The Motion of a Single Molecule, the λ-Receptor, in the Bacterial Outer Membrane

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ABSTRACT Using optical tweezers and single particle tracking, we have revealed the motion of a single protein, the λ-receptor, in the outer membrane of living *Escherichia coli* bacteria. We genetically modified the λ-receptor placing a biotin on an extracellular site of the receptor in vivo. The efficiency of this in vivo biotinylation is very low, thus enabling the attachment of a streptavidin-coated bead binding specifically to a single biotinylated λ-receptor. The bead was used as a handle for the optical tweezers and as a marker for the single particle tracking routine. We propose a model that allows extraction of the motion of the protein from measurements of the mobility of the bead-molecule complex; these results are equally applicable to analyze bead-protein complexes in other membrane systems. Within a domain of radius $\simeq$ 25 nm, the receptor diffuses with a diffusion constant of $(1.5 \pm 1.0) \times 10^{-9}$ cm^2/s and sits in a harmonic potential as if it were tethered by an elastic spring of spring constant of $\sim 1.0 \times 10^{-2}$ pN/nm to the bacterial membrane. The purpose of the protein motion might be to facilitate transport of maltodextrins through the outer bacterial membrane.

INTRODUCTION

The bacterial outer membrane is a complex structure containing proteins, lipids, carbohydrates, and the peptidoglycan layer. Diffusion in the bacterial outer membrane is crucial for a number of vital functions as, e.g., passive transport through pores. Little is known about the mobility of proteins in live bacterial outer membranes, but there have been several intriguing studies of the motion of proteins in the lipid membrane of eucaryotic cells. In Edidin et al. (1991), membrane proteins were labeled with gold particles and dragged with optical tweezers through lipid membranes. It was found that within the membrane, primarily on the cytoplasmic one-half, there exists dynamic barriers to lateral movement. Kusumi and coworkers (1993) have used single particle tracking (SPT) and fluorescence photo bleaching recovery to study how receptors move in the plasma membrane and have found four characteristic types of motion; one of these they denote as a "confined diffusion" mode where the molecule is confined within a compartment of diameter 300 to 600 nm and within which the diffusion coefficient is between 4.6×10^{-12} cm^2/s and 1×10^{-9} cm^2/s. In a later study (Sako and Kusumi, 1994), video-enhanced contrast optical microscopy is used to reveal the motion of single proteins embedded in the plasma membrane. They observe mainly confined diffusion with a confinement diameter of 500 to 700 nm and a diffusion constant of $\sim 10^{-9}$ cm^2/s. Also, the same authors (Sako et al., 1995) used laser tweezers to determine the motion of a protein in the plasma membrane and propose the "fenced versus tethered" models of membrane protein motion. Pralle et al. (2000) have identified how proteins embedded in lipid rafts move across the plasma membrane and find typical diffusion coefficients in the range 1 to 4×10^{-8} cm^2/s (dependent on the amount of cholesterol in the membrane); also, these authors provide an elegant way of measuring a local diffusion constant as the movement of the protein is restricted to a linear dimension of $\sim$100 nm. Suzuki et al. (2000) have dragged membrane-bound proteins across the plasma membrane using optical tweezers. This is a more "global" way of determining diffusion constants as the protein is dragged a long way compared with its size through parts of the membrane, which could be inhomogeneous. Doing this, diffusion constants in the range 1.5 to 13×10^{-11} cm^2/s are obtained (Suzuki et al., 2000). Also, Peters et al. (1999) have studied adhesion proteins by dragging them through the plasma membrane with optical tweezers. They find diffusion constants between 10^{-9} cm^2/s and 10^{-12} cm^2/s. Furthermore, they find that if the actin cytoskeleton is disrupted by pretreatment with cytochalasin D, the proteins appear to be harmonically attached to the plasma membrane with spring constants of $\sim 5 \times 10^{-3}$ pN/nm.

To our knowledge, there has never before been a single molecule study of mobility of a protein in the membrane of bacteria and, in particular, no previous studies of the mobility of the λ-receptor at the single molecule level. The λ-receptor, also called LamB or maltoporin, is a channel in the outer membrane of *Escherichia coli* responsible for transporting maltodextrins to the extracytoplasmic space or periplasm. Also, it is the surface receptor of the virus, bacteriophage λ. Our results reveal that the receptor performs a characteristic wiggling type of motion, and we have

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characterized this motion whereby we obtained information on the physical properties of the membrane. Furthermore, we gain information on how the protein is connected to the membrane; the protein appears to be sitting in a harmonic potential and as Gabay and Yasunaka (1980) showed that the λ-receptor binds to the peptidoglycan layer, it is likely that it is this binding that is observed and that has a hookian spring behavior. The λ-receptor also extends through the outer membrane layer of lipopolysaccarides and phospholipids. But as this layer is much softer than the peptidoglycan layer, the restriction of the λ-receptor is probably caused by its connection to the peptidoglycan layer. AFM measurements of the stiffness of an isolated hydrated peptidoglycan layer in a direction orthogonal to the layer have been reported (Yao et al., 1999), yielding an elastic modulus of 2.5×10^7 N/m^2. The square root of this elastic modulus is several orders of magnitude larger than the spring constants describing the motion of the λ-receptor within the plane of the bacterial membrane reported here.

MATERIALS AND METHODS

Bacterial assay

To ensure specific binding of the bead to the λ-receptor, we make use of a biotin-streptavidin binding, which is known to be very specific and sufficiently strong (Merkel et al. 1999) that a single bond is enough to attach the streptavidin coated bead to the biotinylated λ-receptor. A strain has been produced where the λ-receptor has been biotinylated in vivo: A biotin-acceptor site (Beckett et al., 1999) was introduced into the λ-receptor by insertion of oligonucleotides between the codons 157 and 158 of mutated *lamB* in pSB2267 (Brown, 1997). The host strain used in all experiments was S2188: F$^-$ *lacI^Q*Δ*lamB106 endA hsdR17 supE44 thi1 relA1 gyrA96* Δ*fimB-H::kan* (Brown, 1997), which lacks an intact gene encoding the λ-receptor. The sequence inserted including the flanking restriction sites was (Operan Technologies, Inc., Alameda, CA): CTGCAGGGTGGCCTGAACGACATCTTCGAAGCTCAGAAAATTGAATGGCACGAGGACCTCGAG.

This sequence was inserted to generate two independent clones, pLO15 and pLO16. To ensure retention of biological function we determined that the engineered proteins permitted fermentation of maltodextrins.

The bacteria were grown for 24 h at 37°C on YT agar (Miller, 1972) supplemented with 25 μg/ml chloramphenicol. A single colony was suspended in an M63 (Miller, 1972) medium containing 1 μg/ml B1, 25 μg/ml chloramphenicol, 0.1% casein hydrolysate, and 0.2% glycerol. The bacteria were grown in shaking water bath for 24 h at 37°C. Thereafter, the bacteria were diluted into fresh broth and grown at 37°C until they were in log-phase then grown for 1/2 h at 37°C in 0.1 mM isopropylthio-β-galactoside (IPTG) to induce the expression of the λ-receptor. One milliliter of this culture was centrifuged for 3 min at $1700 \times g$, and the bacteria were resuspended with buffer. The buffer used throughout the experiment was a KCl-potassium phosphate (10 mM potassium phosphate, 0.1 M KCl, pH 7) buffer with 0.2 mg/ml gelatin.

The beads were streptavidin-coated polystyrene beads from Bangs Laboratories, Inc. (Fishers, IN) with a diameter of 0.53 μm. To wash the beads they were suspended in millipore water for ~10 min and thereafter centrifuged at $1700 \times g$ for 10 min. They were resuspended in buffer and put in an ultrasonic bath for at least 15 min to dissociate agglomerates.

A perfusion chamber was made by attaching a clean coverslip coated with poly-L-lysine to a microscope slide by two pieces of double-sided tape. The chamber was washed twice with millipore water. Bacteria were incubated at room temperature in the perfusion chamber for 15 min, allowing them to adhere to the poly-L-lysine-coated coverslip. Heparin (12.5 μg/ml) was then perfused into the chamber and incubated at room temperature for 15 min. A layer of heparin passivates the charge on the poly-L-lysine thereby diminishing the attraction between the poly-L-lysine-coated coverslip and the streptavidin-coated beads. Subsequently, the chamber was washed with buffer four times. The washed streptavidin coated beads were added and allowed to incubate at room temperature for 15 min. The chamber was then washed with M63 media as described above with the only exception that it contained 0.2% glucose and no glycerol. It was washed until it appeared clear (after rinsing approximately five times) where after the chamber was sealed with valap and stored at 5°C until it was used within a few hours. Glucose was used in the final perfusion chamber to support anaerobic growth. We have observed that the number of moving coincident beads was increased by a factor of six when glucose was added. The residual movement, which was observed when glucose was not added to the bacteria, may be an intrinsic property of the λ-receptor or may be due to incomplete starvation of the bacteria. The increased diffusion observed in the presence of glucose is consistent with the measurements described by Ryter et al. (1975).

Attaching a bead to the biotinylated λ-receptor might alter its biological function. As the fraction of in vivo biotinylated receptors is very low, any result from probing the function of the λ-receptors in general would be dominated by the response from receptors with no bead attached. Therefore, we are not able to check the degree of retention of biological function of a λ-receptor with a bead attached to it.

Immunofluorescence experiments

Bacterial samples of both the bioclone (harboring pLO15 or pLO16) and the control (harboring pSB2267) were prepared as described in the Bacterial assay section but instead of mixing with streptavidin-coated beads, the bioclone and the control were both incubated for 10 min with a streptavidin-fluorescein isothiocyanate (FITC) conjugate allowing for an attachment of the fluorophore to the biotinylated receptors. Then, the bacteria were resuspended in M63 with glucose and put into perfusion chambers where they were immobilized by poly-L-lysin as previously described. Each streptavidin-FITC conjugate contains three to nine fluorophores (product no. S3762, Sigma, St. Louis, MO). The samples were investigated under a microscope equipped with a Hg lamp and a SONY XC-E150 charge-coupled device camera. The experiments were carried out without the addition of IPTG and for IPTG concentrations of 0.1 and 1 mM.

Optical tweezers

Our optical tweezers setup is based on a Nd:YVO_4 laser and is capable of measuring corresponding forces and distances in the picoNewton and nanometer regimes with a time resolution of microseconds using a quadrant photodiode system as detection method. This setup is described in detail in Oddershede et al. (2001), and our detection routine is basically similar to the one used by Pralle et al. (2000).

SPT

In the SPT analysis of the motion of the λ-receptor, bright field microscopy images of a bead attached to the protein were saved on S-VHS using a NI-IMAQ utility integrated in LabView. The charge-coupled device camera was a Sony XC-75CE with 752×582 pixels. The pixel resolution was 50 to 60 nm/pixel, depending on the exact distance between the camera and the sample. We used the method of finding the position of the bead attached to the protein described by Gelles et al. (1988): In one particular frame, a subregion containing only the bead is stored. This is called a mask. For each image, the cross-correlation matrix of the mask and the image of

interest is calculated and normalized to have values in the range [−1, 1]. The cross-correlation matrix has high values where the image resembles the mask and the position of the bead in the image will be seen as a prominent peak in the cross-correlation matrix. A threshold-value, $t = 0.5$, is subtracted from all entries in the cross-correlation matrix, and the *x*- and *y*-coordinates of the bead are then found as the "center of mass" of the positive entries of the cross-correlation matrix. The same mask and threshold are used during the analysis of all images in the recording. Within the field of view is also included a still object, typically a bead stuck to the cover glass. The coordinates of this still object are subtracted from the (moving) object of interest to eliminate drift of the sample, which can be substantial during measurements of up to 15 min.

Measuring the relative distance between two stationary beads stuck to the cover glass of the specimen gave a standard deviation of 7 nm. This is interpreted as the spatial resolution of our SPT routine. The time resolution is 25 Hz determined by the frame rate of the charge-coupled device camera.

A BEAD IN AN OPTICAL TRAP

If a bead is held in an optical trap, its motion in one dimension x is well described by the Langevin equation:

$$m\ddot{x} = -\kappa x - \gamma\dot{x} + F(T) \tag{1}$$

in which κ is the stiffness of the optical trap, γ is the friction of the surrounding liquid on the bead, and $F(T)$ is the stochastic force resulting from the Brownian motion of the liquid molecules at temperature T. As inertial forces can be neglected, the left side can be set to zero. The motion of the bead in the liquid can be treated as a simple Stokes flow with friction coefficient $\gamma = 6\pi\eta r$, r being the radius of the bead and η the viscosity of the liquid.

From Eq. 1 the power spectrum of the position x is found to be Lorentzian (Gittes and Schmidt, 1998):

$$P_x(f) = \frac{k_B T}{\gamma\pi^2(f^2 + f_c^2)} \tag{2}$$

with a corner frequency $f_c = \kappa/2\pi\gamma$ from which κ can be found.

The optical tweezers constitute a harmonic potential for the trapped bead, as demonstrated in Simmons et al. (1996). Thus, the distribution of the bead's position in the trap is a Gaussian distribution: In thermal equilibrium, the distribution of positions $p(x)$ is

$$p(x)dx \propto \exp\left(-\frac{U(x)}{k_B T}\right)dx, \tag{3}$$

in which $U(x)$ is the potential felt by the bead. Thus, a Gaussian distribution corresponds to a harmonic potential, $U(x) = (1/2)\kappa x^2$, provided the measurements are made over sufficiently long time. This result applies for both the temporal resolution of the optical tweezers and for that of our SPT routine. The width of the distribution σ is related to κ by:

$$\sigma^2 = \frac{k_B T}{\kappa}. \tag{4}$$

There are two independent ways of determining σ: 1) from the power spectrum of $x(t)$ (Eq. 2) f_c, and hence κ is found and used in Eq. 3 to find σ in meters. 2) The quadrant photodiode measures position in volts. From the histogram of these positions, σ is found in volts. These two values of σ can be compared to give a conversion factor between volts and meters describing the output from the photodiode (e.g., see Oddershede et al., 2001). To find the conversion factor and κ the optical trap is always calibrated with a bead unattached to a bacterium at the relevant height above the coverslip in every sample and for every laser intensity used.

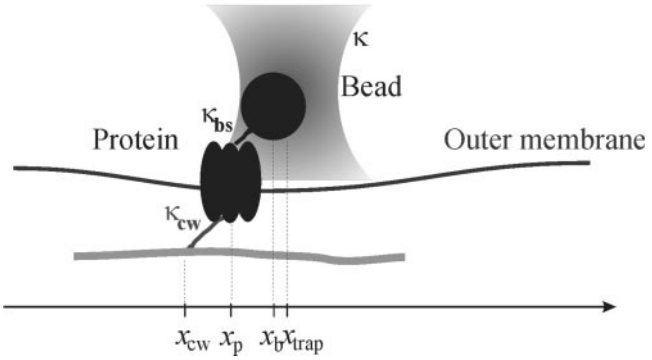

FIGURE 1 Sketch of the one-dimensional model on which the data analysis is based. The protein is located in the outer membrane and attached to the cell wall by a spring of spring constant κ_{cw}. Furthermore, it is attached to the bead by a spring of spring constant κ_{bs}, and the bead is trapped by the optical tweezers, described by a spring constant κ. The position of the bead is x_b, and that of the protein is x_p. The equilibrium position of the optical trap is at x_{trap}, and the equilibrium position of the attachment between protein and cell wall is at x_{cw}. The components in this figure are not drawn to scale.

MODELING THE SYSTEM

The purpose of this model of the motion of the bead-protein complex is to extract a series of parameters describing the biological system: a value for the friction coefficient describing the friction, which the protein feels in the bacterial membrane, γ_p, and from this quantity the diffusion coefficient of the protein in the membrane, D. As will be shown in the Results section, we have experimental evidence that the protein feels a harmonic potential from the cell wall. To describe this interaction, we extract a value for the apparent spring constant of the attachment to the cell wall, κ_{cw}.

Consider a one-dimensional model of the motion of the protein in the bacterial membrane. The protein is attached to a dielectric bead, and the protein is embedded in and somehow attached to the bacterial membrane (Fig. 1). We treat

the coupling between bead and protein through the biotin-streptavidin binding as a stiff hookian spring with spring constant κ_{bs}. Also, the attachment of the protein to the cell wall is treated as a hookian spring with spring constant κ_{cw}, which we assume frequency independent as discussed in the Results section. Finally, the coupling between bead and optical trap is treated as a spring with spring constant κ. The motion of the protein in the bacterial membrane is described as a frictional motion with friction coefficient γ_p.

The above assumptions and definitions lead to the following set of equations of motion for the bead with spatial coordinate x_b and for the protein with spatial coordinate x_p:

$$M_b\ddot{x}_b = -\kappa(x_b - x_{trap}) + \kappa_{bs}(x_p - x_b) - \gamma_b\dot{x}_b + F_b \quad (5)$$

$$M_p\ddot{x}_p = -\kappa_{cw}(x_p - x_{cw}) - \kappa_{bs}(x_p - x_b) - \gamma_p\dot{x}_p + F_p \quad (6)$$

Here, M_b and M_p are the masses of bead and protein, x_{trap} is the equilibrium position of the optical trap, and x_{cw} is the equilibrium position of the attachment of the protein to the cell wall. F_b and F_p are stochastic, time-dependent forces resulting in Brownian motion of the bead and the protein, respectively. We assume that F_b and F_p can be represented by white noise terms, i.e., they have vanishing mean and are δ-correlated. As both the protein and the bead have very small Reynolds numbers, we can neglect the inertial term in each of Eqs. 4 and 5, making the left side vanish.

In the experiments, the position of the bead, x_b, is observed, and from this we wish to extract information on how the protein moves in the bacterial membrane. To obtain the power spectrum of the bead position, Eqs. 6 and 7 are Fourier transformed:

$$0 = -\kappa\tilde{x}_b + \kappa_{bs}(\tilde{x}_p - \tilde{x}_b) + i2\pi f\gamma_b\tilde{x}_b + \tilde{F}_b \quad (7)$$

$$0 = -\kappa_{cw}\tilde{x}_p - \kappa_{bs}(\tilde{x}_p - \tilde{x}_b) + i2\pi f\gamma_p\tilde{x}_p + \tilde{F}_p \quad (8)$$

in which, e.g., the Fourier transformed of the position of the bead, x_b, is denoted $\tilde{x}_b$. Note that the unknown constants, x_{trap} and x_{cw}, disappear from the expressions.

It is now straightforward to deduce the power spectrum of the position of the bead, $P_b(f) = \langle|\tilde{x}_b(f)|^2\rangle$. The result is a fraction between a second and a fourth order polynomium in f, which simplifies considerably if we assume that the spring connecting the bead and the protein, of spring constant κ_{bs}, is much stiffer than the other two springs, of spring constants κ_{cw} and κ. A crude estimate for the value of κ_{bs} can be obtained: the association constant, K_a, for the streptavidin-biotin binding is roughly $K_a = 10^{15}\ M^{-1}$ (stated in, e.g., Livnah et al., 1993) from which we find the height of the energy barrier as $\Delta E = k_BT \ln K_a \simeq 35\ k_BT$. Furthermore, we note that the extension of the inner barrier, x_{barr}, in the interaction potential is roughly 5 Å (Merkel et al., 1999; Grubmüller et al., 1996). If we approximate the interaction potential as being harmonic out to the position of this inner barrier, we may estimate the spring constant κ_{bs} based on $\Delta E \simeq \frac{1}{2}\kappa_{bs}x_{barr}^2$ providing the desired value: $\kappa_{bs} \simeq 1 \times 10^3$ pN/nm.

Because a typical spring constant of the optical trap is considerably less, on the order of $10^{-4} - 10^{-2}$ pN/nm, and we find that $\kappa_{cw} \simeq 10^{-2}$ pN/nm (see Results), we assume that $\kappa_{bs} \gg \kappa, \kappa_{cw}$. In that case, and in the relevant frequency range ($f \ll \kappa_{bs}/2\pi\gamma_p \simeq 10^7$ Hz) the power spectrum reduces to

$$P_b(f) \simeq \frac{k_BT}{\pi(\gamma_p + \gamma_b)(f^2 + f_{c,\lambda}^2)} \quad (9)$$

with corner frequency

$$f_{c,\lambda} = \frac{\kappa + \kappa_{cw}}{2\pi(\gamma_p + \gamma_b)}. \quad (10)$$

This result for the power spectrum can be compared with that of a bead alone (Eq. 2) to realize that the protein-bead complex has a power spectrum as if it were a single object moving in a liquid with a friction coefficient $\gamma_p + \gamma_b$, held in a harmonic potential of spring constant $\kappa_{cw} + \kappa$, and with a corner frequency equal to $f_{c,}\lambda$.

The power spectrum of the position, Eq. 9, of the bead contains two unknowns, κ_{cw} and γ_p. To determine κ_{cw}, we consider the position histogram of the bead on the protein. Experimentally, we find that this histogram fits well to a Gaussian distribution (Results section, see Fig. 3), which implies that the bead attached to the protein feels a harmonic potential. The spring constant of this harmonic potential, κ_{tot}, is simply the sum: $\kappa_{tot} = \kappa + \kappa_{cw}$. The width of the distribution can be determined directly from the measured positions as $\sigma_\lambda^2 = \langle x_b^2\rangle - \langle x_b\rangle^2$ from which κ_{cw} can be extracted because:

$$\sigma_\lambda^2 = \frac{k_BT}{\kappa + \kappa_{cw}}. \quad (11)$$

When κ_{cw} has been determined, γ_p can be extracted by fitting Eq. 10 to the experimental data. Finally, the diffusion constant of the protein in the bacterial membrane, D can be found:

$$D = k_BT/\gamma_p. \quad (12)$$

Depending on the size of the bead and the precision obtained in the experiments, it can be quite important to account for the motion of the bead when extracting physical quantities describing the motion of the protein. The larger the bead and the larger the diffusion constant, the larger the influence of the bead on the total friction coefficient $\gamma_{tot} = \gamma_p + \gamma_b$. Thus, for small diffusion coefficients and small beads, as in the work of Sako and Kusumi (1994), we found the effect of the bead to be smaller than 1%, whereas for the largest diffusion coefficients measured in later work of the same authors (Sako and Kusumi, 1995), we estimated the effect to be roughly 33%.

For further modeling we approximate the motion of the λ-receptor to take place in two dimensions. This is justified because the λ-receptor stays within a region of diameter of ~50 nm, and the dimensions of the bacterium are ~2000 × 500 nm.

The orientation of the bacterium in a given measurement was random relative to the coordinate system defined by the detection method. We have transformed this random coordinate system into the bacterial coordinate system and into principal axes. When transforming into principal axes the maximal deviation of the measured quantities in two orthogonal directions is found. Sometimes, this gave a difference of up to a factor of two in, e.g., diffusion coefficients D, and the values of D_x and D_y thus seemed to depend upon the orientation of the coordinate system in which they were measured. When the direction of the principal axes were compared with the coordinate system of the bacteria, however, no correlation was observed, and thereby we have no evidence for any preferred direction of the anisotropy with respect to bacterial axes.

In the forthcoming, the x and y directions are random with respect to bacterial and principal axes. In the lack of a natural coordinate system, the results we present for κ_{cw}, σ, and diffusion constant D are defined as to be independent of choice of coordinate system. This is accomplished by noting that $\langle (r - r_0)^2 \rangle = \langle (x(t) - x_0)^2 \rangle + \langle (y(t) - y_0)^2 \rangle$ is independent of the orientation and origin of the particular coordinate system used. For a particle diffusing in two dimensions we have:

$$\langle (r(t) - r(0))^2 \rangle = 4Dt = 2D_x t + 2D_y t, \qquad (13)$$

making D a simple average: $D = \frac{1}{2}(D_x + D_y)$. Following the relation between D and γ (Eq. 11), a generalized friction coefficient, γ_p can be defined:

$$\frac{2}{\gamma_p} = \frac{1}{\gamma_{px}} + \frac{1}{\gamma_{py}}. \qquad (14)$$

Similarly, κ_{cw} can be defined as:

$$\frac{2}{\kappa_{cw}} = \frac{1}{\kappa_{cwx}} + \frac{1}{\kappa_{cwy}}. \qquad (15)$$

The definitions of γ and κ_{cw} share the property that if the values of the quantities in the two directions are identical, then the generalized value is also equal to this number. For the above definitions and Eq. 12 to hold in two dimensions and to get a value for σ_λ, which is independent of choice of coordinate system, we have chosen to define σ_λ as:

$$\sigma_\lambda = \frac{1}{\sqrt{2}} \sqrt{\sigma_x^2 + \sigma_y^2}. \qquad (16)$$

RESULTS

Our first results concern the efficiency of the in vivo biotinylation described in Materials and Methods. To avoid having more than a single receptor bound to each streptavidin coated bead, the biotinylation efficiency of secreted proteins must be very low. Reed and Cronan (1991) show that only a small fraction of the exported proteins were biotinylated due to the rapid kinetics of protein export. Jander et al. (1996) observe that the efficiency of the in vivo biotinylation of secreted proteins is as rare as to be below their limit of detection. Therefore, we expected the fraction of biotinylated λ-receptors to be very low and verified this prediction in the following manner. With IPTG, we controlled the number of expressed receptors, and if no IPTG was used we saw no increase in the number of streptavidin coated beads attaching to the bacteria harboring pLO15 or pLO16 compared with the number of beads attaching unspecifically to the controls harboring pSB2267. This absence of attached beads must have been due to inefficient biotinylation because the bacteria had λ-receptors in their outer membranes as they were able to transport and ferment maltodextrins. For the concentration of IPTG used in our experiments, the number of coincident beads and bacteria was four times larger for the bacteria expressing the modified λ-receptor than for the controls. Also, the beads associated with the bacteria expressing the modified λ-receptor showed a motion that was qualitatively different from those associated with the controls. They performed a clearly visible and characteristic "wiggling" motion. The frequency of beads performing wiggling motion on bacteria was ~20-fold higher with bacteria harboring pLO15 or pLO16 than with those harboring pSB2267. Using the optical tweezers to monitor the motion of one of the rare beads, which seemed to move on the controls, we saw a power spectrum that was not fitted well by a Lorentzian function and therefore was not the same signal as from the motion of a bead moving on bacteria expressing the modified λ-receptor. This fact ensures that what we are observing is indeed a bead on a moving, engineered λ-receptor and not just the motion of the bead attached to something residing on the cell surface (as a "balloon on a stick"). After induction with IPTG, the average number of diffusing beads on each engineered bacteria is only 0.27, supporting our conclusion that the efficiency of biotinylation is very low.

As a further check on the efficiency of the biotinylation, we measured the frequency of fluorescent bacteria after adding fluorescine-labeled streptavidin (Materials and Methods). As we are not equipped for single molecule fluorescence spectroscopy, we cannot determine how many fluorophores a particular signal originates from, and we might not be able to detect the signal from a single fluorophore. To test our equipment, we prepared a sample with a dilution of the streptavidin-FITC conjugate to a degree where we expected to have ~10 fluorophores within the field of view of our microscope objective. The number of observed bright areas are approximately in accordance with the expected number, which suggests that we are able to observe the signal from a single streptavidin-FITC conju-

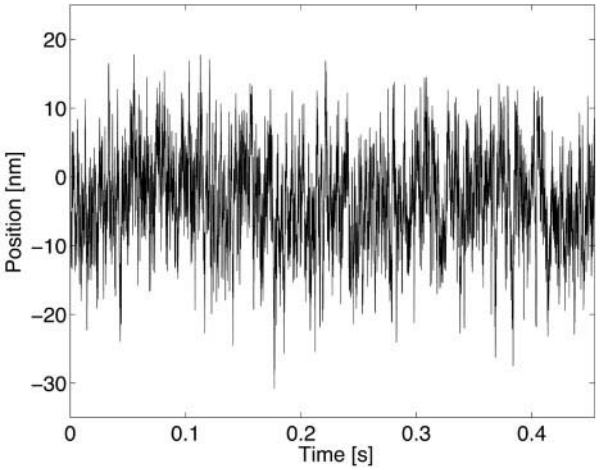

FIGURE 2 Position of a bead on a λ-receptor versus time as detected by optical tweezers. The optical tweezers serve both to trap the bead and as a detection method in conjunction with a quadrant photodiode.

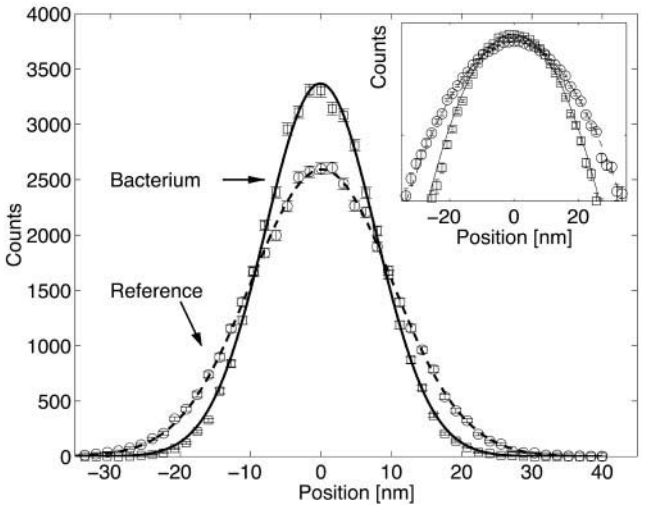

FIGURE 3 Position histogram of a bead on λ-receptor (*squares*) and of a bead unattached to a bacterium (*circles*) found by optical tweezers. The data from the unattached bead are referred to as "reference" on the figure and are needed for calibration purposes. The lines are Gaussian fits to the data (*full line*, bead on λ-receptor; *dashed line*, unattached bead). From these fits the standard deviation in one dimension for the bead on the bacterium is found to be $\sigma_\lambda = 7.8$ nm and for the unattached bead $\sigma = 10.1$ nm. The inset shows the same data on semilogarithmic axes.

gate. We have detected that some bacteria fluoresce weakly, whereas others do not and we can compare the detected fluorescence among the different samples. The numbers of fluorescent bacteria from the bioclones having undergone the in vivo biotinylation was, respectively, 5%, 12%, and 31% for IPTG concentrations of 0, 0.1, and 1 mM. These values correlate well with our measurements of binding streptavidin-coated beads. Strains harboring pSB2267 failed to fluoresce. Using the streptavidin-coated bead as a marker, there were on average 0.27 beads per bacteria for [IPTG] = 0.1 mM, which is the concentration used in the reported experiments. This should be compared with the 12% of the in vivo biotinylated bacteria that appear to be fluorescently marked. The reason that the beads appear to be more efficient markers for the biotinylation frequency might be that we are able to exactly count the number of beads on a bacteria, and there might be one, two, or even occasionally three beads wiggling on a single bacterium, but we are not able to determine the number of streptavidin-FITC conjugates on each bacterium. Alltogether, the immunofluorescence data support very low efficiency of the in vivo biotinylation, which is also suggested from our experiments using the beads as markers and reported in literature (Reed and Cronan, 1991; Jander et al., 1996).

With the optical tweezers setup, the position of the bead on the λ-receptor versus time is measured. An example of such a measurement is shown in Fig. 2. The duration of a typical optical tweezers measurement is on the order of seconds, and the sampling frequency was 22,000 Hz giving a temporal resolution of 46 μs for this particular measurement. The sampling frequency could easily be chosen higher if needed. Histograms as shown in Fig. 3 are obtained by binning the position data. The narrower position histogram is from a bead attached to a λ-receptor (position data of which are shown in Fig. 2), and the wider histogram stems from that of a bead unattacted to a bacterium from the same sample in an optical trap. The inset shows the same data on semilogarithmic axes. The lines in Fig. 3 are Gaussian fits to data. Fig. 3 confirms that a single bead in an optical trap experiences a harmonic potential and furthermore shows the intriguing fact that a bead bound to a λ-receptor also has a Gaussian position distribution, indicating that it, too, sits in a harmonic potential. This observation led to the assumptions behind Eq. 13 in the model section.

Fig. 4 shows the power spectrum of the position data both for a bead unattached to a bacterium (full thin line) in the optical tweezers and from a bead attached to a λ-receptor in the optical trap (dashed thin line). Both data sets are fitted with Lorentzian functions according to Eqs. 14 (full thick line) and 15 (dashed thick line). Attaching the bead to the bacterial system lowers the corner frequency, $f_{c,\lambda} < f_c$, and also shifts the horizontal asymptotic behavior at low frequencies. In the model leading to Eqs. 16 and 17, κ_{cw} is assumed to be a constant, however, it might be frequency dependent. Gittes et al. (1997) and Helfer et al. (2001) reported on the frequency-dependent viscoelasticity of some soft materials giving rise to a deviation from normal Brownian motion and a corresponding shift in the exponent. But as our power spectrum for frequencies above the corner frequency fit well to an exponent of -2, we do not have experimental evidence to prove viscoelastic behavior, which would give rise to a numerically lower exponent, on of the order of -1.75, in that frequency range.

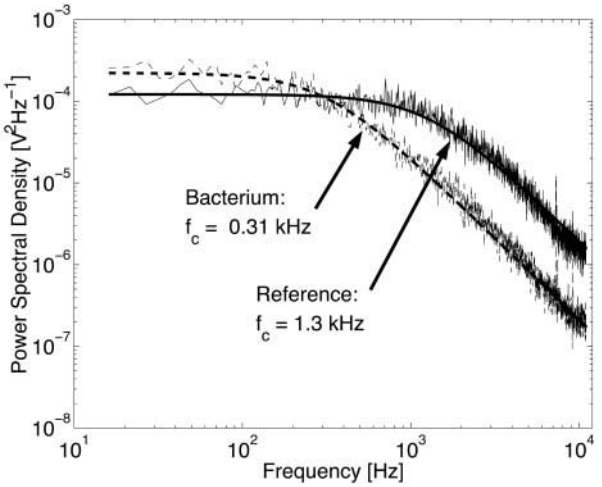

FIGURE 4 Power spectrum of $x(t)$ for a bead on λ-receptor (referred to as bacterium) and for an unattached bead (reference) held by optical tweezers. Lines are fits to Lorentzian functions (*full thick line* is Eq. 22 and *dashed thick line* is Eq. 23).

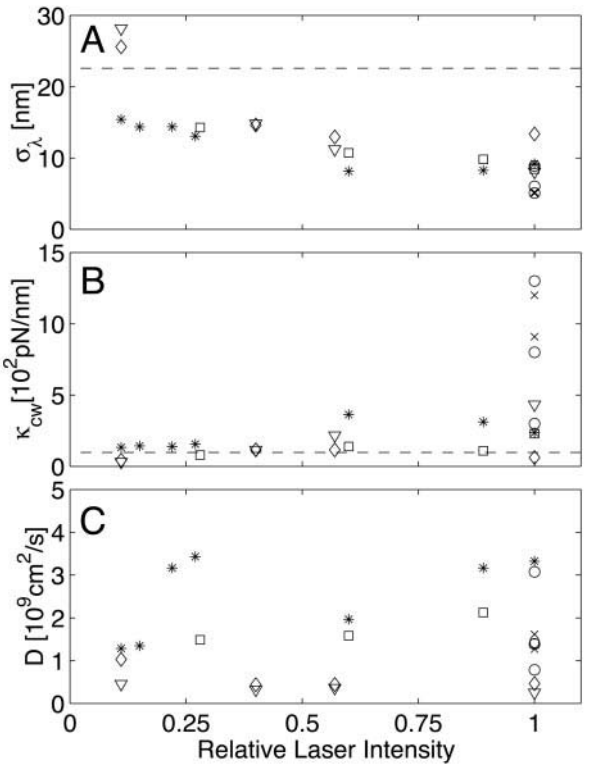

FIGURE 5 Physical parameters versus relative laser intensity from bead-protein complexes from six independent samples (each sample is shown by one type of marker). (*a*) Standard deviation σ_λ of the Gaussian position distribution for a bead bound to a λ-receptor. The horizontal dashed line is the average value, $\bar{\sigma}_{SPT} = 22.6 \pm 8.1$ nm, obtained from SPT analyses with the optical tweezer off. (*b*) Spring constant κ_{cw} characterizing the harmonic tethering of the λ-receptor in the bacterial membrane. The horizontal dashed line is the average value, $\bar{\kappa}_{cw,SPT} = (0.98 \pm 0.44) \times 10^{-2}$ pN/nm, obtained from SPT analyses with the optical tweezer off. (*c*) Diffusion constant D of the λ-receptor in the bacterial membrane as a function of laser intensity.

The standard deviation σ_λ (defined in Eq. 16) that is a measure of the radius of the area, which the λ-receptor-bead complex scans during a measurement, is found from plots like Fig. 3. Fig. 5 *a* shows σ_λ obtained with various laser intensities for six bead-protein complexes from six independent samples (each sample is shown by one type of marker). As we do not know the exact amount of power delivered by the laser in the actual trap, the abscissa is given in relative intensities instead. As expected, the motion of the bead is more confined at high laser intensities than for low laser intensities and vice versa. The horizontal dashed line is the average of the standard deviations obtained from the SPT measurements giving the "natural" value of the unperturbed system (see below). The value of σ_λ measured by optical tweezers approaches that of the SPT measurements as the laser intensity is decreased.

Knowing σ_λ and κ, κ_{cw} can be found via Eq. 18. Fig. 5 *b* shows κ_{cw} as a function of laser intensity. For laser intensities below a certain threshold value, κ_{cw} seems independent of applied intensity. If this threshold value is chosen to be at a relative laser intensity of 0.5, the average value of κ_{cw} found by the optical tweezers measurements with intensities below this threshold is $\bar{\kappa}_{cw} = 1.1 \times 10^{-2}$ pN/nm. The averaged value of κ_{cw} found by SPT (see below) is shown as the dashed horizontal line in Fig. 5 *b*.

From power spectrum analyses as shown in Fig. 4, $f_{c,\lambda}$ can be found using Eq. 19. As κ and κ_{cw} are known from the previous analysis, the total friction coefficient $\gamma_p + \gamma_b$ can be found from Eq. 20. As γ_b is found from Stokes equation to be 5.0×10^{-9} Ns/m, we can find γ_p and hence, from Eq. 21 the diffusion constant D of the λ-receptor in the membrane. Fig. 5 *c* shows D as a function of laser intensity. Within the precision of current measurements D seems to be independent of applied laser intensity. The average value thus obtained for D from the optical tweezers measurements at all laser intensities is $\bar{D} = (1.5 \pm 1.0) \times 10^{-9}$ cm²/s. Within one time step the receptor approximately moves $\Delta x_{OT} = \sqrt{D \times \Delta t_{OT}} = 2.6$ nm in a linear direction. Hence, in the unlikely case that its motion is in one direction only, it takes ~20 time steps (sampling at 22 kHz) to move from one end of the 50-nm domain to the other. Thus, the temporal resolution of our optical tweezers setup allows for a determination of D. A similar evaluation for SPT gives $\Delta x_{SPT} = 77$ nm showing that our SPT data cannot be used to determine D.

As SPT is an almost noninvasive detection method, the results of the SPT routine can be used to see how and if the bacterial system is perturbed during the optical tweezers measurements. SPT was done on six independent samples, four of which were the same as those used in the optical tweezers measurements shown in Fig. 5. For technical reasons we did not always succeed in investigating the exact same bead on bacteria with both techniques. Fig. 6 shows

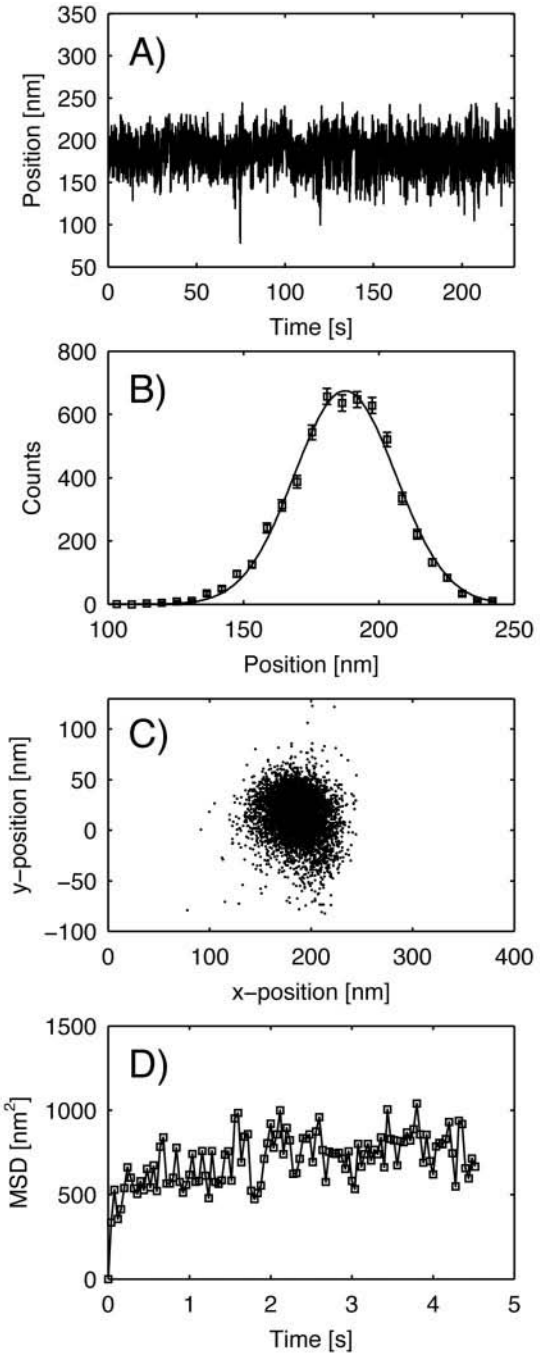

FIGURE 6 Data from a bead attached to a λ-receptor in the bacterial membrane obtained by SPT. (*a*) The *x*-position of the bead. (*b*) Histogram of the position distribution. The position histogram is fitted to a Gaussian distribution (*full line*) and has a standard deviation of $\sigma_{SPT} = 19$ nm for this experiment. (*c*) Scatter plot, showing the distribution of locations of the bead within the plane of the bacterial membrane. (*d*) Mean square displacement (MSD) as a function of time taken over 50 averages.

data obtained by SPT on the same bead bound to a λ-receptor of which data obtained by optical tweezers are shown in Figs. 2, 3, and 4. Fig. 6 *a* shows the position obtained by SPT in an experiment where the position of the bead was tracked for 4 min. Fig. 6 *b* shows the position histogram of the data shown in Fig. 6 *a*. Fitting these data by a Gaussian function yields a value of $\sigma_{SPT} = 19$ nm directly comparable with the squares in Fig. 5 *a* showing σ_λ obtained by optical tweezers measurements with various laser intensities on the same sample. The average value for σ_{SPT} using six independent samples is found to be $\bar{\sigma}_{SPT} = 22.6 \pm 8.1$ nm. Thus, the average value of $\kappa_{cw,\,SPT}$, the spring constant of a nonperturbed system is $\bar{\kappa}_{cw,\,SPT} = (0.98 \pm 0.44) \times 10^{-2}$ pN/nm. Fig. 6 *c* shows a scatter plot of the data obtained by SPT analysis. The motion of the λ-receptor is restricted within a certain domain for time scales at least on the order of minutes (we never saw a receptor leave this region although watching for more than 15 min). Also, the motion is fairly isotropic. Fig. 6 *d* shows the mean square displacement defined as $\langle r^2 \rangle = \langle (x(t) - x(0))^2 \rangle$, as a function of time taken over 50 averages within the same time series. This is a method suggested by Saxton (1993) to find the confinement radius of a confined diffusion. The square root of the asymptotic value, which $\langle r^2 \rangle$ approaches gives a confinement radius of ~26 nm ($= \sqrt{2}\sigma$), which was consistently seen for the other samples as well. If the SPT time resolution had been better, the increase in mean square displacement at short time lags would give a value for *D* and show possible anomalous diffusion.

DISCUSSION

The invoked in vivo biotinylation scheme results in a very low efficiency of biotinylation, thus enhancing the probability that the bead is only attached to a single receptor. This is in accordance with the literature. Also, it is supported by the fact that the observed spring constant κ_{cw} describing the attachment of the protein to the cell wall does not vary between measurements on independent samples, neither while using the SPT routine or while using the optical tweezers as detection method provided that the laser intensity is below the threshold value. If the bead occasionally had been bound to two or more receptors, the observed κ_{cw} would vary accordingly.

The value of the standard deviation σ_λ of the position measurements of a bead attached to a protein determined by SPT was 23 ± 8 nm. In the optical tweezers measurements a similar value is approached as the laser intensity is lowered. The limit as to how low laser intensity we can apply in the measurements is determined by the fact that the trap must be strong enough to trap a single bead for calibration purposes.

In the optical tweezers measurements with high laser intensities we occasionally observed some anisotropy of the measured physical parameters. The direction of the principal vector appeared random with respect to the bacterial axes, and at lower laser intensities no anisotropy was observed. This suggests that the anisotropy could be a result of

optical damage of the bacterial system at high laser intensities.

Apparently, the λ-receptor was harmonically bound in the bacterial outer membrane. Using the optical tweezers with laser intensity below a certain threshold, the value of the spring constant was found to be $\bar{\kappa}_{cw} = (1.1 \pm 0.4) \times 10^{-2}$ pN/nm. Above this threshold, κ_{cw} increased significantly, which might be a sign that some biological process, restricting the motion of the protein, is being enforced by the optical tweezers. Using SPT we found $\bar{\kappa}_{cw,SPT} = (0.98 \pm 0.44) \times 10^{-2}$ pN/nm. Within the uncertainties, the value of κ_{cw} found by the two different methods are identical. By dragging a protein through the membrane of eucaryotic cells without a cytoskeleton, Peters et al. (1999) obtain spring constants on the order of 0.5×10^{-2} pN/nm, approximately one-half of what we find for the bacterial system. That the two values of κ_{cw} are different is not surprising considering the different nature of the two types of membranes.

Within the accuracy of our measurements the value of the friction coefficient γ_p and of the diffusion constant D of the protein in the membrane seemed independent of the applied laser intensity. These values have only been determined by optical tweezers measurements as our SPT routine was too slow to permit a measurement hereof. The obtained value of $\gamma_p = 2.7 \times 10^{-8}$ Ns/m, was larger than γ_b by approximately a factor of 5. If we, in lack of earlier work on the mobility of single molecules in the bacterial outer membranes, instead compare to the work done on plasma membranes, our value of $D = (1.5 \pm 1.0) \times 10^{-9}$ cm^2/s is consistent with the findings of Kusumi et al. (1993) and Sako et al. (1994). If we consider the work by Pralle et al. (2000) who are using the same local method as we are to measure diffusion constants, we see that our diffusion constants were lower than what they find, suggesting that the part of the bacterial outer membrane to which the λ-receptor is attached is more viscous than a plasma membrane. Our result for D was higher than observed by Peters et al. (1999) and Suzuki et al. (2000), but they are using a different detection method, which yields a "global" measurement by dragging a protein across the plasma membrane. Therefore, these results may not be directly comparable with our results where the protein stayed within a diameter of 50 nm in bacterial outer membranes rather than eucaryotic plasma membranes. With the diffusion constant we observed, the λ-receptor would go from one end of the bacterium to the other within a few seconds. The fact that this did not happen was most likely due to the attachment of the λ-receptor to the fairly rigid peptidoglycan layer (Gabay and Yasunaka, 1980) giving rise to the observed harmonic potential.

The motion of the λ-receptor appears to be energy dependent, and we speculate that the purpose of this motion is to facilitate transport of the maltodextrin polymers through the porin spanning the outer membrane.

Finally, while studying bacteria by optical tweezers, one should also be aware of the possible photo damage that one might induce in the biological system (Neuman et al., 1999). As our results seem independent of applied laser intensity (below a certain intensity threshold) and as they coincide with our results from SPT, which is an almost noninvasive and independent method, we believe that photo damage can be ignored for most of the results presented here.

CONCLUSIONS

We have studied the motion of a single λ-receptor protein in the outer membrane of an *E. coli* bacteria. We have presented a method for the in vivo biotinylation of an extracellular site of the λ-receptor, and in agreement with previous literature, the efficiency of the in vivo biotinylation was found to be extremely low. By attaching a streptavidin coated bead to the λ-receptor, the motion of a single λ-receptor protein in the outer membrane of an *E. coli* has been revealed using the techniques of SPT and optical tweezers. We propose a model of the bead-protein complex allowing the extraction of several physical parameters describing the motion of the protein in the membrane and its connection to the bacterial membrane. The assumptions in the model that the bead and protein are tightly attached to each other and that the protein is attached in a spring-like fashion is supported by experimental evidence. This model can equally well be used to analyze motion of protein movements in other membrane systems.

The λ-receptor stays within an area with a radius of roughly 25 nm for at least several minutes. Within this area it performs a motion as if it were held by a spring of spring constant $\kappa_{cw} \simeq 1.0 \times 10^{-2}$ pN/nm and sat in a very viscous liquid within which it moved with a diffusion constant $D = (1.5 \pm 1.0) \times 10^{-9}$ cm^2/s.

The results of this paper open many directions for future work, e.g., the exploration of possible anisotropy in the motion of the λ-receptor, the dependence of the motion on modulation of the peptidoglycan layer, and the dependence on bacterial metabolism.

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