

Optical trapping and fluorescence microscopy

Single-molecule DNA helicase reactions were performed using an optical trapping system as reported⁷ with some modifications³⁸. The system is constructed around a Nikon Eclipse microscope (Nikon, Tokyo, Japan). A high-pressure mercury lamp (100 W; USHIO America, Inc., Cypress, CA) and Y-FL 4-cube Epi-Fluorescence (Nikon) attachment were used for illumination. Images were captured using a high sensitivity electron bombardment CCD camera (EB-CCD C7190, Hamamatsu Photonics, Hamamatsu City, Japan) and digitalized on-line using an LG-3 frame grabber (Scion Corporation, Frederick, MD) at 30 frame/s. The optical trap was created by focusing a 1064 nm laser (Nd:YVO₄, 6 W max, J-series power supply, Spectra Physics, Mountain View, CA) through a high numerical aperture (NA) objective (100× / 1.3 oil DICH, Nikon). A high NA objective is necessary to create an intensity gradient sufficiently large to form the trap³⁹. The laser is expanded with a 20× beam expander (HB-20XAR.33, Newport, Irvine CA) to fill the back aperture of the objective. The laser is collimated and aligned using a 1× telescope. The laser is reflected along the optical axis of the microscope by means of a low pass dichroic mirror placed between the objective and the fluorescence cube.

Experiments were carried out in a multi-channel microfluidic flow cell secured on a computer controlled motorized stage (MS-2000, Applied Scientific Instruments, Eugene,

OR) mounted on the microscope. The design of the flow cell allows laminar flow of different solutions without mixing. The solutions are introduced into the flow cell by a syringe pump with multiple syringes (KD Scientific, Holliston, MA), generating a flow rate of ~100–150 $\mu\text{m/s}$. PEEK tubing (Upchurch Scientific) is used to connect the syringes to the flow cell. The microfluidic system permits imaging of protein-DNA complexes on a single molecule of flow stretched DNA; it also enables the rapid movement of the sample to the different buffers in the channels of the flow cell. The position of the stage and, hence the flow cell, is controlled using a custom-built program. Bead-DNA complexes can be moved between adjacent solution channels within 1 s via the movement of the stage. For the pausing experiments, a three-channel flow cell was used.

Single-molecule DNA helicase reactions

The protocol used for DNA-bead preparation was modified from that used previously^{34,40}. Biotinylated λ DNA (100 pM in 1–2 μl) was incubated with 1–2 μl of 1 μM *ProActive*[®] streptavidin-coated microspheres (~35 pM; Bangs Laboratories) for 1 hr on ice or at 37 °C. Bead-DNA complexes were then transferred into 0.5 ml of degassed sample solution containing 45 mM NaHCO_3 (pH 8.3), 20% (w/v) sucrose, 50 mM DTT, and 20 nM YOYO-1 dye (Molecular Probes). DNA was incubated with the dye for at least 1 hr in the dark at room temperature (~23 °C). Immediately before transfer to the sample syringe, magnesium acetate and RecBCD, to final concentrations of 2 mM and 10–60 nM, respectively, were added to the sample mixture. For the control, the RecBCD storage buffer without RecBCD was used to replace the enzyme solution. The reaction solution contained 45 mM NaHCO_3 (pH 8.3), 20% (w/v) sucrose, 50 mM DTT, 1 mM ATP, 2 mM magnesium acetate and 20 nM YOYO-1 dye. For the pausing experiments, the three-channels were as follows. The first channel contained bead–DNA complexes and 2 mM $\text{Mg}(\text{OAc})_2$ in SMB (45 mM NaHCO_3 (pH 8.3), 15% (w/v) sucrose, 50 mM dithiothreitol and 20 nM YOYO-1 dye). The second channel contained 1 mM ATP and 2 mM $\text{Mg}(\text{OAc})_2$ in SMB. The third channel contained either 10 mM EDTA or 2 mM $\text{Mg}(\text{OAc})_2$ in SMB; the two solutions used as indicated were either: 45 mM NaHCO_3 (pH 8.3), 15% (w/v) sucrose, 50 mM DTT, 10 mM EDTA and 20 nM YOYO-1 dye, or 45 mM NaHCO_3 (pH 8.3), 15% (w/v) sucrose, 50 mM DTT, 2 mM magnesium acetate and 20 nM YOYO-1 dye.

Single-molecule data analysis

Videos were digitalized through an LG-3 frame-grabber card using an ImageJ plugin. Images were then averaged and the length of the DNA molecule in each frame was measured using a custom-built ImageJ plugin³⁸. The experimental data were fitted to either a line or a three-segment line using Origin 7.5 (OriginLab Corp., Northampton, MA) or GraphPad Prism 5.02 (GraphPad Software, Inc.). The translocation rates of RecBCD were calculated from the slopes of the corresponding segments and the standard error of the best-fit values are reported. Unless otherwise indicated, standard deviation is reported for statistical analysis of a number of molecules. The analysis method has an estimated resolution of 50 bp/s. The difference in the unwinding rates between the fast and slow populations is significantly beyond the experimental uncertainty. When a distribution of unwinding rates was plotted, the rates were grouped in 100 bp/s bins. The distributions were fit to the sum of two Gaussian curves, unless otherwise noted. Error bars in Fig. 2d represent

the expected bounds assuming a binomial distribution of switching events for the given sample size. For comparison of the rate distributions, the two-sample Kolmogorov–Smirnov test was used. For correlation analysis, Spearman rank correlation test was used. All P values reported for statistical analysis refer to the two-tailed probability of the tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Moffitt JR, Chemla YR, Smith SB, Bustamante C. Recent advances in optical tweezers. *Annu. Rev. Biochem.* 2008; 77:205–228. [PubMed: 18307407]
2. Ha T. Single-molecule fluorescence resonance energy transfer. *Methods.* 2001; 25:78–86. [PubMed: 11558999]
3. Xie XS, Lu HP. Single-molecule enzymology. *J. Biol. Chem.* 1999; 274:15967–15970. [PubMed: 10347141]
4. Spies M, et al. A molecular throttle: the recombination hotspot χ controls DNA translocation by the RecBCD helicase. *Cell.* 2003; 114:647–654. [PubMed: 13678587]
5. Spies M, Amitani I, Baskin RJ, Kowalczykowski SC. RecBCD enzyme switches lead motor subunits in response to χ recognition. *Cell.* 2007; 131:694–705. [PubMed: 18022364]
6. Handa N, Bianco PR, Baskin RJ, Kowalczykowski SC. Direct visualization of RecBCD movement reveals cotranslocation of the RecD motor after χ recognition. *Mol. Cell.* 2005; 17:745–750. [PubMed: 15749023]
7. Bianco PR, et al. Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature.* 2001; 409:374–378. [PubMed: 11201750]
8. Dillingham MS, Kowalczykowski SC. RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.* 2008; 72:642–671. [PubMed: 19052323]
9. Lam ST, Stahl MM, McMilin KD, Stahl FW. Rec-mediated recombinational hot spot activity in bacteriophage lambda. II. A mutation which causes hot spot activity. *Genetics.* 1974; 77:425–433. [PubMed: 4415485]
10. Dillingham MS, Spies M, Kowalczykowski SC. RecBCD enzyme is a bipolar DNA helicase. *Nature.* 2003; 423:893–897. [PubMed: 12815438]
11. Taylor AF, Smith GR. RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature.* 2003; 423:889–893. [PubMed: 12815437]
12. Handa N, et al. Molecular determinants responsible for recognition of the single-stranded DNA regulatory sequence, χ , by RecBCD enzyme. *Proc. Natl. Acad. Sci. USA.* 2012; 109:8901–8906. [PubMed: 22603794]
13. Singleton MR, Dillingham MS, Gaudier M, Kowalczykowski SC, Wigley DB. Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks. *Nature.* 2004; 432:187–193. [PubMed: 15538360]
14. Dillingham MS, Webb MR, Kowalczykowski SC. Bipolar DNA translocation contributes to highly processive DNA unwinding by RecBCD enzyme. *J. Biol. Chem.* 2005; 280:37069–37077. [PubMed: 16041061]
15. Frauenfelder H, McMahon BH, Austin RH, Chu K, Groves JT. The role of structure, energy landscape, dynamics, and allostery in the enzymatic function of myoglobin. *Proc. Natl. Acad. Sci. USA.* 2001; 98:2370–2374. [PubMed: 11226246]

16. Lu HP, Xun L, Xie XS. Single-molecule enzymatic dynamics. *Science*. 1998; 282:1877–1882. [PubMed: 9836635]
17. Xue Q, Yeung ES. Differences in the chemical reactivity of individual molecules of an enzyme. *Nature*. 1995; 373:681–683. [PubMed: 7854448]
18. Craig DB, Arriaga EA, Wong JCY, Lu H, Dovichi NJ. Studies on single alkaline phosphatase molecules: Reaction rate and activation energy of a reaction catalyzed by a single molecule and the effect of thermal denaturation - The death of an enzyme. *J. Am. Chem. Soc.* 1996; 118:5245–5253.
19. Shi J, et al. Multiple states of the Tyr318Leu mutant of dihydroorotate dehydrogenase revealed by single-molecule kinetics. *J. Am. Chem. Soc.* 2004; 126:6914–6922. [PubMed: 15174861]
20. Wolynes PG, Onuchic JN, Thirumalai D. Navigating the folding routes. *Science*. 1995; 267:1619–1620. [PubMed: 7886447]
21. Onuchic JN, Wolynes PG, Luthey-Schulten Z, Socci ND. Toward an outline of the topography of a realistic protein-folding funnel. *Proc. Natl. Acad. Sci. USA*. 1995; 92:3626–3630. [PubMed: 7724609]
22. Dill KA, Ozkan SB, Shell MS, Weikl TR. The protein folding problem. *Annu. Rev. Biophys.* 2008; 37:289–316. [PubMed: 18573083]
23. Nguyen HD, Hall CK. Effect of rate of chemical or thermal renaturation on refolding and aggregation of a simple lattice protein. *Biotechnol. Bioeng.* 2002; 80:823–834. [PubMed: 12402328]
24. Ikai A, Tanford C. Kinetic evidence for incorrectly folded intermediate states in the refolding of denatured proteins. *Nature*. 1971; 230:100–102. [PubMed: 4927005]
25. Sela M, White FH Jr, Anfinsen CB. Reductive cleavage of disulfide bridges in ribonuclease. *Science*. 1957; 125:691–692. [PubMed: 13421663]
26. Frauenfelder H, Sligar SG, Wolynes PG. The energy landscapes and motions of proteins. *Science*. 1991; 254:1598–1603. [PubMed: 1749933]
27. Ma B, Nussinov R. Enzyme dynamics point to stepwise conformational selection in catalysis. *Curr. Opin. Chem. Biol.* 2010; 14:652–659. [PubMed: 20822947]
28. Boehr DD, Nussinov R, Wright PE. The role of dynamic conformational ensembles in biomolecular recognition. *Nat. Chem. Biol.* 2009; 5:789–796. [PubMed: 19841628]
29. Perkins TT, Li HW, Dalal RV, Gelles J, Block SM. Forward and reverse motion of single RecBCD molecules on DNA. *Biophys. J.* 2004; 86:1640–1648. [PubMed: 14990491]
30. Amitani I, Liu B, Dombrowski CC, Baskin RJ, Kowalczykowski SC. Watching individual proteins acting on single molecules of DNA. *Meth. Enzymol.* 2010; 472:261–291. [PubMed: 20580968]

Methods References

31. Roman LJ, Kowalczykowski SC. Characterization of the helicase activity of the *Escherichia coli* RecBCD enzyme using a novel helicase assay. *Biochemistry*. 1989; 28:2863–2873. [PubMed: 2545238]
32. Bianco PR, Kowalczykowski SC. The recombination hotspot Chi is recognized by the translocating RecBCD enzyme as the single strand of DNA containing the sequence 5'-GCTGGTGG-3'. *Proc. Natl. Acad. Sci. USA*. 1997; 94:6706–6711. [PubMed: 9192629]
33. Dillingham MS, Spies M, Kowalczykowski SC. RecBCD enzyme is a bipolar DNA helicase. *Nature*. 2003; 423:893–897. [PubMed: 12815438]
34. Bianco PR, et al. Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature*. 2001; 409:374–378. [PubMed: 11201750]
35. Spies M, Dillingham MS, Kowalczykowski SC. Translocation by the RecB motor is an absolute requirement for χ -recognition and RecA protein loading by RecBCD enzyme. *J. Biol. Chem.* 2005; 280:37078–37087. [PubMed: 16041060]
36. Kreuzer KN, Jongeneel C. V *Escherichia coli* phage T4 topoisomerase. *Meth. Enzymol.* 1983; 100:144–160. [PubMed: 6312256]

37. Dillingham MS, Webb MR, Kowalczykowski SC. Bipolar DNA translocation contributes to highly processive DNA unwinding by RecBCD enzyme. *J. Biol. Chem.* 2005; 280:37069–37077. [PubMed: 16041061]
38. Amitani I, Liu B, Dombrowski CC, Baskin RJ, Kowalczykowski SC. Watching individual proteins acting on single molecules of DNA. *Meth. Enzymol.* 2010; 472:261–291. [PubMed: 20580968]
39. Neuman KC, Block SM. Optical trapping. *Rev. Sci. Instrum.* 2004; 75:2787–2809. [PubMed: 16878180]
40. Spies M, et al. A molecular throttle: the recombination hotspot χ controls DNA translocation by the RecBCD helicase. *Cell.* 2003; 114:647–654. [PubMed: 13678587]

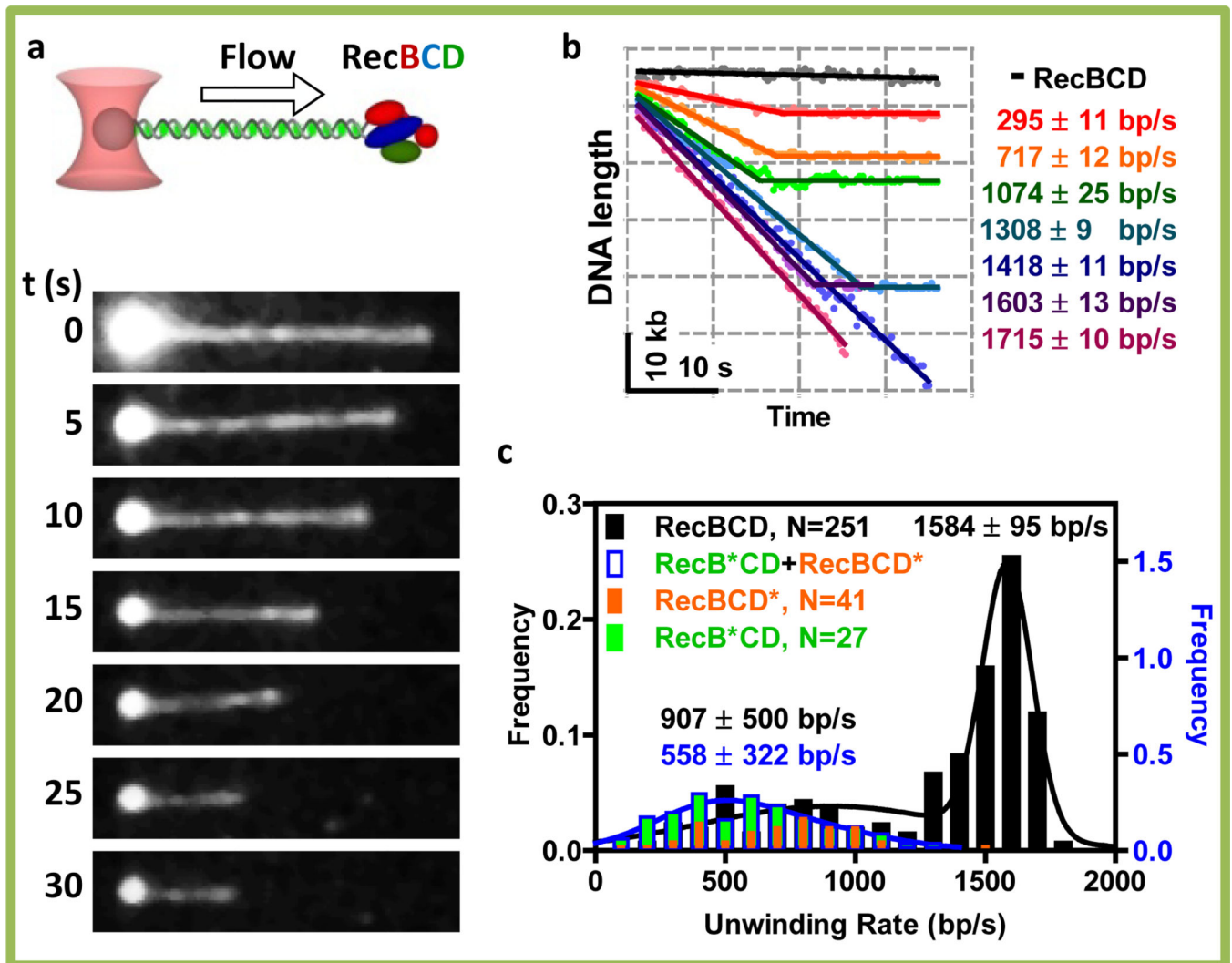


Figure 1. Unwinding of DNA by individual RecBCD molecules is heterogeneous, with a fixed rate for the duration of DNA translocation

a, Visualization of a RecBCD unwinding an individual DNA molecule: (*top*) experimental scheme; (*bottom*) sequential images. **b**, Time courses for unwinding DNA (lacking a χ sequence) by different RecBCD molecules: black: absence of RecBCD; colors: individual RecBCD enzymes. Errors are shown as standard error of the fit. **c**, Distribution of unwinding rates for wild-type RecBCD and motor-mutants fit to the sum of two Gaussian functions and a single Gaussian, respectively. The distribution of the motor mutants was summed to represent equal numbers of each protein. Errors are the s.d.

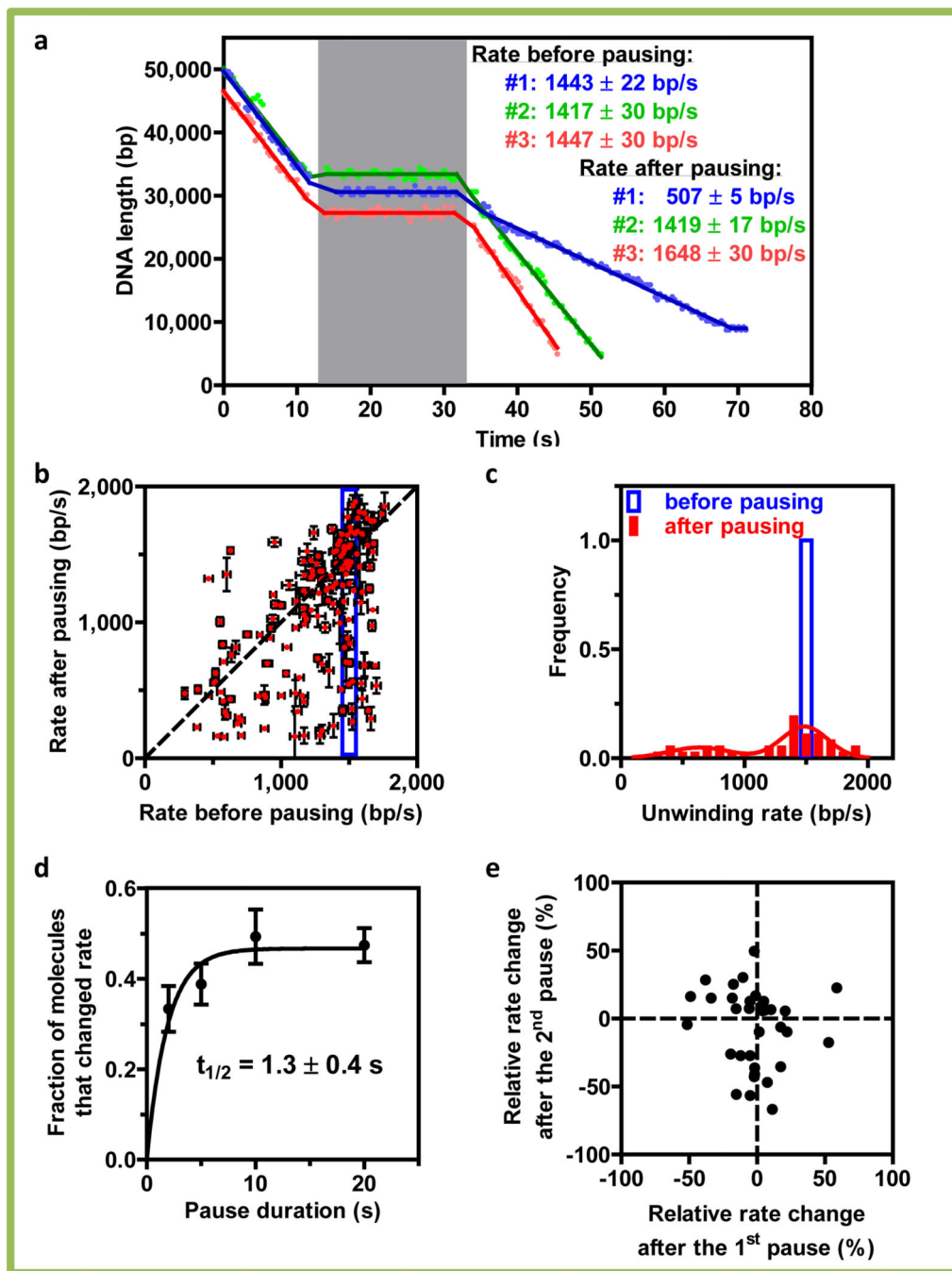


Figure 2. The DNA unwinding rate of single enzymes is stochastically changed to a velocity within the original distribution, after transient depletion of Mg^{2+} -ATP

a, DNA unwinding by three representative RecBCD enzymes. The gray block indicates the pause duration. Errors are shown as standard error of the fit. **b**, The rates before and after pausing ($N = 173$). Error bars represent the standard error of the fit. **c**, Distribution of rates before (blue) and after (red) pausing for molecules with an initial rate of 1450 to 1550 bp/s (blue box, panel b; $N = 36$); before pausing, the selected bin had a mean velocity of 1493 ± 27 bp/s (SD); after pausing and redistribution, the mean velocity was 1245 ± 453 bp/s (SD)

(median = 1411 bp/s). **d**, Proportion of molecules that changed rates after pausing plotted versus pause duration and fitted to an exponential curve; error bars are expected bounds assuming a binomial distribution of switching events. **e**, Scatter plot of the relative rate changes after two pauses ($N = 34$).