

# DNA unwinding heterogeneity by RecBCD results from static molecules able to equilibrate

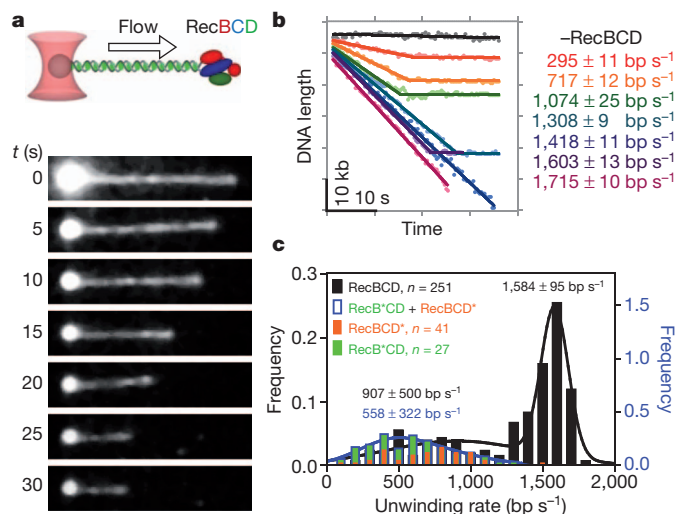
Bian Liu<sup>1,2,3</sup>, Ronald J. Baskin<sup>2</sup> & Stephen C. Kowalczykowski<sup>1,2,3</sup>

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<sup>1–3</sup>. 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 behaviour<sup>4–6</sup>. Enigmatically, the DNA unwinding rates of individual enzyme molecules are seen to vary considerably<sup>6–8</sup>, but the origin of this heterogeneity remains unknown. Here we investigate the physical basis for this behaviour. Although any individual RecBCD molecule unwound DNA at a constant rate for an average of approximately 30,000 steps, we discover that transiently halting a single enzyme–DNA complex by depleting Mg<sup>2+</sup>-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 that 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 behaviour of each RecBCD molecule. Each stabilized sub-state can persist for the duration (approximately 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 behaviour. This ligand-dependent stabilization of rate-defining conformational sub-states results in seemingly static molecule-to-molecule variation in 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 recombination<sup>8</sup>. RecBCD initiates homologous recombination by processing dsDNA to generate 3'-ended single-stranded DNA (ssDNA) upon recognition of the recombination hotspot sequence  $\chi$  (crossover hotspot instigator (Chi); 5'-GCTGGTGG-3'). The RecB and RecD subunits are SF1 helicases with 3'→5' and 5'→3' translocation polarities, respectively<sup>10,11</sup>. RecC holds the complex together and recognizes  $\chi$ <sup>12</sup>. 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 DNA<sup>13</sup>.

Earlier single-molecule studies of DNA unwinding by RecBCD revealed considerable variation in the unwinding rates of each molecule<sup>4–7</sup>. To understand the molecular origin of this intrinsic heterogeneity, we analysed the unwinding behaviour of a larger set of individual RecBCD molecules on bacteriophage  $\lambda$  DNA lacking  $\chi$  (Fig. 1a, b). A total of 251 molecules were initially analysed (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<sup>4,5</sup>, the sizes of those data sets 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  $1,584 \pm 95$  base pairs (bp) s<sup>-1</sup> ( $\pm$  standard deviation (s.d.)) whereas the minor population (29%) has a mean rate of  $907 \pm 500$  bp s<sup>-1</sup> ( $\pm$  s.d.). 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  $\chi$ -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 the slow ones (Supplementary Fig. 2). Both the rate and processivity of the slow species are comparable to the behaviour of RecBCD mutants with a defective motor subunit<sup>14</sup>, leading us to examine the single-molecule behaviour of two such single-motor mutant enzymes. DNA unwinding by RecBCD<sup>K177Q</sup> (RecBCD\* in Fig. 1c) is manifest as a single Gaussian distribution with a mean rate of  $729 \pm 290$  bp s<sup>-1</sup>, and for RecB<sup>K29Q</sup>CD



**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: experimental scheme (top) and sequential images (bottom). **b**, Time courses for unwinding DNA (lacking a  $\chi$  sequence) by different RecBCD molecules: black, absence of RecBCD; colours, individual RecBCD enzymes. Errors are 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.

<sup>1</sup>Department of Microbiology and Molecular Genetics, University of California, Davis, California 95616, USA. <sup>2</sup>Department of Molecular and Cellular Biology, University of California, Davis, California 95616, USA. <sup>3</sup>Biophysics Graduate Group, University of California, Davis, California 95616, USA.

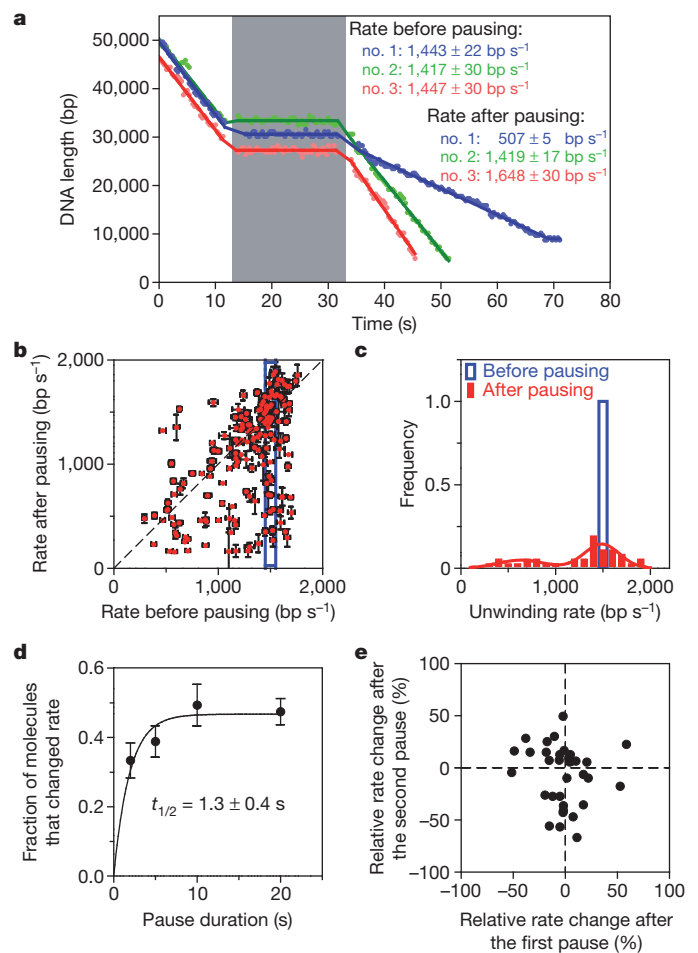
(RecB\*CD in Fig. 1c) it is  $432 \pm 227 \text{ bp s}^{-1}$  (see also Supplementary Fig. 2). These findings suggest that, for the wild-type enzyme, the slow species represents enzymes wherein one motor subunit is initially not engaged, but can be reversibly re-engaged when halted (see below).

The origin of heterogeneity can be dynamic or static<sup>15–17</sup>. 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 owing to the presence of multiple related genes, or from post-translational modifications<sup>18</sup>. It can also result from enzyme molecules with identical chemical composition that have different stable conformational sub-states in equilibrium<sup>15,17,19</sup> or that are kinetically trapped in non-equilibrium states capable of multiple turnovers<sup>20–22</sup>. We initiated experiments designed to distinguish between these possible origins. Although the protein preparation contained 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 used thermal annealing<sup>23</sup>. Ensemble assays showed that RecBCD could be heated to a maximum of  $45^\circ\text{C}$  for 10 min, with no loss of activity (Supplementary Fig. 5a, b). Therefore, an enzyme population that was treated at  $45^\circ\text{C}$ , and slowly cooled at a rate of  $1^\circ\text{C min}^{-1}$ , was analysed 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<sup>24,25</sup>. 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  $1,736 \pm 133 \text{ bp s}^{-1}$  for the fast population versus  $1,773 \pm 104 \text{ bp s}^{-1}$  for the control (Supplementary Fig. 6b), which is the same within experimental uncertainty. The mean of the treated slow population is  $556 \pm 451 \text{ bp s}^{-1}$  versus  $793 \pm 307 \text{ bp s}^{-1}$  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, indicating 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 owing to the presence of multiple conformations of similar free energy<sup>26</sup>, but the binding of substrates could lock an enzyme in a given conformation<sup>27,28</sup>. 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 selection<sup>27</sup>. 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 ( $\sim 10 \text{ s}$ ), the complex was moved to a third channel that contained 10 mM EDTA, but neither  $\text{Mg}^{2+}$  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  $1,443 \text{ bp s}^{-1}$  to  $507 \text{ bp s}^{-1}$ ; for molecule 2, it was the same upon resumption; and for molecule 3, it increased from  $1,447 \text{ bp s}^{-1}$  to  $1,648 \text{ bp s}^{-1}$ . After the 20-s incubation in EDTA, 53% (91 out of 173) of the molecules



**Figure 2** | The DNA unwinding rate of single enzymes is stochastically changed to a velocity within the original distribution, after transient depletion of  $\text{Mg}^{2+}$ -ATP. **a**, DNA unwinding by three representative RecBCD enzymes. The grey block indicates the pause duration. Errors are 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  $1,450$ – $1,550 \text{ bp s}^{-1}$  (blue box, panel **b**;  $n = 36$ ). Before pausing, the selected bin had a mean velocity of  $1,493 \pm 27 \text{ bp s}^{-1}$  (s.d.); after pausing and redistribution, the mean velocity was  $1,245 \pm 453 \text{ bp s}^{-1}$  (s.d.) (median =  $1,411 \text{ bp s}^{-1}$ ). **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 ( $n = 34$ ).

continued unwinding with the same rate (within a 20% difference), whereas 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. By 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 behaviour 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 conformational 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 1,450 and 1,550 bp s<sup>-1</sup> before pausing. After incubation in EDTA, the velocities became broadly redistributed, ranging from 300 bp s<sup>-1</sup> to 1,900 bp s<sup>-1</sup>. 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 (that is, 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 indicate 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 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<sup>2+</sup>. 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  $1.3 \pm 0.4$  s (Fig. 2d), suggesting that a conformational change responsible for the change in velocity in the absence of ATP requires  $\sim 1$  s. The combined data set for all pauses (Supplementary 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 that spontaneous switching is rare. Our interpretation is in accord with an earlier study which found that a few

individual RecBCD enzymes can spontaneously change velocity when examined at low (15  $\mu$ M) ATP<sup>29</sup>. 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  $\sim 67$ -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 were 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 behaviour of other single enzymes have reported static heterogeneity in catalytic rates owing to variation in the covalent structures<sup>18</sup>, the presence of metastable conformations<sup>15,17,19</sup> or dynamic heterogeneity caused by conformational fluctuation<sup>16</sup>. 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 behaviour 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 behaviour. 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, that is, made seemingly static, upon ligand binding<sup>27</sup>. These findings help us 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 base pairs per second 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 to 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 behaviour affords a molecular plasticity in the cellular functions of RecBCD to respond to unpredictable needs. RecBCD has two seemingly contradictory functions: one is the degradation of



foreign duplex DNA (for example, DNA viruses) and the other is the repair of broken chromosomal DNA<sup>8</sup>. The regulation of these activities is controlled by recognition of the DNA regulatory sequence  $\chi$ . Each *E. coli* cell contains only ten 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 conformational 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 SUMMARY

Single-molecule DNA helicase reactions were performed using an optical trapping and microfluidics system as reported<sup>7,30</sup> with minor modification. For the pausing experiments, a three-channel flow cell was used. The first channel contained bead-DNA complexes and 2 mM Mg(OAc)<sub>2</sub> in single-molecule buffer (SMB; 45 mM NaHCO<sub>3</sub> (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 Mg(OAc)<sub>2</sub> in SMB. The third channel contained 10 mM EDTA or 2 mM Mg(OAc)<sub>2</sub> in SMB.

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.

**Full Methods** and any associated references are available in the online version of the paper.

Received 14 October 2012; accepted 22 May 2013.

Published online 14 July 2013.

- Moffitt, J. R., Chemla, Y. R., Smith, S. B. & Bustamante, C. Recent advances in optical tweezers. *Annu. Rev. Biochem.* **77**, 205–228 (2008).
- Ha, T. Single-molecule fluorescence resonance energy transfer. *Methods* **25**, 78–86 (2001).
- Xie, X. S. & Lu, H. P. Single-molecule enzymology. *J. Biol. Chem.* **274**, 15967–15970 (1999).
- Spies, M. *et al.* A molecular throttle: the recombination hotspot  $\chi$  controls DNA translocation by the RecBCD helicase. *Cell* **114**, 647–654 (2003).
- Spies, M., Amitani, I., Baskin, R. J. & Kowalczykowski, S. C. RecBCD enzyme switches lead motor subunits in response to  $\chi$  recognition. *Cell* **131**, 694–705 (2007).
- Handa, N., Bianco, P. R., Baskin, R. J. & Kowalczykowski, S. C. Direct visualization of RecBCD movement reveals cotranslocation of the RecD motor after  $\chi$  recognition. *Mol. Cell* **17**, 745–750 (2005).
- Bianco, P. R. *et al.* Processive translocation and DNA unwinding by individual RecBCD enzyme molecules. *Nature* **409**, 374–378 (2001).
- Dillingham, M. S. & Kowalczykowski, S. C. RecBCD enzyme and the repair of double-stranded DNA breaks. *Microbiol. Mol. Biol. Rev.* **72**, 642–671 (2008).
- Lam, S. T., Stahl, M. M., McMiliin, K. D. & Stahl, F. W. Rec-mediated recombinational hot spot activity in bacteriophage lambda. II. A mutation which causes hot spot activity. *Genetics* **77**, 425–433 (1974).
- Dillingham, M. S., Spies, M. & Kowalczykowski, S. C. RecBCD enzyme is a bipolar DNA helicase. *Nature* **423**, 893–897 (2003).
- Taylor, A. F. & Smith, G. R. RecBCD enzyme is a DNA helicase with fast and slow motors of opposite polarity. *Nature* **423**, 889–893 (2003).
- Handa, N. *et al.* Molecular determinants responsible for recognition of the single-stranded DNA regulatory sequence,  $\chi$ , by RecBCD enzyme. *Proc. Natl Acad. Sci. USA* **109**, 8901–8906 (2012).
- Singleton, M. R., Dillingham, M. S., Gaudier, M., Kowalczykowski, S. C. & Wigley, D. B. Crystal structure of RecBCD enzyme reveals a machine for processing DNA breaks. *Nature* **432**, 187–193 (2004).
- Dillingham, M. S., Webb, M. R. & Kowalczykowski, S. C. Bipolar DNA translocation contributes to highly processive DNA unwinding by RecBCD enzyme. *J. Biol. Chem.* **280**, 37069–37077 (2005).
- Frauenfelder, H., McMahon, B. H., Austin, R. H., Chu, K. & Groves, J. T. The role of structure, energy landscape, dynamics, and allostery in the enzymatic function of myoglobin. *Proc. Natl Acad. Sci. USA* **98**, 2370–2374 (2001).
- Lu, H. P., Xun, L. & Xie, X. S. Single-molecule enzymatic dynamics. *Science* **282**, 1877–1882 (1998).
- Xue, Q. & Yeung, E. S. Differences in the chemical reactivity of individual molecules of an enzyme. *Nature* **373**, 681–683 (1995).
- Craig, D. B., Arriaga, E. A., Wong, J. C. Y., Lu, H. & Dovichi, N. J. 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.* **118**, 5245–5253 (1996).
- Shi, J. *et al.* Multiple states of the Tyr318Leu mutant of dihydroorotate dehydrogenase revealed by single-molecule kinetics. *J. Am. Chem. Soc.* **126**, 6914–6922 (2004).
- Wolynes, P. G., Onuchic, J. N. & Thirumalai, D. Navigating the folding routes. *Science* **267**, 1619–1620 (1995).
- Onuchic, J. N., Wolynes, P. G., Luthey-Schulten, Z. & Socci, N. D. Toward an outline of the topography of a realistic protein-folding funnel. *Proc. Natl Acad. Sci. USA* **92**, 3626–3630 (1995).
- Dill, K. A., Ozkan, S. B., Shell, M. S. & Weikel, T. R. The protein folding problem. *Annu. Rev. Biophys.* **37**, 289–316 (2008).
- Nguyen, H. D. & Hall, C. K. Effect of rate of chemical or thermal renaturation on refolding and aggregation of a simple lattice protein. *Biotechnol. Bioeng.* **80**, 823–834 (2002).
- Ikai, A. & Tanford, C. Kinetic evidence for incorrectly folded intermediate states in the refolding of denatured proteins. *Nature* **230**, 100–102 (1971).
- Sela, M., White, F. H. Jr & Anfinsen, C. B. Reductive cleavage of disulfide bridges in ribonuclease. *Science* **125**, 691–692 (1957).
- Frauenfelder, H., Sligar, S. G. & Wolynes, P. G. The energy landscapes and motions of proteins. *Science* **254**, 1598–1603 (1991).
- Ma, B. & Nussinov, R. Enzyme dynamics point to stepwise conformational selection in catalysis. *Curr. Opin. Chem. Biol.* **14**, 652–659 (2010).
- Boehr, D. D., Nussinov, R. & Wright, P. E. The role of dynamic conformational ensembles in biomolecular recognition. *Nature Chem. Biol.* **5**, 789–796 (2009).
- Perkins, T. T., Li, H. W., Dalal, R. V., Gelles, J. & Block, S. M. Forward and reverse motion of single RecBCD molecules on DNA. *Biophys. J.* **86**, 1640–1648 (2004).
- Amitani, I., Liu, B., Dombrowski, C. C., Baskin, R. J. & Kowalczykowski, S. C. Watching individual proteins acting on single molecules of DNA. *Methods Enzymol.* **472**, 261–291 (2010).

**Supplementary Information** is available in the online version of the paper.

**Acknowledgements** We are grateful to members of the laboratory for their comments on this work. S.C.K. was supported by the National Institutes of Health (GM-62653 and GM-64745).

**Author Contributions** B.L., R.J.B. and S.C.K. conceived the general ideas, designed the experiments and interpreted the data. B.L. performed experiments. B.L. and S.C.K. analysed the data and wrote the manuscript. R.J.B. passed away on July 3, 2010; this work is dedicated to his collegiality and contributions.

**Author Information** Reprints and permissions information is available at [www.nature.com/reprints](http://www.nature.com/reprints). The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to S.C.K. ([sckowalczykowski@ucdavis.edu](mailto:sckowalczykowski@ucdavis.edu)).

## METHODS

**Proteins and DNA substrates.** *E. coli* RecBCD enzyme was expressed and purified as described previously<sup>31,32</sup>. To check purity, protein was analysed 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 AlphaInnotech gel documentation system. The two mutant enzymes, RecBCD<sup>K177Q</sup> (RecBCD\*) and RecB<sup>K29Q</sup>CD (RecB\*CD), were purified as described<sup>10</sup>.

Bacteriophage  $\lambda$  DNA (*N*<sup>6</sup>-methyladenine-free lambda DNA, New England Biolabs) was biotinylated by ligation to a 3'-biotinylated 12-mer oligonucleotide (*cosA*: 5'-GGGCGGCACCT-3' or *cosB*: 5'-AGTTCGCCGCC-3', Operon Technologies) that is complementary to one of the cohesive ends of  $\lambda$  DNA<sup>7</sup>; except for the thermal re-annealing and control experiments, where *cosA* was used, all other experiments used the *cosB* oligonucleotide.

The pUC19 plasmid DNA was purified by caesium 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 6,330 M<sup>-1</sup> (nucleotides) cm<sup>-1</sup>.

**ATP hydrolysis assays.** The ATP hydrolysis activity of the enzyme was measured spectrophotometrically as reported<sup>33</sup> by coupling ATP hydrolysis to NADH oxidation<sup>34</sup> 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<sup>-1</sup> NADH, 30 U ml<sup>-1</sup> pyruvate kinase, 30 U ml<sup>-1</sup> lactate dehydrogenase and 50  $\mu$ 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 A<sub>340 nm</sub> decrease (s<sup>-1</sup>)  $\times$  9,820  $\div$  0.0005 ( $\mu$ M RecBCD)  $\div$  60 = rate of ATP hydrolysis (s<sup>-1</sup>)<sup>33</sup>.

**Re-purification of RecBCD.** RecBCD enzyme (0.1 mg) from -80 °C freezer stock was thawed on ice, diluted fivefold 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  $\mu$ l each) on one side of the peak in ultraviolet absorbance were used immediately for single-molecule helicase assays.

**Stopped-flow dye-displacement helicase assay.** Essentially, the protocols used previously<sup>14</sup> 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 ssDNA-binding protein (SSB). The RecBCD enzyme, at the final concentration indicated, was incubated with 0.05 nM (molecules) NdeI-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 analysed using GraphPad Prism 5.02 (GraphPad Software). 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 and 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<sup>-1</sup> 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 (~23 °C) for 1 h, the sample was dialysed 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 h 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 reported<sup>7</sup> with some modifications<sup>30</sup>. The system is constructed around a Nikon Eclipse microscope (Nikon). A high-pressure mercury lamp (100 W; USHIO America) and Y-FL-4-cube Epi-Fluorescence (Nikon) attachment were used for illumination. Images were captured using a high sensitivity electron bombardment couple-charged device (CCD) camera (EB-CCD C7190; Hamamatsu Photonics) and digitalized online using an LG-3 frame grabber (Scion Corporation) at 30 frames s<sup>-1</sup>. The optical trap was created by focusing a 1,064 nm laser (Nd:YVO<sub>4</sub>, 6 W max, J-series power supply; Spectra Physics) through a high numerical aperture (NA) objective ( $\times$ 100/1.3 oil

DICH; Nikon). A high NA objective is necessary to create an intensity gradient sufficiently large to form the trap<sup>35</sup>. The laser is expanded with a 20 $\times$  beam expander (HB-20XAR.33; Newport) to fill the back aperture of the objective. The laser is collimated and aligned using a 1 $\times$  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) 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), generating a flow rate of ~100–150  $\mu$ m s<sup>-1</sup>. 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<sup>4,7</sup>. Biotinylated  $\lambda$  DNA (100 pM in 1–2  $\mu$ l) was incubated with 1–2  $\mu$ l of 1  $\mu$ m ProActive streptavidin-coated microspheres (~35 pM; Bangs Laboratories) for 1 h on ice or at 37 °C. Bead–DNA complexes were then transferred into 0.5 ml of de-gassed sample solution containing 45 mM NaHCO<sub>3</sub> (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 h in the dark at room temperature. 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<sub>3</sub> (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 Mg(OAc)<sub>2</sub> in SMB (45 mM NaHCO<sub>3</sub> (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 Mg(OAc)<sub>2</sub> in SMB. The third channel contained either 10 mM EDTA or 2 mM Mg(OAc)<sub>2</sub> in SMB; the two solutions used as indicated were either: 45 mM NaHCO<sub>3</sub> (pH 8.3), 15% (w/v) sucrose, 50 mM DTT, 10 mM EDTA and 20 nM YOYO-1 dye, or 45 mM NaHCO<sub>3</sub> (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 plugin<sup>30</sup>. The experimental data were fitted to either a line or a three-segment line using Origin 7.5 (OriginLab Corp.) 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<sup>-1</sup>. 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<sup>-1</sup> 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.

- Roman, L. J. & Kowalczykowski, S. C. Characterization of the helicase activity of the *Escherichia coli* RecBCD enzyme using a novel helicase assay. *Biochemistry* **28**, 2863–2873 (1989).
- Bianco, P. R. & Kowalczykowski, S. C. 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* **94**, 6706–6711 (1997).
- Spies, M., Dillingham, M. S. & Kowalczykowski, S. C. Translocation by the RecB motor is an absolute requirement for  $\gamma$ -recognition and RecA protein loading by RecBCD enzyme. *J. Biol. Chem.* **280**, 37078–37087 (2005).
- Kreuzer, K. N. & Jongeneel, C. V. *Escherichia coli* phage T4 topoisomerase. *Methods Enzymol.* **100**, 144–160 (1983).
- Neuman, K. C. & Block, S. M. Optical trapping. *Rev. Sci. Instrum.* **75**, 2787–2809 (2004).