Motor step size and ATP coupling efficiency of the dsDNA translocase EcoR124I

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The Type I restriction-modification enzyme EcoR124I is an archetypical helicase-based dsDNA translocase that moves unidirectionally along the 3′–5′ strand of intact duplex DNA. Using a combination of ensemble and single-molecule measurements, we provide estimates of two physico-chemical constants that are fundamental to a full description of motor protein activity—the ATP coupling efficiency (the number of ATP consumed per base pair) and the step size (the number of base pairs transported per motor step). Our data indicate that EcoR124I makes small steps along the DNA of 1 bp in length with 1 ATP consumed per step, but with some uncoupling of the ATPase and translocase cycles occurring so that the average number of ATP consumed per base pair slightly exceeds unity. Our observations form a framework for understanding energy coupling in a great many other motors that translocate along dsDNA rather than ssDNA.

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Introduction

Molecular motor proteins have numerous roles in DNA metabolism and are central to replication, recombination and repair. An important class of nucleic acid motors are the helicases, which can be classified on the basis of homologous amino-acid motifs into superfamilies (SF) 1–5 (Singleton et al., 2007). For many helicases—all those characterized thus far from SF1 for example—directional translocation takes place on a single-stranded polynucleotide. Accordingly, a duplex DNA (or RNA) must be melted to gain access to the linear track. This structural transition in a nucleic acid is the classical role for which helicases are known, and indeed named. However, there are also a great many enzymes—among those found in SF2 for example—that, although classified as helicases, are actually dsDNA translocases that move along intact duplex nucleotides (Hopfner and Michaelis, 2007; Seidel and Dekker, 2007; Singleton et al., 2007). These enzymes do not directly unwind DNA and are thus not helicases in the eponymous sense. One such example is the chromatin-remodelling factors (Lia et al., 2006; Zhang et al., 2006; Seidel and Dekker, 2007). Compared to the ssDNA motors, very little is known about dsDNA translocation. A key piece of information in developing a mechanistic framework for understanding these enzymes is how efficiently the motor couples ATP hydrolysis to protein stepping on dsDNA: how much energy in the form of ATP is required to move a step along DNA and how big is that step?

Knowing this information puts a physical constraint on how domain motions of the helicase motor core are coupled to the nucleic acid.

To solve this fundamental puzzle for the dsDNA translocases, we have examined the motor activity of an archetype dsDNA motor (Singleton et al., 2007), the ATP-dependent Type I restriction-modification (RM) enzyme EcoR124I. The extensive characterization of the dynamics of initiation, translocation and termination of this enzyme (Seidel et al., 2005), alongside extensive kinetic analysis of translocation (Firman and Szczelkun, 2000; Seidel et al., 2004; McClelland et al., 2005; Stanley et al., 2006), means that EcoR124I is the best test system for addressing this question.

The Type I RM enzymes are widely distributed in both bacteria and archaea, and have a beneficial role in protecting a host cell from invasion by foreign DNA such as bacteriophages and plasmids (Murray, 2000). Type I RM enzymes comprise three protein subunits: HsdS, HsdM and HsdR. HsdS and HsdM (HsdS1HsdM2) form independently active methyltransferases (MTase) that recognize specific, bipartite sequences (e.g., GAAmnRRCG for EcoR124I) (Janscak et al., 1996). The HsdR subunits are fusions of PDE(x)K nuclease and SF2 helicase domains (McClelland and Szczelkun, 2004). To cleave DNA, HsdR must bind the MTase and use its helicase motor to translocate along intact dsDNA using ATP hydrolysis (Stanley et al., 2006). As the HsdR motor remains bound to the MTase during translocation, a growing loop of DNA is extruded (van Noort et al., 2004). The MTase core can recruit two HsdRs to form an HsdS1HsdM2HsdR2 complex where each HsdR acts independently (Janscak et al., 1996; Seidel et al., 2004, 2005). As a consequence, translocation by both HsdRs in a complex
results in the extrusion of two DNA loops, one on each side of the site (van Noort et al., 2004). We have shown previously that EcoR124I moves on intact dsDNA without unwinding, using principal motor contacts to the 3′–5′ strand (Stanley et al., 2006). The motor can still translocate on 3′–5′ ssDNA if loaded via dsDNA, although the processivity is greatly reduced (Stanley et al., 2006). The polar motion of EcoR124I on dsDNA has been highlighted as a paradigm for many other dsDNA translocases (Hopfner and Michaelis, 2007), for example, the chromatin-remodelling enzymes (Durr et al., 2006). Using a combination of fast-mixing ensemble and single-molecule techniques, we have now extended the understanding of this enzyme to include the ATP coupling efficiency and motor step size. Our data are consistent with structural models for dsDNA translocation and support the proposal that dsDNA and ssDNA translocases share a similar gross mechanism for motion along different polynucleotide tracks (Hopfner and Michaelis, 2007).

**Results**

**Measurement of the temperature dependence of ATP hydrolysis by stopped-flow fluorimetry**

The ratiometric ATP coupling efficiency (ATP hydrolysed per base pair moved) can be determined simply by comparing the rates of ATP hydrolysis and dsDNA translocation. To ensure that our observations are not biased by a particular set of experimental conditions, we carried out our ATPase and translocase measurements at a range of ATP concentrations and temperatures. Any change in the mechanism that in turn changes the coupling can then be identified (Lucius and Lohman, 2004). Previous studies of Type I enzymes reported a large range of different ATP couplings of ~1–3.1 ATP/bp (Endlich and Linn, 1985; Davies et al., 1999; Bianco and Hurley, 2005). The principle caveat of these measurements was that while measuring the ATPase activity, the translocase activity was typically not measured but inferred indirectly from cleavage experiments or from published data, taken under different experimental conditions. For example, a recent and rather extensive ATPase study reported an inefficient coupling of 3.1 ATP/bp (Bianco and Hurley, 2005). One way that the coupling was calculated was by dividing an ATPase rate (in units of ATP/min) by a processivity parameter (in units of bp). However, this actually gives a value in units of ATP/bp/min, which does not represent a true coupling constant. Another way that the coupling was calculated was by dividing the maximum ATPase rate measured at 37°C by a maximum translocation rate measured at 20°C in a separate study. However, as shown below, over such a temperature difference, the translocation rate changes by a factor of ~3. Therefore, to obtain a precise coupling efficiency, it is crucial to measure both activities directly under the same experimental conditions, which even for SF1 helicases has only been accomplished very recently (Tomko et al., 2007).

To measure both activities with the best accuracy, we utilized a rapid-mix stopped-flow fluorimeter. We were thus able to initiate our reactions under reproducible conditions, that is, identical mixing times, identical mixing conditions, etc. To measure the EcoR124I ATPase activity, we utilized a coumarin-labelled phosphate binding protein (PBP) sensor developed by Martin Webb (Figure 1A; Materials and methods; Supplementary Figures S1–S3; Supplementary data) (Webb, 2003, 2007). The binding of phosphate to the sensor is both fast and tight, allowing rapid ATPase kinetics on a millisecond timescale to be followed. Unlike enzyme-coupled ATPase assays (e.g., the LDH-coupled assays used in other EcoR124I studies; Bianco and Hurley, 2005), the sensor does not have a time lag and the kinetics observed can be related directly to the ATPase kinetics immediately upon starting the reaction (see Supplementary Figure S3). Using the stopped-flow fluorimeter, we preincubated EcoR124I with linear DNA substrates, initiated the reaction by rapid mixing with ATP and then followed the increase in free phosphate released during ATP hydrolysis from the increase in fluorescence of the sensor (Figure 1A and B; Materials and methods; Webb, 2003). Because of the considerable site-independent ATPase activity of EcoR124I (Zinkevich et al., 1997), all data were corrected for background activity measured using a non-specific DNA that is the parental vector for all our specific substrates (Figure 1B, inset; Materials and methods). Hydrolysis of the specific DNA does not occur because on linear substrates collision with a second translocating enzyme is required to activate the nuclease activity (Szczelkun et al., 1996).

The ATPase kinetics recorded under a large variety of conditions (Figure 1B–D) is always characterized by very low initial rate, followed by an acceleration phase until finally a constant steady-state rate is achieved. This behaviour can actually be understood on a quantitative level, as the dynamics of initiation, termination and reinitiation of EcoR124I translocation have been previously quantitatively defined using rigorous kinetic analysis (Figure 1A; Seidel et al., 2005): the MTase forms a stable, long-lived complex on the DNA (t1/2 > 30 min) that, in a second-order process, recruits free HsdR onto the adjacent DNA; the loaded HsdR then starts to translocate a DNA loop, preceded by initiation step(s); at any stage during translocation, the HsdR may detach from the DNA and MTase, terminating the loop; a second HsdR can then be recruited from solution by the MTase to repeat the cycle. Knowing this cycle on a quantitative level allows us to correctly model our ATPase kinetics by numeric integration (Supplementary Figure S4; Seidel et al., 2005). Consistent with the empirical data, the resulting profiles are characterized by a slow exponential lag in the kinetics until a linear steady-state phase is reached, during which the majority of MTase–DNA complexes have HsdRs attached, which are occupied in loop translocation (Supplementary Figures S4 and S6; Frieden, 1979). The lag represents slow loop initiation and recycling of dissociated motors.

Overall, our experimental profiles match those expected from the model (Figure 1B and C) and can be almost completely described by a single exponential approach to a linear steady state (Supplementary Figure S5). But we note that there are small but nonetheless systematic deviations from the fit during the initiation stages, and the reasons are discussed in Supplementary data (see Supplementary Figure S5). Irrespective of the exact details of the initiation pathway, we can use linear regression to fit the steady-state segment of the data to give the global steady-state kcat (Figure 1C; Materials and methods; Frieden, 1979); this is a macroscopic constant that averages over all the motors translocating but also over the various rates during one stepping cycle—ATP binding, hydrolysis and product release.
The temperature dependence of ATP hydrolysis during steady-state loop extrusion. (A) Cartoon showing the cycle of initiation, translocation and termination of DNA translocation by an EcoR124I HsdR subunit (white oval). For clarity, translocation on one side of the MTase only is shown. DNA is shown as a black line and the EcoR124I MTase as a black oval. The binding of free inorganic phosphate by PBP is indicated. (B) Dependence of phosphate release on the availability of free HsdR (concentrations indicated). One-site linearized pLKS5 (S) was corrected by the background level using a single-site linearized pLYB11 (NS) to generate the corrected profile (C). Example kinetic profiles at 10 and 30°C. The steady-state phase of the reaction was fitted to equation (3) to give $k_{\text{ATP}}$, the x-axis intercept gives the initiation time (data not shown; Frieden, 1979). (D) Example of an ATP titration (μM concentrations of ATP indicated), with each profile fitted to equation (3). (E) Hyperbolic dependence of $k_{\text{ATP}}$ on [ATP] as a function of temperature. Solid lines show fits to equation (4). Statistical error bars are smaller than the scatter of the data points. Fitting to equation (4) was not weighted to either the statistical or systematic errors (discussed in Supplementary data). (inset) Dependence of $k_{\text{ATP}}$ on temperature. Error bars are the standard error of the mean (s.e.m.) of the fitted values. The dotted line shows the average $k_{\text{ATP}}$ across the temperature range and the grey box the associated standard deviation (s.d.). $V_{\text{max,calc}}$ and s.e.m. (bp/s) calculated from the fits were 246 ± 3 (10°C), 376 ± 7 (15°C), 503 ± 25 (20°C), 807 ± 11 (25°C) and 998 ± 7 (30°C).

Measurement of the temperature dependence of DNA translocation by stopped-flow fluorimetry

DNA translocation can be measured in bulk solution by the triplex displacement assay (Firman and Szczelkun, 2000; McClelland et al., 2005), which allows variation in both temperature and ATP concentration. Using the stopped-flow fluorimeter, we preincubated our enzyme with specific linear DNA substrates containing a fluorescent triplex bound at varying distances downstream of the recognition site, initiated the reaction by rapid mixing with ATP and then followed the increase in the fluorescence of the triplex probe as it was displaced following collision of the HsdR (Figure 2A; Materials and methods). By following this protocol...
col, we could measure translocation under exactly the same conditions used above for the ATPase measurements. Data from five different triplex spacings were fitted to the global kinetic model considered above in the ATPase data to obtain \( k_{\text{step}} \) (Materials and methods; Seidel et al., 2005). As with \( k_{\text{ATP}} \), this is a macroscopic constant that averages over ATP binding/hydrolysis/release and motor steps in one stepping cycle.

Translocation rates were examined at four temperatures (10, 15, 20 and 30°C) and across a range of ATP concentrations above and below the \( K_{M,\text{app}} \). Similar measurements at 25°C were obtained previously (Seidel et al., 2005). Examples of displacement profiles are shown in Figure 2B. The dependence of \( K_{M,\text{app}} \) on the ATP concentration and temperature is shown in Figure 2C. At all temperatures, the data show that the rates increase hyperbolically with increasing ATP. As above, the temperature dependence gives a \( Q_1 \approx 2.0 \) (data not shown). The apparent \( K_M \) values obtained from fits to equation (4) are, within error, equal to the values obtained in the ATPase assays (Figure 2C, inset).

**ATP coupling efficiency of EcoR124I**

To compare directly the ATPase and translocation rates obtained above, we must ensure that changes in ATP concentration and/or temperature do not result in changes in the kinetic pathway (Lucius and Lohman, 2004; Tomko et al., 2007). If these were to occur such that the ATPase and translocase reactions were rate-limited by different steps in the pathway, then the coupling ratio observed would be specific to a particular solution condition. The experimental \( k_{\text{ATP}} \) and \( k_{\text{step}} \) values presented in Figures 1E and 2C and the \( V_{\text{max,calc}} \) values obtained from the corresponding fits were compared using the Arrhenius relationship:

\[
v = v_0 e^{-E_a/k_B T} \]  

where \( v \) is the observed rate, \( v_0 \) is the pre-exponential factor, \( E_a \) is the activation energy barrier height and \( k_B T \) is the energy of the surrounding heat bath. ATPase and translocase data at each ATP concentration were fitted to equation (1) independently (Figure 3A; Supplementary Figures S7 and S8). No significant deviations from equation (1) were observed over the temperature ranges examined, indicating that the same kinetic pathway exists under all conditions. Within experimental error, the \( E_a \) values obtained for the ATPase and translocase activities were similar and invariant with ATP concentration (Figure 3B). These observations can be justified as follows.

For our measurements, the translocation pathway can be described in a simplified manner by the kinetic scheme below:

\[
\text{ATP} + ED_n \xrightarrow{k_1} \text{ATP} \cdot ED_n \xrightarrow{k_1} ED_{n+1} \xrightarrow{k_1} \cdots \]  

(Scheme 1)

where \( ED_n \) and \( ED_{n+1} \) represent the motor (E) bound to the DNA (D) at positions \( n \) and \( n+1 \), respectively, \( k_1 \) and \( k_{-1} \) represent the reversible ATP association and dissociation rates, respectively. If this were to occur such that the ATPase and translocase rates to obtain an apparent coupling efficiency that is valid over a wide range of solution conditions, we are confident that the kinetic mechanism does not vary appreciably across the conditions examined. Consequently, we are also confident in directly comparing the ATPase and translocase rates to obtain an apparent coupling efficiency that is valid over a wide range of solution conditions.
important source of error in this value comes from the systematic errors in the $k_{\text{ATP}}$ values (see above and Supplementary data). To quantify the precision of the sample mean, we calculated the error propagation from the sum of the 95% confidence interval ($\pm 4\%$) and the additive systematic errors in $k_{\text{ATP}}$ (+11% and −6%; Supplementary data), to give a coupling constant of $1.26 \pm 0.19$ ATP/bp. Given the wide range of solution conditions explored and the error range in our measurements, we can assert that EcoR124I hydrolyses between 1.1 and 1.5 ATPs for every base pair of dsDNA translocated.

### Measurement of the temperature dependence of DNA translocation by single-molecule measurements

One of the most compelling reasons to use EcoR124I as an archetype dsDNA translocase in these studies is that we can measure the activity of single enzyme molecules using a well-established magnetic tweezers setup (Figure 4A; Seidel et al., 2004, 2005; Stanley et al., 2006). Motor events can be observed as a transient decrease in the end-to-end length of the DNA due to the formation of DNA loops. Both the rate and lifetime of a translocation event can be scored. As the reported rates correspond to the total velocity of shortening of the end-to-end length of the DNA, events with only one motor running ($R_1$) can be distinguished from those with two motors running ($R_2$), as the latter cause a temporary doubling of the rate. As in the ensemble experiments, the use of a one-site linear DNA substrate significantly reduces the chance of DNA cleavage occurring (Szczelkun et al., 1996; Seidel et al., 2004).

To assess the physical step size of the enzyme, which is required to obtain the ATP coupling efficiency, it would be ideal if one could resolve individual steps directly from the tracking profiles. However, EcoR124I motor events can only be observed for forces <5 pN (Seidel et al., 2004), conditions where large Brownian fluctuations of the DNA in combination with the high motor velocity limit the spatial resolution of individual steps to step sizes of >10 bp at 10 Hz. But it is possible to calculate a smaller step size from a noise analysis of a measured translocation profile following many thousands of steps (Charvin et al., 2002; Neuman et al., 2005). When the motor cycle is governed by a single rate-limiting step (i.e., as in Scheme 1), the stochastic nature of stepping produces a random deviation from a straight line in the position–time relationship. This stepping noise, which is proportional to the step size, has distinct spectral characteristics at low frequencies compared to the approximately Gaussian noise of the magnetic bead fluctuations (Charvin et al., 2002; Neuman et al., 2005). Consequently, it is possible to extract the step size from a Fourier analysis of multiple steps during a translocation run in the magnetic tweezers. For example, this method has been used to determine an unwinding step size for the UvrD helicase of 6 bp (Dessinges et al., 2004).

To carry out this analysis on EcoR124I, we assume that Scheme 1 holds, that is, each enzymatic cycle is governed by a single rate-limiting step. (Multiple rate-limiting steps would lead to a decrease in the stepping randomness and thus a lower stepping signal compared to background noise.) To validate this under the single-molecule conditions, tweezers experiments were performed at different temperatures with a fixed, saturating concentration of ATP (Materials and

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**Figure 3** Coupling efficiency of ATP hydrolysis to DNA translocation. (A) Temperature dependence of the rate of ATP hydrolysis (left panel) and translocation (right panel) at ATP concentrations (bottom to top in both panels) 12.5, 25, 48, 96, 170, 320, 600 and 1200 μM. The topmost line in each panel represents the $V_{\text{max,calc}}$ data obtained from the hyperbolic fits in Figures 1E and 2C, and Seidel et al (2005). Solid lines represent independent fits to equation (1). Statistical error bars are smaller than the scatter of the data points. Fitting to equation (1) was not weighted to the statistical errors (discussed in Supplementary data). Each individual fit is shown in Supplementary Figures S7 and S8. (B) Dependence of the activation energies ($E_a$) obtained in (A) on [ATP]. Error bars are the s.e.m. $V_{\text{max,calc}}$ values are arbitrarily plotted at 8 mM ATP. Dotted lines represent the average $E_a$ values and the grey boxes the associated s.d. values. (C) The effect of temperature and [ATP] on the ATP coupling ratio ($k_{\text{ATP}}$/$k_{\text{step}}$). Grey lines show the ratios of ATP hydrolysed to base pairs translocated, as indicated. The dotted line shows the mean coupling ratio, 1.26 ATP/bp. The s.d. of the sample mean is ±0.14 (N=35). The grey box represents the precision of the mean ($\pm 1\sigma$) and is calculated by error propagation as the sum of the 95% confidence interval of the sample and the systematic error in $k_{\text{ATP}}$ (discussed in Supplementary data).
methods); example profiles at two temperatures are shown in Figure 4B.

For each temperature examined, data were collated for the translocation velocity and lifetime for both R1 and R2 events and fitted to equation (1) (Figure 4C and D). In agreement with the stopped-flow measurements, linear Arrhenius relationships were obtained, which supports the assumption that speed is limited by a single rate (modelling additional temperature-dependent and temperature-independent rates causes significant deviations from the data; Supplementary Figure S9). The activation energies for the R1 and R2 events are similar, whereas the absolute speeds are two-fold different across the temperature range examined (Figure 4C).

Comparison of the data from the tweezers assay with that from the triplex assay shows that there is excellent agreement in the HsdR translocation rates (Supplementary Figure S10). The duration of both R1 and R2 motor events

Figure 4

Temperature dependence of EcoR124I step size measured using single-molecule magnetic tweezers assays. (A) Magnetic tweezers set-up (Seidel et al., 2004). A DNA molecule with a single EcoR124I site is anchored between a glass coverslip and a magnetic bead. Magnets are used to maintain the DNA in a stretched conformation. Translocation of one (not shown) or two (shown) HsdR results in the formation of expanding DNA loops, which reduce the apparent end-to-end DNA length and this is monitored by video microscopy. (B) Example time traces recorded at two different temperatures with MTase at 20 nM, HsdR at 160 nM, ATP at 4 mM and the applied stretching force at 3 pN. Each trace shows a similar profile, with the initiation of the first motor to form an R1 event (1), the initiation of the second motor to form an R2 event (2), the dissociation of one or other motor with the concomitant release of a trapped DNA loop to return to an R1 event (3) and finally the dissociation of the remaining motor (4). The graphs below the time traces show the development of the translocation rate, which has been determined from a straight line fit to the data (data not shown) and calculating its derivative. R2 events have a rate that is twice that of R1 events (Seidel et al., 2004). (C) Temperature dependence of the R1 or combined R2 translocation velocity from the magnetic tweezers. Solid lines represent independent fits to equation (1). Error bars in the y-axis represent s.e.m. and errors bars in the x-axis represent the systematic error in the temperature. (D) Temperature dependence of the motor lifetime (t\text{motor}) for single HsdR within an R1 or R2 complex. Solid lines represent independent fits to equation (1) where v = 1/t\text{motor}. Error bars as in (C). (E) Example of a long-duration R2 translocation event recorded at 18°C. The average spectral density of such events was examined to obtain estimates of the step size. (F) Power spectrum averaged over 14 translocation events at 18°C. The blue line represents the fit to equation (2). The two dotted lines indicate step sizes of precisely 1 or 3 bp. The green data are the power spectrum of the noise during periods of inactivity. Error bars represent s.e.m. (G) The temperature dependence of the step size. The dotted line represents the mean across the temperature range. Error bars represent the s.e. returned from the fit (see (F)).
(\(r_{\text{motor}}\)) decreased with increasing temperature. Linear Arrhenius relationships were again obtained, with activation energies for the \(R_1\) and \(R_2\) species, which are two-fold different (Figure 4D). These results are also consistent with previous work, which showed that translocation termination (defined as the dissociation of a single HsdR from the MTase loader complex) is governed by a single rate (Seidel et al, 2004, 2005).

**Step size of EcoR124I measured by magnetic tweezers**

To determine the step size, we examined long-lived \(R_2\) translocation events at different temperatures (Materials and Methods). The activity of each motor in an \(R_2\) complex is statistically independent (Figure 4C; Szczelkun, 2002; Seidel et al, 2004, 2005), and the resulting stepping profiles will be indistinguishable from those of one motor in an \(R_1\) complex (except for a doubling in the overall rate). To provide the best balance between decreasing instrumental noise (by minimizing Brownian motion of the bead) while increasing the run length (which in turn maximizes the occurrence of low-frequency noise), we chose to examine \(R_2\) tweezers data for conditions of [ATP] = 4 mM and \(F = 3\) pN. Note that we can neglect any influence of the applied force on the apparent step size, as the stepping rate is independent of force up to 5 pN, most likely because the force-producing step never becomes rate limiting before the complex collapses (Seidel et al, 2004). A typical profile under these conditions is shown (Figure 4E). Under the assumption, validated above, of a single rate-limiting step per stepping cycle, the power spectrum of a translocation run can be analysed by

\[
\langle S_x(f) \rangle = \frac{\epsilon \langle v \rangle}{2\pi f^2} + b
\]

where \(f\) is the frequency, \(\epsilon\) the step size, \(v\) the speed and \(b\) the experimental background noise (Charvin et al, 2002; Neuman et al, 2005). To improve precision further, the power spectra of multiple translocation events were averaged (Figure 4F). The power spectrum of instrumental noise (i.e., during periods of motor inactivity) is shown for comparison. For this example, a step size of 2.4 bp can be estimated from a fit to equation (2). The dashed lines in Figure 4F show the relationship expected for steps of 1 or 3 bp. Clearly, these do not describe the data well.

Step sizes estimated in this way were determined at a range of temperatures (Figure 4G). Although enzyme speed changes considerably over the temperature range (Figure 4C), the step sizes are, within experimental limits, constant. From this, we can estimate a temperature-independent mean step size of 1.9 \pm 0.6 bp. We note that any additional source of error—nonlinearities in the length measurements—increase the low-frequency noise (additional deviation from a straight line in the position–time relationship) and therefore might lead to a slight overestimation of the real step size. Moreover, if EcoR124I occasionally makes short pauses during translocation (see discussion about uncoupling below) or exhibits a slight sequence dependence of its translocation rate, then again the step size would be somewhat overestimated. Our data thus clearly exclude step sizes exceeding 2. EcoR124I most likely makes steps of 2 bp or perhaps 1 bp at a time along the dsDNA track.

**Discussion**

We have shown above that motion of the dsDNA motor EcoR124I along intact dsDNA can occur in steps of 1–2 bp, with \(\sim 1\) ATP consumed for each base pair moved. Most notably, both results are consistent with a model in which the motor makes small steps on DNA of \(\leq 2\) bp. Much larger differences between the coupling ratio and the apparent step size have been found for other helicase motors (see below).

A small step size explains a number of features of the EcoR124I mechanism: (1) DNA translocation produces changes in DNA twist, with one supercoil induced every 11 \pm 2 bp (Seidel et al, 2004). This is consistent with a small step size where the motor will track closely the helical pitch of the DNA. (2) The translocation rate is independent of force up to \(\sim 5\) pN (above which DNA tension significantly reduces the lifetime of translocation; Seidel et al, 2004). This is consistent with a small step size, which would only slow down any force-producing step by a factor of \(\sim 1.4\) (calculated assuming a force-dependent Arrhenius relationship (Seidel et al, 2004), a step size of 1 bp and a force of 4 pN). (3) DNA translocation is sensitive to changes in the DNA backbone as minor as a single missing phosphate group at a nick (Stanley et al, 2006). This is consistent with the enzyme tracking the DNA backbone closely. (4) EcoR124I is a true dsDNA translocase (Stanley et al, 2006). Any transient DNA unwinding during translocation would most likely lead to a strong sequence-dependent translocation rate, as seen for some true helicases (Johnson et al, 2007), which in turn should significantly increase the observed stepping noise. However, our small step size is inconsistent with large sequence-dependent effects.

Despite a rather close match between ATPase coupling and apparent physical step size, both measured values are, however, not entirely in agreement with a simple uniform stepping model in which 1 or 2 bp steps are made by the enzyme with precisely 1 ATP hydrolysed per step. On the one hand, the ATPase coupling is significantly larger than 1:1 (Figure 3C) and, on the other hand, the apparent step size is close to 2 and thus larger than the coupling ratio (Figure 4G). In the following discussion, we will therefore consider several models, each of which can explain this.

**2.2 Tightly coupled Model**

The motor makes a 2 bp step along the DNA lattice after hydrolysis of 2 ATP (Figure 5A). Within this model, two sequential ATP-binding–hydrolysis–release cycles must occur; simultaneous, cooperative ATP binding at two independent ATP binding sites is not supported by the strictly hyperbolic ATP dependence of \(k_{\text{ATP}}\) and \(k_{\text{ATP}}\) (Figures 1E and 2C). This in turn implies that the energy of the first hydrolysis event must be stored (e.g., within a strained conformational intermediate state of the motor) to be made available following the second hydrolysis event. Note that this model can only give rise to an apparent step size of 2 bp from a Fourier analysis if one allows the rates of the two ATP hydrolysis events to be different. Otherwise, a step size of 1 bp would be obtained (this corresponds to the Clockwork Model with \(k_{\text{ATP}}k_{\text{ATP}} = 1\), which is discussed below and in Figure 6A).

**1:1 Spring-loaded Model**

The motor makes 1 bp steps along the DNA lattice with 1 ATP hydrolysed per step, but with an elastic linkage between the
HsdR and, for example, the MTase, such that measurable DNA shortening is observed only after a threshold strain has accumulated. This model has been adapted from the recently proposed mechanism of HCV NS3 helicase (Myong et al., 2007). The threshold could be reached every 2 bp, as depicted in Figure 5B. As noted above, this model only returns an apparent step size > 1 bp from a Fourier analysis if there are rate differences within the cycle before DNA shortening.

1:1 Clockwork Model

The motor makes 1 bp steps along the DNA lattice with 1 ATP hydrolysed per step, but with a pause every \( n \) steps (Figure 5C). This model has been recently proposed for the translocation of UvrD along ssDNA to explain the difference between an apparent kinetic step size of 4.6 nt and an ATPase coupling of 1 ATP/nt (Tomko et al., 2007). Depending on the ratio between the fast stepping rate and the slow pausing rate, one could obtain an apparent step size anywhere between 1 and \( n \) from a Fourier analysis (Figure 6A).

1:1 Uncoupled Model

Based on the coupling of > 1 ATP/bp observed here, we propose a new model that has not been discussed previously. It assumes 1 bp steps along the DNA lattice with 1 ATP hydrolysed per step, but additionally allows non-productive (uncoupled) steps. In the latter case, the motor enters a cycle of ATP hydrolysis that does not lead to movement of the enzyme (Figure 5D). Consequently, a coupling of > 1 ATP/bp is observed. If one allows the rate of the uncoupled cycle to be slower than the stepping rate, an apparent step size significantly larger than 1 can also be observed (Figure 6B).

Based on the data presented here, we cannot absolutely rule out any of these models, as, under particular conditions, all can return an apparent step size of ~ 2 bp. Although there is some value in attempting to discuss the plausibility of the different models, one has to keep in mind that our current knowledge about the dynamics and mechanisms of SF2 helicases, and helicases in general, is rather limited. Although there are now a large number of structural and biochemical studies of SF1 and SF2 helicases that provide strong support for a coupling ratio of 1 ATP/bp (Roman and Kowalczykowski, 1989; Dillingham et al., 2000; Soutelas and Wigley, 2001; Lee and Yang, 2006; Büttner et al., 2007, Hopfner and Michaelis, 2007), model-based step size determinations (Dessinges et al., 2004; Tomko et al., 2007) and single-molecule experiments resolving individual steps (Dumont et al., 2006; Myong et al., 2007) have provided evidence that are not in agreement with a simple uniform stepping model with a step size of 1 bp. Our study of dsDNA translocation also provides support for a non-uniform stepping mechanism. However, in addition to the previously suggested mechanisms, that is, the Spring-loaded and Clockwork Models (see above), we propose that one should also consider models in which uncoupling between hydrolysis and forward stepping is allowed.

From the examination of SF1 and SF2 structures, it has been proposed that helicases have evolved to use the same underlying inchworm-like motion of two RecA domains for motion on both ssDNA and dsDNA (Hopfner and Michaelis, 2007; Singleton et al., 2007). Given that the magnitude of domain motions between ATP-bound and nucleotide-free conformations has been determined to be ~ 0.34 nm (Büttner et al., 2007; Hopfner and Michaelis, 2007), we favour...
mechanisms where the smallest step taken on DNA is 1 bp. The 1:1 Uncoupled Model (Figure 5D) is the only one considered here that can also accommodate a coupling of >1 ATP/bp, as depicted in Figure 3C (even higher errors of up to 10% in the DNA concentration would still return a coupling >1). However, there is no reason why uncoupled ATPase activity could not occur within the context of a Spring-loaded and/or Clockwork mechanism. What could be the structural rationale for uncoupling? Comparison of processive and non-processive SF2 helicases has revealed that duplex unwinding is powered by very similar RecA domain motions in both systems (Sengoku et al., 2006; Büttner et al., 2007). The additional structural element that assists processive motion has been identified as a so-called ratchet domain, which clamps the nucleic acid template during forward movement (Büttner et al., 2007). Potentially, this clamping may not be 100% efficient, leading to occasional ATP binding and hydrolysis without forward motion. A definitive view of the stepping mechanism(s) of dsDNA translocases such as EcoR124I will probably be achieved only by simultaneously resolving the base-pair-sized motor steps and ATPase cycle in single-molecule experiments, where, in particular, the latter measurements are challenging.

Materials and methods

Preparation of proteins and DNA

EcoR124I was purified, reconstituted and tested as previously described (Stanley et al., 2006). For magnetic tweezers experiments, DNA substrates were prepared from p8Stap (Stanley et al. 2006) into which a 1.4 kbp PCR fragment from pSFVI (3780–5140 bp) carrying a single EcoR124I site was cloned between the XhoI and PciI sites of the plasmid. This provides a 9.7 kbp DNA construct with the EcoR124I site close to the centre of the molecule. For phosphate release experiments, Apul-linearized versions of single-site specific (pLK5; Stanley and Szczelkun, 2006) and non-specific (pTYB11; New England Biolabs) plasmids were prepared as described (Seidel et al., 2005). For the triplex assays, Apul-linearized versions of the single-site specific plasmids pMD543a, 44a, 45a, 46a and 47a were prepared as described (McClelland et al., 2005). Both this family of plasmids and pLK5 are based on the parental vector pTYB11 and show >98% sequence identity. DNA for the ensemble experiments was highly purified by density gradient centrifugation in CsCl-ethidium bromide (Vipond et al., 1995). DNA concentrations were measured by absorbance at 260 nm in a Perkin Elmer Lambda 14 spectrophotometer using an extinction coefficient for dsDNA of 0.02 ml/μg/cm (Sambrook and Russell, 2001). The specific DNA and non-specific DNA were checked by ethidium bromide fluorescence following agarose gel electrophoresis to ensure that the concentrations matched. The errors associated with the determination of DNA concentration are discussed in Supplementary data.

Phosphate release assay

Coumarin-labelled PBP was prepared, characterized, calibrated and tested according to Webb (2003) (Supplementary data; Supplementary Figures S1–S3). Rapid mixing fluorescence was performed using an SF61-DX2 stopped-flow fluorimeter (TgK Scientific Limited, Bradford-on-Avon, UK) with λex = 435 nm (~2 nm bandwidth) with a 455 nm band pass filter placed between the sample housing and photomultiplier tube (PMT). Temperature was maintained between 10 and 30°C by a waterbath connected to the chamber housing the syringes and flow cell. Pre-equilibrated samples in reaction buffer (50 mM Tris–HCl, pH 8.0, 10 mM MgCl2, 1 mM DTT) containing 1 nm linear DNA, 120 nM Mtase, 0–400 nM HsdR, 12 μM PBP, 30 mM 7-methylguanosine (Sigma) and 0.05–0.005 U/ml purine nucleoside phosphorylase (Sigma) were loaded into syringe ‘D’ of the SF61-DX2. Pre-equilibrated samples in reaction buffer containing 0–2400 μM ATP, 12 μM PBP, 30 mM 7-methylguanosine and 0.05–0.005 U/ml purine nucleoside phosphorylase were loaded into syringe ‘D’. (Mg2+ ions are required for both the ATPase (and thus translocate) and nuclease activities. The Mg2+ ion concentration was chosen to be saturating with respect to the in vitro nucleotide concentration, which parallels cellular conditions in Escherichia coli (Kuzminov, 1999).) Equal volumes of 50 μl of C and D were mixed and the progress of the reaction was monitored using default hardware settings. For the standard reactions, the final conditions were 0.5 nM linear DNA, 60 nM Mtase, 200 nM HsdR and 0–1200 μM ATP. The PMT response was calibrated using titration of a P1 standard according to Webb (2003) and was shown to be linear at all temperatures (Supplementary data; Supplementary Figure S2; Supplementary Table S1). The accuracy of the assay was validated by comparison to a radioactive ATPase assay (Supplementary Figure S3).
Triplex displacement assay
Triplex displacement assays were carried out in the SF61-DX2 stopped-flow fluorimeter as described (McClelland et al., 2005; Seidel et al., 2005). Temperature was maintained between 10 and 30°C by a water bath connected to the chamber housing the syringes and flow cell. Final reaction conditions were 1 mM linear DNA (0.5 nM EMBO J. 2004–2002), 60 nM MDase, 200 nM HsdR, 12 nM phosphocreatine, 10 U/mL creatine phosphokinase and 0–1200 μM ATP in reaction buffer. \( k_{\text{off}} \) values were determined from reactions at each of five different triplex spacings (484, 917, 1414, 2054 and 2774 bp).

Magnetic tweezers assays
Magnetic tweezers experiments have been carried out as previously described using M-280 dynabeads (Invitrogen) (Seidel et al., 2004). Enzyme reactions have been carried out in reaction buffer using 20 nM Mtase, 160 nM HsdR and a DNA stretching force of 3 ± 0.3 pN in all experiments. Under these conditions, the translocation rate is independent of the force (Seidel et al., 2004). The temperature during the measurements was varied by controlling the temperature of the surrounding room or by simultaneously controlling the temperature of the magnetic tweezers (Seidel et al. 2005; Stanley et al., 2006). Linear steady-state phases of the ATPase rate profiles were fitted to

\[
C_p = \frac{1}{k_{\text{ATP}}} t + t_{\text{init}}
\]

where \( C_p \) is the phosphate concentration, \( k_{\text{ATP}} \) is the macroscopic steady-state ATPase rate, \( t_{\text{init}} \) is the macroscopic initiation delay time and \( t \) is the reaction time. ATP dependence data were fit to a Michaelis–Menten relationship:

\[
V = \frac{V_{\text{max}}[\text{ATP}]}{K_{\text{ATP}} + [\text{ATP}]}
\]

Fits to equations (1) and (4) were not weighted to statistical or systematic errors (the statistical and systematic errors associated with the ensemble experiments are discussed in Supplementary data). Systematic errors associated with \( k_{\text{off}} \) were incorporated in the analysis of the average coupling ratio (see above and Figure 3C).

Single-molecule translocation events were analysed as described previously (Seidel et al., 2004). Segments of constant enzyme velocity were fitted with straight lines to extract translocation rates and processivities. For step size estimation by noise analysis, we recorded at each temperature typically 10–20 translocation events containing a segment of constant \( R_2 \) velocity of more than 6.7 s (or 400 data points at 60 Hz sampling rate). From each segment, the constant slope was subtracted and the resulting data were subdivided into 6.7 s subsegments that were slightly overlapping. For each subsegment, the power spectrum was calculated and all power spectra from all subsegments at a given temperature were averaged to reduce the statistical error. For power spectrum calculations, a Hanning window was applied, but similar results were obtained if no window was applied (data not shown). The averaged power spectrum was subsequently fit by equation (2) to extract the step size. Background noise was treated the same way by analysing segments where no enzymatic activity has been observed.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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