

The rotation-coupled sliding of EcoRV

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ABSTRACT

It has been proposed that certain type II restriction enzymes (REs), such as EcoRV, track the helical pitch of DNA as they diffuse along DNA, a so-called rotation-coupled sliding. As of yet, there is no direct experimental observation of this phenomenon, but mounting indirect evidence gained from single-molecule imaging of RE-DNA complexes support the hypothesis. We address this issue by conjugating fluorescent labels of varying size (organic dyes, proteins and quantum dots) to EcoRV, and by fusing it to the engineered Rop protein scRM6. Single-molecule imaging of these modified EcoRVs sliding along DNA provides us with their linear diffusion constant (D_1), revealing a significant size dependency. To account for the dependence of D_1 on the size of the EcoRV label, we have developed four theoretical models describing different types of motion along DNA and find that our experimental results are best described by rotation-coupled sliding of the protein. The similarity of EcoRV to other type II REs and DNA binding proteins suggests that this type of motion could be widely preserved in other biological contexts.

INTRODUCTION

Proteins that interact with DNA are essential for maintenance and expression of the genetic information. For some of these proteins, it is crucial to rapidly find a recognition site amidst a large excess of competitor DNA. As direct binding to a recognition site is unlikely, the process of target site location begins with the formation of a non-specific protein-DNA complex, followed by protein translocation along the DNA to the specific binding site. Apart from ATP-dependent directional

motion, translocation can be achieved by random mechanisms, so-called facilitated diffusion (1), that include sliding, hopping and/or jumping, and by intersegment transfer (2–4).

In the past few years, high-resolution fluorescence microscopy techniques, down to the single-molecule level, have been developed to directly visualize the mechanisms involved in target site location (5–7). Using these techniques, the facilitated diffusion of a growing number of DNA binding proteins has been characterized, and the 1D diffusion constant (D_1) of proteins labeled with organic dyes (8), other proteins or quantum dot nanoparticles (QDs) (9) has been measured.

Restriction enzymes (REs) were among the first proteins whose facilitated diffusion was studied (10). These enzymes are part of restriction-modification systems in bacteria, and mainly defend the host organism against foreign DNA (11–14). EcoRV is one of the best studied RE that belongs to the Type II family, whose members do not require ATP for translocation along DNA (15). EcoRV searches for its target site by random 1D walks along non-specific DNA (sliding) and 3D translocations (hopping or jumping). This is in contrast to directional translocations performed by Type I REs (16) or RNA polymerases (17,18). Due to its important role in the defense of the host, EcoRV must reconcile two objectives: it must find its target site rapidly, but a careful probing of the sequence information presented in the major and minor groove of DNA is necessary to avoid missing the target. One way of accomplishing such a careful search is for EcoRV to follow the helical pitch of the DNA during its diffusion. So far, direct observation of such a rotation-coupled sliding has not been reported, certainly because of the speed and randomness of the subnanometer sized steps of the translocation. Despite lack of hard evidence, several arguments are in favor of this type of motion for EcoRV. A rotation-coupled motion would slow down the effective diffusion of the enzyme, which is consistent with value of D_1 observed for EcoRV (8).

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Indirect evidence that REs do not overlook recognition sites, and that they pause upon encountering ‘star’ sites (i.e. sites that differ in 1 bp from the canonical recognition sequence), also supports rotation-coupled sliding (19), as well as the dependence of D_1 on the protein size reported by Blainey *et al.* (20) who studied the diffusion of various DNA binding proteins and one RE.

Here, we employed a new approach to investigate a potential rotation-coupled motion of EcoRV as it slides along DNA. To modulate the effective size of EcoRV, we conjugated the enzyme to five fluorescent labels ranging from small organic dyes (radius, $r \sim 0.5$ nm) to large QDs ($r \sim 15.4$ nm) and we produced a variant of EcoRV fused to an engineered fluorescent Rop protein scRM6 (21). Using single-molecule imaging, we measured the diffusion constants of these modified EcoRV sliding on elongated DNA. To understand the observed motion of the enzymes, we compared our experimental results with theoretical diffusion constants predicted by four different models of EcoRV motion. These models consider a purely linear diffusion or a rotation-coupled diffusion of the enzyme with the fluorescent label attached to EcoRV by either a rigid or a flexible linker (22). The comparison between our data and the dependence of D_1 on the label size predicted by these models strongly supports a rotation-coupled sliding of EcoRV along the DNA helix.

MATERIALS AND METHODS

EcoRV purification and labeling

A single-cysteine, His₆-tagged EcoRV K58C variant was purified as described previously (23). The enzyme was biotinylated at the cysteine residue with a maleimide derivative [Maleimide-PEG₂ (or PEG₁₁)-Biotin, Pierce] by incubating it in a 1:1 ratio for 1 h at 4°C in PBS buffer. The biotin molecule was introduced through a flexible polyethylene glycol (PEG) linker of $n = 2$ or 11 U (PEG₂ or PEG₁₁), which provides flexibility between the protein and the label (Supplementary Data). Biotinylated EcoRV was incubated with commercial polymer QD-streptavidin conjugate (Qdot605 Streptavidin, Invitrogen) in a 1:1 ratio for 30 min at 4°C in PBS buffer. Then, a 4-fold excess of biocytin (Sigma Aldrich) was added to the reaction mixture, in order to block the unreacted streptavidin on the QD surface. The labeling of EcoRV with peptide-coated QDEO6 was achieved by interaction of the His₆-tag of EcoRV with the surface of QD nanoparticle (24). The enzyme and QDs were incubated at a 1:3 molar ratio for 30 min at 4°C in PBS buffer. The conjugation efficiency and stoichiometry of EcoRV and QDEO6 was confirmed using gel electrophoresis as previously described (24). As with the PEG linkers, the terminal His₆-tag provides flexibility (25,26) between the QD label and EcoRV (Supplementary Data).

In experiments using QD655 and savCy3 as fluorescent labels, we used the same K58C EcoRV variant biotinylated with the same Maleimide-PEG₂-Biotin (Pierce) derivative.

EcoRV-scRM6 fusion protein purification and labeling

The EcoRV-scRM6 fusion protein was created by fusing the single cysteine (sc) variant scRM6 D54C via its C-terminus to the N-terminus of the cysteine free variant of EcoRV. A modified gene coding for scRM6, in which eight native cysteines had been replaced by alanines (positions 33, 47, 90, 104, 147, 161, 204 and 218), was synthesized by GeneArt, and a single cysteine was introduced at position 54 in the first loop for fluorescent labeling. The genes coding for scRM6 D54C and cysteine-free EcoRV were consecutively introduced into the vector pET28a coding for an N-terminal His₆-tag. In this way, two scRM6 proteins were fused to the EcoRV homodimer, each extending the N-terminal α -helix of the EcoRV subunits, thereby forming a rigid connection. A noticeable influence of the scRM6 domain on the activity of EcoRV was not observed, which suggests that the specific binding and cleavage of the fusion construct is only slightly affected compared to wild-type EcoRV.

The single-cysteine EcoRV-scRM6 fusion protein was purified using Ni-NTA Agarose (Qiagen) similarly as described previously (27). The labeling with the Cy3B fluorophore (GE Healthcare) was achieved by incubating the fusion enzyme with Cy3B in a 1:10 molar ratio overnight at 4°C in 1 × PBS buffer. The free fluorophore and impurities from protein purification were removed using a HiTrap Heparin HP column (GE Healthcare) connected to the AKTApurifier (GE Healthcare). The fractions containing labeled enzyme were collected and dialyzed overnight against the storage buffer (30 mM K-phosphate pH 7.4, 5 mM DTT, 200 mM NaCl, 1 mM EDTA, 60% glycerol).

DNA preparation and stretching

DNA with biotinylated ends and without any recognition site for EcoRV was prepared as previously described (8). The DNA molecules were stretched to $\sim 70\%$ of their contour length and attached to a streptavidin functionalized coverslip by their ends, ensuring that most of a stretched DNA molecule was freely accessible in solution. Non-specific interactions between the enzymes, labels and residual streptavidin on the surface were prevented by incubation with casein blocking reagent (Roche Diagnostics) for 15 min. The measurements with EcoRV labeled with different QDs were performed at pH 7.4 using a 20-mM phosphate buffer containing 20 mM NaCl, 2 mM MgCl₂, 1 mM DTT and 0.1 mg/ml blocking reagent. The measurements with EcoRV-scRM6 fusion protein were performed at pH 7.4 using 1 × KGB buffer (100 mM κ -glutamate, 20 mM NaCl, 25 mM Tris-acetate pH 7.4, 10 mM Mg-acetate, 1 mM DTT and 0.1 mg/ml blocking reagent), as the DNA/EcoRV-scRM6 interaction time in phosphate buffer was too short for an accurate measurement of the diffusion constant.

Optical set-up

For detection of individual labeled enzymes, we used a TIRFM setup, previously described in detail (8). Briefly, the stretched DNA molecules were first stained with

SybrGold and visualized through a 480DF40 excitation filter and a 505-LP emission filter (Omega Optical) after excitation using a mercury lamp. Once the DNA was located in the field of view, SybrGold was removed using a buffer containing 20 mM MgCl₂. The labeled enzymes were then injected into the flow cell, and the flow was stopped. Labeled enzymes were excited using a laser at 532 nm (intensity ~ 100 W/cm²), visualized through a bandpass filter appropriate for the label, and imaged on an EMCCD camera (Ixon, Andor Technology) with an exposure time of 20 ms. To locate the proteins on the DNA, the point-spread function of the fluorescent spots was fit using a 2D Gaussian function in each frame of the image sequence, using a home-made software.

Data analysis

The diffusion constant D_1 of the labeled enzymes was derived from the mean square displacement (MSD) calculated from enzyme trajectories longer than 20 successive frames (28 trajectories for QD605-PEG₂-EcoRV, 24 trajectories for QD605-PEG₁₁-EcoRV, 27 trajectories for QDEO6-EcoRV and 45 trajectories for EcoRV-scRM6). The main error in the estimation of D_1 stems from the inaccuracy in determining the position of the DNA ends, and thus the DNA stretch rate. This rate is included in the calculation of the MSD for taking into account the effective translocation of the enzyme along the DNA. Assuming a localization accuracy of ~ 120 nm for the end-to-end distance (~ 2 μ m), we obtained $\Delta D/D \sim 0.15$, which was translated into error bars on the MSD plots (Figure 3).

RESULTS

First, we conjugated fluorescent labels varying in size by a factor of 30 (Figure 1A) to the K58C variant of EcoRV. We estimated the hydrodynamic radius (r_1) of the different labels using fluorescence correlation spectroscopy (FCS) (Supplementary Figure S1, Supplementary Table S1). The organic dye Cy3B ($r_1 \sim 0.5$ nm) was attached to EcoRV via a single cysteine residue. The fluorescent protein streptavidin-Cy3 (savCy3, $r_1 \sim 2.1$ nm), and commercial streptavidin/polymer coated QDs (QD605, $r_1 \sim 10.0$ nm; QD655, $r_1 \sim 15.4$ nm) were attached to EcoRV by biotinylating EcoRV at the same cysteine residue (C58). Smaller QDs (QDEO6, $r_1 \sim 7.2$ nm) than those available commercially were prepared with a coating of short hydrophilic peptides, and EcoRV was directly conjugated through its N-terminal His₆-tag to the nanoparticle surface (24).

Next, we fused two engineered Rop proteins (scRM6) to one EcoRV protein, each at the N-terminal α -helix of the two EcoRV subunits, and further labeled this fusion construct with Cy3B (Figure 2A). From the hydrodynamic radius of the fusion construct, measured using FCS (Supplementary Figure S2), we derived an effective radius $r_1 \sim 3.1$ nm for each of the scRM6 proteins.

We optimized an optical system to observe individual EcoRV interacting with DNA (8). These experiments were performed on a TIRF microscope with laser excitation,

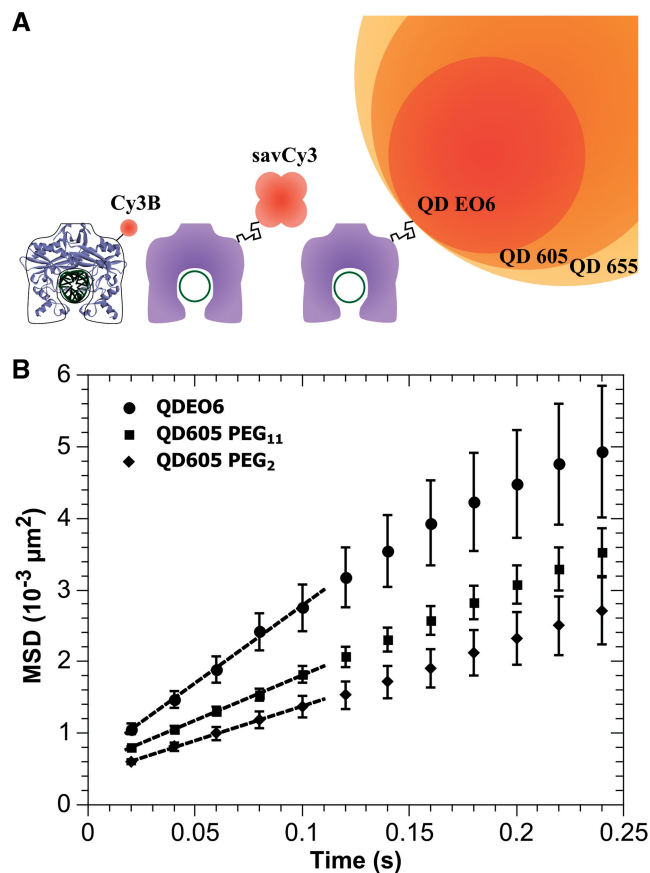


Figure 1. (A) A schematic display of different labels attached to EcoRV. The 2D representation of EcoRV and DNA is drawn around the crystal structure of the EcoRV/DNA complex (protein data bank 4rve). The labels are attached to the enzyme via flexible polyethylene glycol linkers (PEG₂ or PEG₁₁) at residue 58, or via the N-terminal polyhistidine His₆-tag (located in the vicinity of the position 58). Radii of different labels are: Cy3B ~ 0.5 nm, sav-Cy3 ~ 2.1 nm, QDEO6 ~ 7.2 nm, QD605 ~ 10 nm, QD655 ~ 15.4 nm. EcoRV and labels are drawn to scale. (B) The longitudinal MSD of EcoRV labeled with QDEO6 or QD605 via PEG linkers of different length. The MSD depends linearly on time, which shows that the QD labeled enzyme slides along the DNA. The linear diffusion constant D_1 is derived from the slope of the curve (dashed lines: linear fits on the first five points of the MSD) using the relation: slope = $2D$.

filters for detecting the labels and an EMCCD camera for imaging single-molecule events. The DNA was elongated on the surface of a glass coverslip using a custom flow cell and tethered via the biotin–streptavidin interaction. Based on this setup, we were able to directly observe individual EcoRV molecules sliding along DNA (Supplementary Figure S4). From the reconstructed trajectories of the enzymes, we calculated MSDs and derived D_1 for the modified EcoRVs.

From our measurements, we calculated the MSD of EcoRV labeled with QDs (Figure 1B) and fusion protein (Figure 2B). For QDEO6 labeled EcoRV, we derived $D_1 = (1.08 \pm 0.16) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$. Similarly, for QD605 labeled EcoRV via the PEG₂ linker, we found $D_1 = (0.48 \pm 0.07) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$. Substitution of the

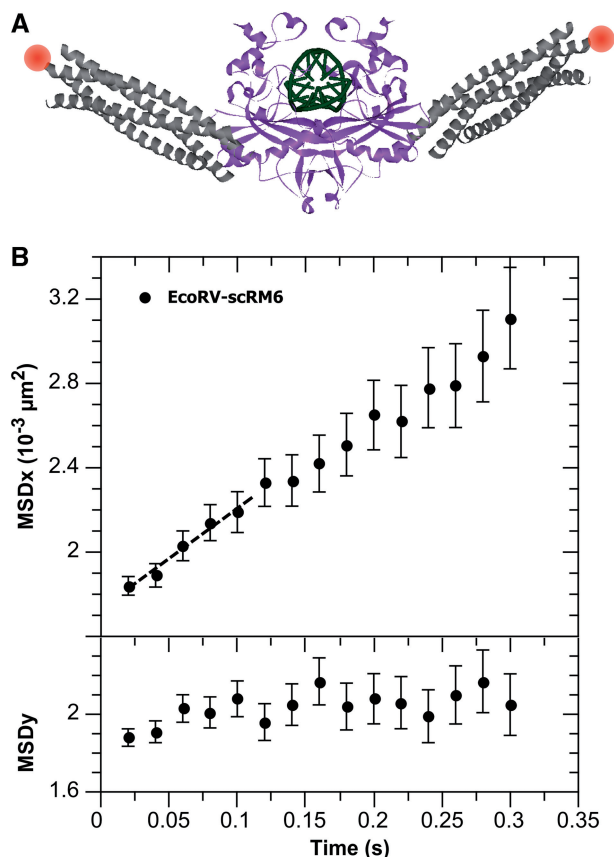


Figure 2. (A) One possible model of EcoRV (protein data bank 4rve) fused to scRM6 protein (protein data bank 1qx8), in which the structure of scRM6 protein is aligned with the N-terminal helix of EcoRV. EcoRV is presented in magenta, DNA in green, scRM6 protein in gray and the label in orange. (B) The longitudinal (along the DNA molecule) and transverse (perpendicular to the DNA molecule) MSD of EcoRV fused to the scRM6 protein labeled with Cy3B. The longitudinal MSD depends linearly on time, which shows that the fusion protein slides along the DNA, while, as expected, the transverse MSD is constant over time. The linear diffusion constant D is derived from the slope of the curve (dashed line: linear fit on the first five points of the MSD) using the relation: slope = $2D$.

PEG₂ linker with longer PEG₁₁ did not significantly alter the diffusion constant of QD605 labeled EcoRV [$D_1 = (0.63 \pm 0.07) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$]. The smallest diffusion constant, in the present series of experiments, was observed for the EcoRV fusion construct, for which we found $D_1 = (0.24 \pm 0.04) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$ (Figure 2B).

In previous studies, we determined the diffusion constant for EcoRV labeled with Cy3B (8), savCy3 (22) and QD655 (9). We found that D_1 was similar for EcoRV labeled with Cy3B [$D_1 = (1.1 \pm 0.2) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$] or savCy3 [$D_1 = (1.2 \pm 0.1) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$], but that D_1 decreased by a factor of three when EcoRV was labeled with QD655 [$D_1 = (0.32 \pm 0.02) \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$]. For the QD655 label, the same EcoRV diffusion constant was measured while the DNA was removed from the surface by optical tweezers (9), suggesting that the surface does not impede the protein diffusion.

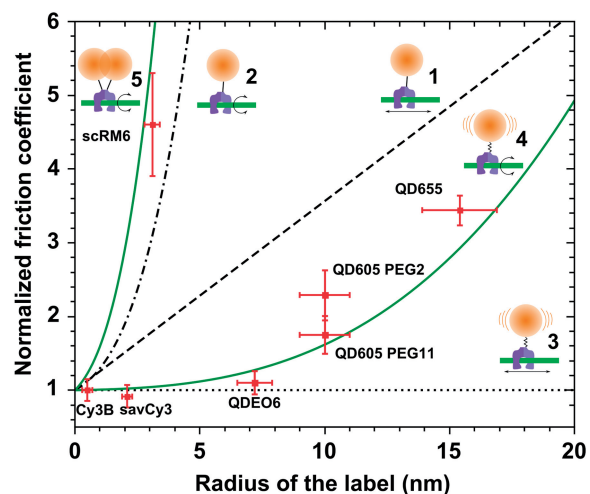


Figure 3. Dependence of the normalized friction coefficient $\xi(r_1)/\xi(0)$ of a single-labeled (Models 1–4) or double-labeled (Model 5) EcoRV on the radius r_1 of the label(s): (1) linear diffusion with a rigid linker, (2) rotational diffusion with a rigid linker, (3) linear diffusion with a flexible linker, (4) rotational diffusion with a flexible linker, (5) rotational diffusion with a rigid linker. EcoRV is presented schematically in magenta, DNA in green and the label in orange. Experimental normalized friction coefficients ξ (in red) are calculated using $\xi(r_1)/\xi(0) = D_{\text{Cy3B}}/D_1$, where D_{Cy3B} is the linear diffusion constant of EcoRV labeled with Cy3B, and D_1 is the linear diffusion constant of a protein variant with one or two label(s) of radius r_1 .

DISCUSSION

A direct observation of a RE rotating while sliding on DNA is a difficult task. Using even the most sophisticated cameras, a few milliseconds of exposure time is necessary to detect the light emitted by a single fluorophore. During this short time, the length over which a protein slides along the DNA is tens of nm (20 nm for EcoRV, with $D_1 \sim 10^{-2} \mu\text{m}^2 \text{s}^{-1}$ and an exposure time of 20 ms), meaning that the protein may circle around the DNA several times during the acquisition of a single image. This limitation makes directly observing rotational motion unrealistic. So until improvements to instrumentation and methodology allow for acquisitions in the microsecond range, an indirect strategy is necessary to study this phenomenon.

Attaching large labels to a protein sliding along the DNA is one of these strategies. By slowing down, the protein in its course, the label decreases the diffusion constant; this is experimentally accessible, and thus may help to find out if the protein rotates while sliding. To further assist in obtaining accurate diffusion constants, we used conditions of low ionic strength that helped achieve DNA–EcoRV interaction time in the range of a second. Although the ionic strength was lower than the physiological one, we do not expect the diffusion constant to depend on the salt concentration (9,28). In addition, at low salt conditions, hopping does not affect the diffusion constant (8); the effect of small jumps can be seen as an increase of the measured diffusion constant only at high salt conditions (>40 mM).

The interpretation of our experimental data required developing models accounting for different types of motion on the DNA. Here, we considered a framework developed by Bonnet *et al.* (22). We derive an effective friction coefficient ξ from the linear diffusion constant D_1 of the modified EcoRV using the Stokes–Einstein relation: $D_1 = k_B T / \xi$, where k_B is the Boltzmann constant and T the absolute temperature. Then, normalized friction coefficients for each of the six-labeled EcoRV enzymes are obtained by dividing ξ by the friction coefficient of EcoRV labeled with Cy3B, which is considered as the friction coefficient of an unlabeled enzyme as the radius of Cy3B is small compared to that of EcoRV. This allows us to compare the dependence of ξ on the label radius with the predictions of four different models.

The models we considered were: (i) linear diffusion of EcoRV along DNA, with a rigid linker between the label and the enzyme; (ii) rotational diffusion with a rigid linker; (iii) linear diffusion with a flexible linker between the label and the enzyme and (iv) rotational diffusion with a flexible linker (Figure 3).

In the first two models, the protein label complex is considered as a solid, as the linker is rigid. In this case, the friction coefficient ξ of the complex is given by $\xi = f \times (\xi_p + \xi_l)$, where ξ_p and ξ_l are the 3D friction coefficients of the protein and of the label, respectively, and $f > 1$ accounts for DNA–protein friction.

In the first model the labeled enzyme diffuses along the DNA without rotating, so ξ_p and ξ_l are translational friction coefficients (denoted as ξ_p^{lin} and ξ_l^{lin}). In this model, the friction coefficient $\xi_{\text{rigid}}^{\text{lin}}$ is given by the Stokes' law:

$$\xi_{\text{rigid}}^{\text{lin}}(r_1) = 6\pi\eta f_{\text{lin}}(r_p + r_1),$$

where η is the viscosity of the solution, and r_p is the hydrodynamic radius of EcoRV. Since $\xi(0) = 6\pi\eta f_{\text{lin}} r_p$ is the friction coefficient of an unlabeled enzyme, the previous equation becomes:

$$\xi_{\text{rigid}}^{\text{lin}}(r_1) = \xi(0) \left(1 + \frac{r_1}{r_p} \right).$$

As the size of the organic dye Cy3B is small compared to the size of EcoRV, $\xi(0)$ is also the friction coefficient of EcoRV labeled with Cy3B. Thus, the numerical value of $\xi(0)$ can be derived from the experimental value of the diffusion constant D_{Cy3B} of EcoRV labeled with Cy3B along DNA, using the Stokes–Einstein relation $\xi(0) = k_B T / D_{\text{Cy3B}}$. In the first model, the normalized friction coefficient $\xi_{\text{rigid}}^{\text{lin}}(r_1) / \xi(0)$ is thus expected to increase linearly with the label radius (Figure 3).

In the second model the EcoRV-label complex rotates while sliding, and the linker is rigid. The friction coefficients ξ_p and ξ_l include both translation and rotation. Yet, they can be equated to the rotational friction coefficients (denoted as ξ_p^{rot} and ξ_l^{rot}), as the rotational contribution to

friction is much larger than translational (29). The friction coefficient $\xi_{\text{rigid}}^{\text{rot}}$ is thus given by (22):

$$\xi_{\text{rigid}}^{\text{rot}}(r_1) = f_{\text{rot}} \left(\xi_p^{\text{rot}} + \xi_l^{\text{rot}} \right) = f_{\text{rot}} \left[8\pi\eta \left(\frac{2\pi}{h} \right)^2 r_p^3 + \left(\frac{2\pi}{h} \right)^2 \times (8\pi\eta r_1^3 + 6\pi\eta r_1(r_1 + r_p)^2) \right]$$

with $h = 3.4$ nm (one helix turn). The term ξ_p^{rot} is proportional to r_p^3 as we assume that the protein's center of mass is located on the DNA axis, as suggested by the crystallographic structure of the non-specific DNA–EcoRV complex (22). The term ξ_l^{rot} refers to the rotation-coupled diffusion of the label, the center of mass of which is supposed to be at a distance $r_p + r_1$ from the DNA axis (22). If $\xi(0)$ is the friction coefficient of the unlabeled enzyme, given by:

$$\xi(0) = f_{\text{rot}} \xi_p^{\text{rot}} = f_{\text{rot}} 8\pi\eta \left(\frac{2\pi}{h} \right)^2 r_p^3$$

then (22):

$$\xi_{\text{rigid}}^{\text{rot}}(r_1) = \xi(0) \left[1 + \left(\frac{r_1}{r_p} \right)^3 \left[1 + \frac{3}{4} \left(\frac{r_1 + r_p}{r_1} \right)^2 \right] \right]$$

and it can be seen that the normalized friction coefficient $\xi_{\text{rigid}}^{\text{rot}}(r_1) / \xi(0)$ of the complex strongly depends on the label radius r_1 (Figure 3). The value of $\xi(0)$ does not depend on the model, since it represents the friction coefficient of the unlabeled enzyme.

The Models 3 and 4 assume a flexible linker between the protein and the label. The Brownian motion of the label can thus be partially decoupled from that of the protein (22), and the friction coefficient ξ of the protein-label complex is given by $\xi = f \xi_p + \xi_l$. In this case, only the friction coefficient of the protein is influenced by the additional factor f because of the decoupling of the protein and label motions.

In the case of linear diffusion of the enzyme along DNA (Model 3), the friction coefficient $\xi_{\text{flexible}}^{\text{lin}}$ is given by the Stokes' law:

$$\xi_{\text{flexible}}^{\text{lin}}(r_1) = 6\pi\eta (f_{\text{lin}} r_p + r_1) = \xi(0) \left(1 + \frac{1}{f_{\text{lin}}} \frac{r_1}{r_p} \right),$$

with $\xi(0) = 6\pi\eta f_{\text{lin}} r_p$ as in the case of a rigid link. $\xi_{\text{flexible}}^{\text{lin}}(r_1)$ differs from $\xi_{\text{rigid}}^{\text{lin}}(r_1)$ because of the additional $1/f_{\text{lin}}$ factor, which must be evaluated for comparison with experimental data. As f_{lin} accounts for the additional friction due to the protein–DNA interaction, it is given by $f_{\text{lin}} = D_p^{3D} / D_{\text{Cy3B}}^{\text{DNA}}$, where $D_p^{3D} = k_B T / 6\pi\eta r_p$ is the 3D diffusion coefficient of EcoRV (22). Assuming $r_p = 3.9$ nm for EcoRV, obtained by FCS (8), and thus $D_p = 54 \mu\text{m}^2 \text{s}^{-1}$, one gets $f_{\text{lin}} \approx 5.3 \times 10^3$ (with $D_{\text{Cy3B}}^{\text{DNA}} = 1.1 \times 10^{-2} \mu\text{m}^2 \text{s}^{-1}$ the diffusion constant along DNA of EcoRV labeled with Cy3B). Thus, while $\xi_{\text{rigid}}^{\text{lin}}$ and $\xi_{\text{flexible}}^{\text{lin}}$ both depend linearly on r_p , the dependence of

$\xi_{\text{flexible}}^{\text{lin}}/\xi(0)$ is much weaker than that of $\xi_{\text{rigid}}^{\text{lin}}/\xi(0)$ (Figure 3).

If the EcoRV-label complex rotates while sliding, assuming a flexible linker (Model 4), the friction coefficient $\xi_{\text{flexible}}^{\text{rot}}$ is given by (22):

$$\xi_{\text{flexible}}^{\text{rot}}(r_1) = f_{\text{rot}} \xi_{\text{p}}^{\text{rot}} + \xi_1^{\text{rot}} = \xi(0) \left[1 + \frac{1}{f_{\text{rot}}} \left(\frac{r_1}{r_{\text{p}}} \right)^3 \left[1 + \frac{3}{4} \left(\frac{r_1 + r_{\text{p}}}{r_1} \right)^2 \right] \right],$$

with

$$\xi(0) = f_{\text{rot}} \xi_{\text{p}}^{\text{rot}} = f_{\text{rot}} 8\pi\eta \left(\frac{2\pi}{h} \right)^2 r_{\text{p}}^3$$

as in the case of a rigid link. Here again, $f_{\text{rot}} = D_{\text{p}}^{\text{rot}}/D_{\text{Cy3B}}$ must be evaluated. It differs from f_{lin} , as it now accounts for the rotation of the enzyme during sliding, so that $D_{\text{p}}^{\text{rot}} = k_{\text{B}}T/(8\pi\eta(2\pi/h)^2 r_{\text{p}}^3)$. Assuming $r_{\text{p}} = 3.9$ nm, one gets $f_{\text{rot}} \approx 74$. Since f_{rot} is much smaller than f_{lin} , the dependence of $\xi_{\text{flexible}}^{\text{rot}}/\xi(0)$ on r_1 is lower than that of $\xi_{\text{rigid}}^{\text{rot}}/\xi(0)$, though noticeable (Figure 3).

For the comparison of our experimental data with the predictions of the models, we distinguish between EcoRV labeled with savCy3 and QDs (via a PEG linker or a His₆-tag) and EcoRV fused to scRM6. When EcoRV is labeled with savCy3 or QDs, the linker can be considered as flexible (see Supplementary Data), and we expect the diffusion constant to be in agreement with the results predicted by the Model 3 or 4 depending on whether or not EcoRV rotates while sliding. For the fusion protein, the modified EcoRV can be considered as a rigid body: (i) the engineered Rop protein (21) is a homotetramer with a heptad sequence periodicity, and thus forms a stable coiled-coil structure, (ii) the two scRM6 proteins were fused to the EcoRV homodimer, each extending the N-terminal α -helix of the one EcoRV subunit and forming a rigid connection because the hydrogen bonds of the continuous α -helix are formed across the junction of the EcoRV N-terminal helix and the scRM6 helix. Thus, we expect the diffusion constant to be in agreement with the results of the Models 1 or 2, depending on the nature of the motion of the enzyme along DNA. In addition, two scRM6 are attached to a single EcoRV, whereas the models consider a single label attached to the enzyme. The models should therefore be amended accordingly.

Our measurements with EcoRV labeled with savCy3 or QDs are in excellent agreement with a model in which EcoRV rotates while diffusing along DNA (Model 4 in Figure 3). The remaining three models simply cannot account for the experimental data. In the case of EcoRV-QD605, we found that the friction coefficients were similar when the linker was either PEG₂ or PEG₁₁. This result confirms that sufficient flexibility for the framework of Bonnet *et al.* (22) to be valid can be achieved even with a 3 nm contour length linker (see Supplementary Data).

For quantitative interpretation of the experimental value of the diffusion constant of EcoRV fused to scRM6, we considered that the fusion protein can be modeled as EcoRV labeled with two spherical labels of radius r_1 rigidly attached to the enzyme (Supplementary

Figure S3), and we derived $r_1 \sim 3.1$ nm from an FCS measurement of the 3D diffusion coefficient of the EcoRV fusion protein (Supplementary Data). A direct comparison of D_1 with the results predicted by the models (Figure 3) cannot be made, as the models consider only one label attached to the enzyme. Yet the value of ξ (2 labels, r_1)/ $\xi(0)$ for a protein with two identical labels of radius r_1 can be easily deduced from the value of ξ (1 label, r_1)/ $\xi(0)$ as derived earlier (Supplementary Data):

$$\frac{\xi(2\text{labels}, r_1)}{\xi(0)} = 1 + 2 \left(\frac{\xi(1\text{label}, r_1)}{\xi(0)} - 1 \right)$$

Once again, we found that D_1 measured for EcoRV fused to scRM6 is in agreement with the model of EcoRV rotating along DNA assuming that scRM6 is rigidly attached to the enzyme (Figure 3), and that the remaining three models cannot account for the experimental data.

Other models could have been considered to account for our experimental data. For instance, it has been proposed that facilitated diffusion could imply a rotation-coupled diffusion alternating with a pure translational diffusion along the DNA (30). At least one additional parameter is needed in this model (e.g. the fraction of time spent sliding without rotating), which is not the case in our approach as adjustable parameters are not necessary to account for the data.

In summary, we used different conjugation strategies to prepare EcoRV REs with fluorescent labels of varying size, with rigid or flexible linkers. This enabled us to compare several models for the motion of EcoRV along DNA with experimental data obtained by single-molecule measurements. Our results show that the behavior of EcoRV can only be predicated by the model in which EcoRV diffuses along DNA following the pitch of the double helix, which provides further evidence for rotation-coupled sliding. We have also shown that not only the size of the label, but the flexibility of the linker must be considered. This may also be relevant when interpreting the diffusion parameters of biomolecules with large labels, like QDs, in other contexts, including the behavior of individual molecular motors or the transport and motion of proteins in living cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1; Supplementary Figures 1–4; Supplementary References (8,22,31–39).

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