

Supplemental data for

Initiation and re-initiation of DNA unwinding by the *E. coli* Rep helicase

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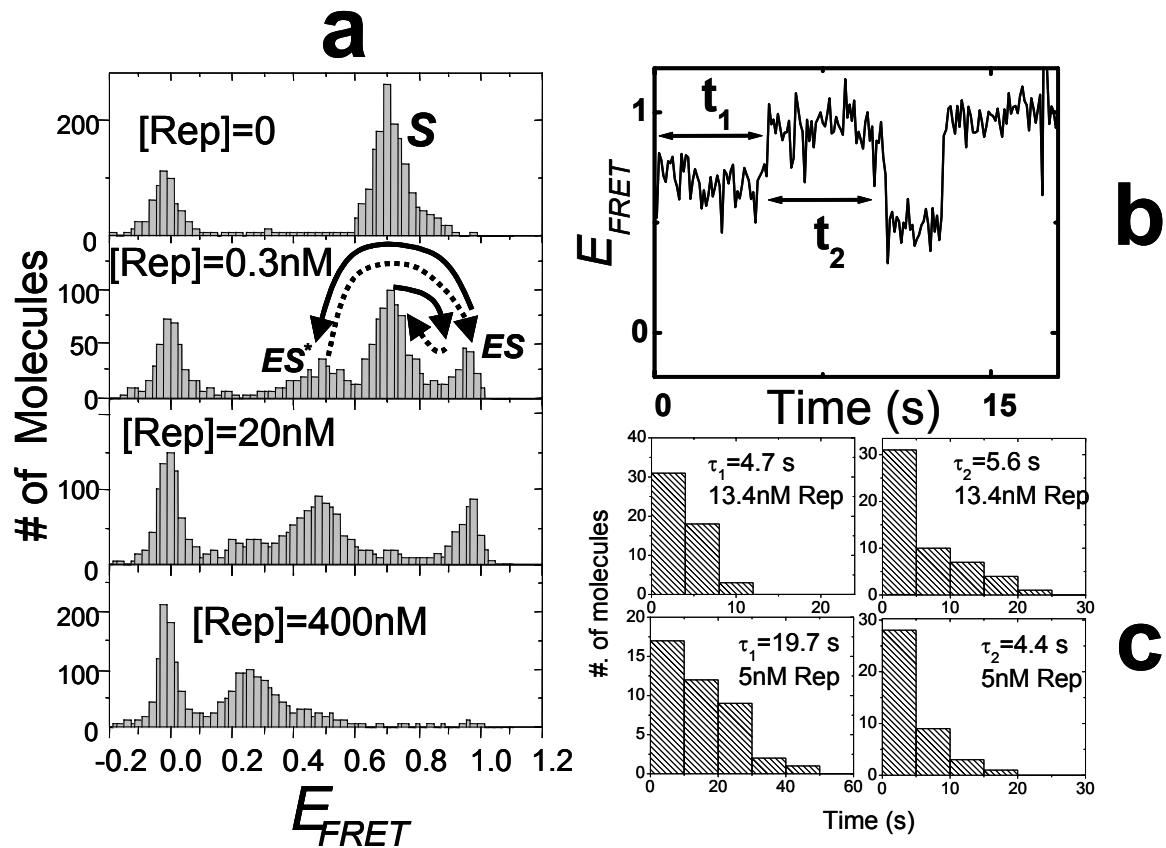


Figure 1. **a.** In the absence of ATP, E_{FRET} distributions of DNA III (Fig. 2) at various [Rep] showed at least *four* conformations. E_{FRET} was determined from each DNA by averaging the first second of observation. The peak at $E_{FRET}=0$ is due to inactive acceptor dyes and will not be discussed further. With no Rep in solution, the distribution is peaked at $E_{FRET}=0.7$, reflecting the DNA-only state (**S**). At sub-nanomolar [Rep], two new peaks appear at $E_{FRET} = 0.95$ and 0.5 (**ES** and **ES*** states, respectively) while the DNA-only peak decreases. The two conformations, **ES** and **ES***, persist up to 20nM Rep, whereas above this concentration, a new broad peak appears near $E_{FRET} = 0.25$ reflecting binding of two or more proteins. **b.** The E_{FRET} time trace upon addition of a Rep solution shows two abrupt transitions: At $t=0$, 13.4nM Rep was added. DNA starts from the **S** state and shows stepwise changes to the **ES** state, and then to the **ES*** state. From each trace,

the time spent in the **S** and **ES** states (t_1 and t_2) were determined and their histograms are shown in **c** for two different [Rep]. Average times (τ_1 and τ_2) are also shown. The first transition, from **S** to **ES**, is dependent on [Rep] and has a rate similar to that for Rep monomer binding to ss-DNA, and the second transition from **ES** to **ES***, occurs with a rate independent of [Rep]. Therefore we interpret the first step as Rep monomer binding and the second step as a conformational change of the monomer-DNA complex. This is further supported by a reciprocal experiment shown in Figure II. These two conformations were also observed for Rep binding to a DNA with a 5' tail instead of a 3' tail, indicating they do not result from direct interactions of protein with the 3'-ss/ds-DNA junction. Therefore, in the absence of ATP, Rep monomer binds to the DNA substrate with little specificity for the ss/ds/DNA junction. In order to test if dipole orientation effects contribute significantly to the observed FRET differences between the two states (**ES** and **ES***), we measured single molecule FRET and polarization anisotropy simultaneously using four channel scheme described by Cognet, Harms, Blab, Lommerse, and Schmidt, Applied Physics Letters 77, 4052-4054 (2000). Then, molecules were grouped into three categories based on E_{FRET} : **S**, **ES** and **ES***. Polarization anisotropy distributions of donor and acceptor for all three were identical, suggesting that the FRET differences are not likely due to dipole effects. Detailed study of the nature of conformations and their functional implications will be reported elsewhere.

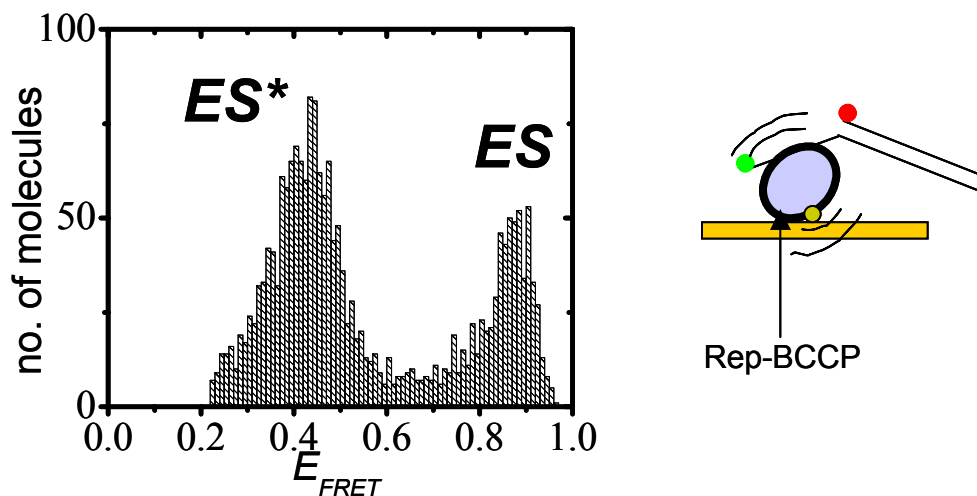


Figure II. In order to further test if the two conformations shown in Figure I is truly due to Rep monomer binding, we performed a reciprocal experiment. Rep-BCCP, expressed by pGG215, is biotinylated in vivo and consists of the 87 C-terminal domain of the biotin carboxyl carrier protein (BCCP) subunit of *E. coli* acetyl-CoA carboxylase fused to the C-terminus of the wild-type Rep protein. The plasmid pGG215 was constructed by the method of PCR overlap extension. The C-terminal portion of the Rep ORF was amplified using primers GHG31, GHG64 and pRepO. The BCCP ORF was amplified using primers GHG63, GHG65 and pEY2 (pEY2 was a gift from Jeff Gelles). The two PCR products were combined in a third PCR reaction using primers GHG31 and GHG65. The third PCR product was digested with *AccI* and *XhoI* and cloned into pGG213, generating pGG215. The portion of pGG215 generated by PCR was confirmed by sequencing. Primer sequences:

GHG31: 5'GGTGGCGGTGAGAGTGAAGAAGAGCTGGATCAGG

GHG63: 5'CGAGGGAAAATGGAAGCGCCAGCAGC

GHG64: 5'CGCTTCCATTTTCCCTCGTTTTGCCGCC

GHG65: 5'TCTAGAGTCCTCGAGTCATTCGATAACAACAA

Rep-BCCP was immobilized to Streptavidin coated PEG surface at 1nM concentration in Tris 20mM, NaCl 1M, MgCl₂ 1.7mM, glycerol 10%, pH 7.5 solution and extensively washed with the same buffer before the measurements. At this concentration, the average distance between proteins on the surface is estimated to be about 1 micron so the chance of having two next to each other is negligible unless dimer is already present in solution at 1nM concentration. The estimated concentration of Streptavidin on the surface is ~50,000 times higher than the Rep-BCCP concentration; hence it is unlikely that multiple Rep-BCCP would bind to the same Streptavidin. Our observation that Rep-BCCP requires 200nM concentration in order to obtain maximum unwinding amplitude in single-turnover unwinding kinetics experiments suggests that dimer of Rep-BCCP cannot be the dominant species at 1nM. Furthermore, we used buffer containing 1M NaCl during surface immobilization to minimize any potential residual interactions among Rep-BCCP monomers. E_{FRET} was determined similarly as in Figure I, when DNA III without biotin (100 pM) is bound to the Rep-BCCP *in the absence of ATP*(see cartoon). Control experiment without Rep-BCCP showed negligible binding of DNA. E_{FRET} histogram shows two peaks corresponding to **ES** and **ES*** state but no significant population of **S** is seen. E_{FRET} peak at zero due to inactive acceptors has been removed from the figure. Slightly different E_{FRET} values from those of Figure I are likely due to different set of dichroic mirrors used. This measurement further supports that the two conformations are due to two different conformations of Rep monomer/DNA complex, rather than due to multiple proteins. Detailed kinetic data for various DNA substrates and solution conditions will be reported elsewhere.

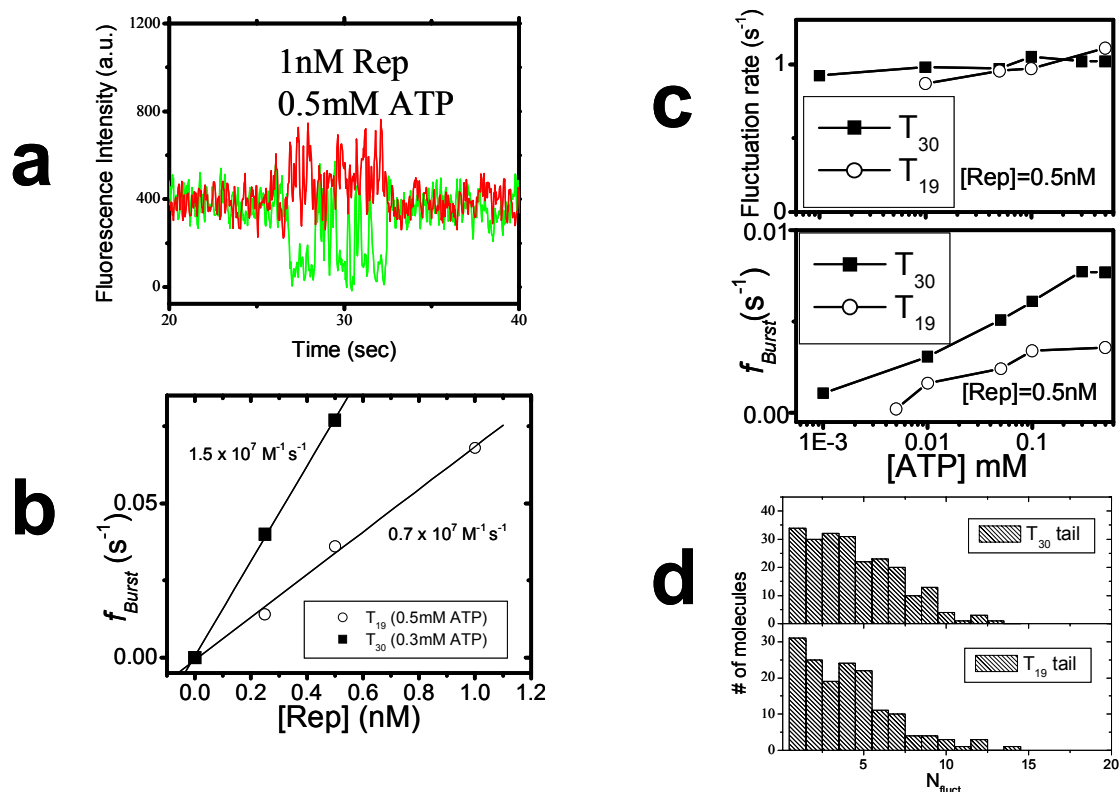


Figure III. From data of type shown in Figure 2(a), we obtained the average fluctuation rates and the burst frequency for various [Rep], [ATP] and two different tail lengths (T_{19} and T_{30}). **a.** Visual inspection of the raw data (donor and acceptor intensity time records, 33 ms bin time) was an effective means for this analysis because high FRET values signified by almost complete disappearance of the donor signal and the increase in acceptor signal is very easy to identify since no such fluctuations were seen in the absence of Rep. We may systematically underestimate the number of fluctuations in a burst due to the limited time resolution, but our main conclusions are based on comparisons between different [ATP], [Rep] and tail length so are likely to hold regardless of this possibility of underestimation. **b.** The burst frequency, f_{Burst} , was obtained by dividing the number of burst events by the observation time from at least 1,000 single molecule traces under each condition. f_{Burst} vs. [Rep] shows a linear dependence and the slopes are $0.7 \times 10^7 M^{-1} s^{-1}$ for T_{19} and $1.5 \times 10^7 M^{-1} s^{-1}$ for T_{30} . **c.** The fluctuation rate, defined as the average number of fluctuations per burst divided by the duration of the burst, depends on [ATP] only weakly, hence ATP binding is not rate-limiting for the fluctuations. f_{Burst} strongly depends on [ATP] consistent with the hypothesis that the burst occurs after ATP-dependent translocation to the junction. f_{Burst} is substantially higher if a DNA possessing a 3'- T_{30} tail is used rather than a 3'- T_{19} tail, likely due to a higher initial binding rate. **d.** Histograms of the number of fluctuations per burst (N_{fluct}) does not show a significant dependence on the tail length for T_{19} and T_{30} , suggesting that the fluctuations do not reflect the translocation process itself and occur after the translocation. N_{fluct} or the burst duration did not show dependence on [Rep] below 2nM. The burst duration is ~ 4 seconds, consistent with the Rep monomer dissociation rate of $\sim 0.24 s^{-1}$ in the presence of ATP measured in bulk solution. Therefore, it is likely that the burst is due to a Rep monomer binding event. Rep-BCCP data (Figure 2(b)) further supports this interpretation.

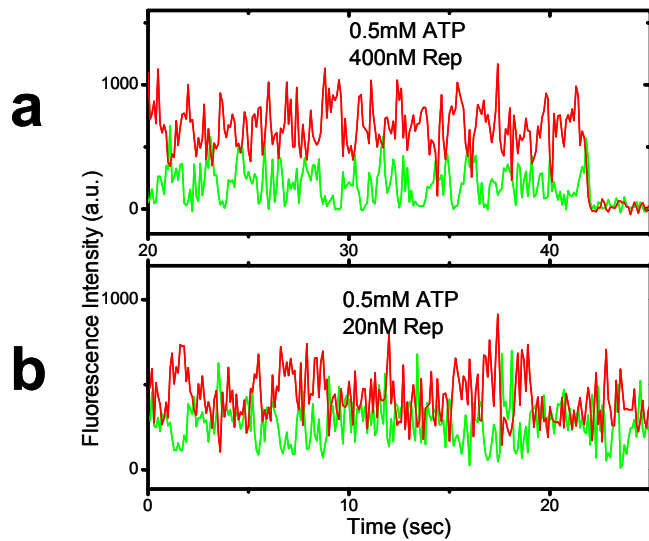


Figure IV. **a.** Continuous E_{FRET} fluctuations of DNA III at 400nM Rep and 0.5mM ATP (100 ms bin time). At $t=42$ s, unwinding occurs and fluorescence signal disappears. **b.** Similar E_{FRET} fluctuations of DNA III at 20nM Rep and 0.5mM ATP (100 ms bin time). Above 20nM Rep, the fluctuations become continuous indicating that the ss/dsDNA junction becomes saturated by Rep monomer.