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Force Spectroscopy and Dynamics of the Biotin-Avidin Bond Studied by Scanning Force Microscopy

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Abstract

Scanning force microscopy (SFM) has been used to measure the strength of bonds between biological receptor molecules and their ligands. Here we report the measurement of the unbinding forces between avidin and biotin a model of the receptor-ligand interactions used in earlier studies- as a function of the loading rate. We have explored the unbinding force over three orders of magnitude in loading rate, and find that the force increases from ~20 pN to ~80 pN with increasing loading rate. We argue that the unbinding forces are connected to the bond lifetime as a function of an applied force. This allows to estimate thermal off-rates from measurements at finite forces. The deduced behavior of the bond lifetime of avidin-biotin in dependence of the force indicates that the dissociation proceeds via an intermediate state. Our data thus also allows to estimate the rate of an intermediate transition of the dissociation process.

Introduction

Recent advances in single-molecule manipulation at room temperature offer new tools for the study of the dissociation kinetics of single molecular ligand-receptor complexes under physiological conditions [1 - 10]. These methods allow to investigate the properties of individual complexes or molecules directly without extrapolation from ensemble assays.

It has recently been observed that the unbinding forces measured between a single ligand/receptor complex are directly related to the off-rate as a function of applied force [11]. This led to the development of the so-called dynamic force spectroscopy (DFS) [9, 10]: Here the force on the complex is increased with a constant rate (the loading rate) until the complex dissociates (unbinds). Since the unbinding is a statistical process the experiment is repeated several times in order to determine the most probable unbinding force. The unbinding force is then determined as a function of the loading rate. The dissociation kinetics can be extracted from these measurements: The derivative of the unbinding force with respect to the loading rate is to a good approximation the lifetime of the bond at the unbinding force [11, 12].

Up to now the above method has been used to determine the dissociation kinetics of different ligand/receptor systems [9, 10, 13 - 18] and the unfolding kinetics of proteins [19]. Interestingly, all ligand-receptor systems investigated so far show an exponential increase of the off-rate with force in the limit of small forces, as

originally stated by Bell [20]. This can also be viewed as a linear decrease of the energy for dissociation, which is expected for a single sharp energy barrier along the dissociation path. However, there are also ligand-receptor systems that show deviations from this behavior for larger forces, which is attributed to the internal structure of their energy landscape [9, 10].

In this paper we present loading rate dependent unbinding force measurements of the avidin-biotin complex with the SFM technique under physiological conditions. As investigated by biomembrane force probe (BFP) [9, 10] this system shows a dependence of the unbinding force on the loading rate that indicates an internal structure of the bond. Because the elasticity of the SFM force probe is fixed during one setup the range of accessible loading rates is smaller than in the above-mentioned experiment. We show that with one experimental setup the loading rate can be varied within three to four orders of magnitude. We compare the rate dependence of the unbinding forces of our experiments with the measurements by the BFP method and discuss our findings with respect to future biological applications of the SFM techniques.

Experimental

Surface and Tip Modification

Ultra-flat template-striped gold surfaces with a mean roughness of < 1 nm over several microns squared were produced as described by Hegner at al. [21, 22]. A amino-reactive self-assembled monolayer of Dithio-bis(succinimidylundecanoate) (DSU) was formed by immersing the gold surface in a 1 mM solution of DSU in Dioxane for 20 min [23]. DSU was synthesized according to the method of Wagner et al. [24]. After rinsing with dioxane and drying under a stream of argon the samples were incubated with 2mg/ml biotinylated bovine serum albumin (Biotin-LC-BSA (BBSA), Pierce, Rockford, IL) in citrate buffer (pH 6.8, 0.4 mM) for 2 h for covalent immobilisation. Alternatively BBSA was immobilized by adsorption on silanized glass surfaces with a concentration of 2mg/ml overnight. However the gold substrates where BBSA is covalently immobilized showed lower unspecific binding in the force experiments. After rinsing with PBS (pH 7.3, Life Technologies, Paisley, UK) the samples were incubated with 1mg/ml Avidin (Sigma, Buchs, Switzerland) in PBS for 4 h. After repeated rinsing with PBS the samples were used for the force measurements.

AFM-cantilevers (Si_3Ni_4-Microlever, Park Scientific Instruments, Sunnyvale, CA) were first irradiated for 30 min with a strong UV source (UV-Clean, Boekel Scientific, Feasterville, PA) and then silanized in a 2% solution of aminopropyltriethoxisilane (Fluka, Buchs, Switzerland) in Toluene for 2h. After rinsing with the solvent and drying under a stream of argon the tips were incubated with a 1mM solution of the α-Biotin, ω-N-hydroxysuccinimidyl ester of poly(ethylene glycol)-carbonate (Biotin-PEG-NHS, Shearwater Polymers, Huntsville, AL) in DMSO for 3h. After rinsing with the solvent and drying under a stream of argon the tips were ready to use in the force experiments. All steps described above were carried out at room temperature and solvents were from Fluka (Buchs, Switzerland).

Force Measurements

Force measurements were performed with the hardware (piezo and optical head) of a commercial instrument (Nanoscope IIIa-Multimode AFM, Veeco-Digital Instruments, Santa Barbara, CA) in a fluid cell in PBS. The acquisition of the cantilever deflection data was done with a 16-Bit AD/DA card (National Instruments, Austin, TX) in connection with an additional personal computer and a home-build high voltage amplifier. The piezo voltage was generated using the card and the high voltage amplifier. A self-developed software (written in LabVIEW, National Instruments, Austin, TX) was used to control the piezo displacement and the acquisition of the force curves.

Our self-developed software allows the independent control of the approach-velocity of the piezo towards the tip and the retract-velocity away from the tip, respectively. Additionally, the retract velocity was varied after each approach/retract cycle in order to acquire a data set of unbinding forces for different loading rates with one experimental set up. In this manner several systematic error sources, e.g. a possible loss of the activity of the tip or variations of the activity of the surface at different points, are eliminated.

The rate dependence of the unbinding forces was measured by approaching the tip with a velocity of 100 nm/s until a repulsive force of up to 200 pN was sensed (higher contact forces were only used to calibrate the deflection sensitivity). The sample was then retracted from the tip with different velocities in the range of 10 nm/s to 5000 nm/s. The effective loading rate was determined as the slope of the force versus time curves before an unbinding event. The spring constant of the cantilevers were determined to be 34 ± 7 pN/nm for the data presented here [25].

Results and Discussion

Theoretical Considerations

For the analysis of our data we briefly review some considerations concerning the dissociation of a complex under an applied force and the connection to the dependence of the unbinding force on the loading rate [11]. In the experiment the force on the complex increases

continuously until the complex unbinds. The unbinding is a statistical process driven by thermal fluctuations. This situation can be described by the probability *N(t)* to be in the bound state at the time t which obeys the equation

$$dN(t)/dt = -k_{off}(F(t))N(t) \quad (1)$$

with the initial condition *N(0)* = 1. Here $k_{off}(F)$ describes the dependence of the off-rate on the force and *F(t)* is the (experimentally observable) increase of the force with time. For most cases a linear increase of the force with a constant loading rate r, i.e. *F = rt*, is a good approximation [26]. The probability *p(F)dF* to observe an unbinding event in the force interval between *F(t)* and *F(t)+dF* is the probability *–dN(t)* to observe an unbinding event per unit time. In the important case where the off-rate increases exponentially from the thermal rate k_{off} with the force, i.e.,

$$k_{off}(F) = k_{off}\exp(F/F_0) \quad (2)$$

the distribution of unbinding forces *p(F)* can be calculated analytically (with a constant loading rate *r*) from the solution of (1). One obtains the important result that the most probable unbinding force *F**, i.e., the maximum of the distribution, increases logarithmically with the loading rate:

$$F^* = F_0 \ln\frac{r}{F_0 k_{off}} \quad (3)$$

The ansatz (2) was originally stated by Bell [20] in the context of cell adhesion: An applied force *F* tilts the energy landscape of a ligand-receptor bond so that in a first approximation the energy barrier for dissociation decreased by *-FΔx*, *Δx* being a characteristic interaction range of the complex, which defines the force scale $F_0 = k_BT/\Delta x$ in (2).

Force measurements

In the upper part of figure 1 a scheme of the experimental setup is drawn. The characters index the geometry of the experiment at different time points during the retract cycle. On the cantilever tip the biotin molecules were attached via an elastic poly(ethylene glycol) spacer. On the ultraflat gold sample surface [21, 22] avidin molecules were immobilized via a bioreactive self-assembled (DSU) monolayer and biotinylated-BSA [7]. At point (a) the cantilever pushed slightly against the surface, then the surface was retracted with a constant velocity and the force curve showed the typical non-linear elastic behavior of the linker PEG polymer upon stretching (b) before the avidin-biotin complex unbinds (c). The lower part of figure 1 shows a typical force versus time curve (for simplicity only the force upon retraction from the sample is shown). The elastic behavior of the 40 nm long polymeric linker upon stretching is observed [6, 27]. The discontinuity in the force curve was caused by the unbinding of an individual avidin-biotin complex. The root mean square thermal noise of a cantilever with spring constant 0.03 N/m is 11 pN. With our data acquisition we were able to resolve unbinding forces of down to 7 pN at small retract velocities by averaging.

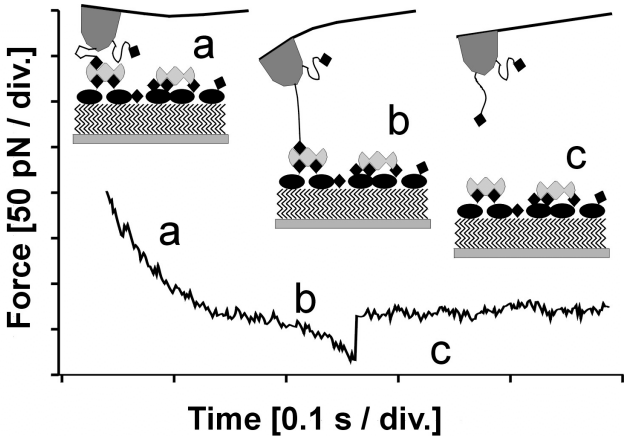

Fig. 1.: *Setup of the dynamic force spectroscopy experiment and force versus time for a cycle of touch and separation with formation and unbinding of an avidin-biotin bond.* **(a)** *Repulsive regime of the force when touching the surface.* **(b)** *Loading of the complex before unbinding of the bond.* **(c)** *Single avidin-biotin complex released and subjected to thermal fluctuations.*

To demonstrate the specificity of the observed forces the samples were blocked with 1 mM biotin in the measurement buffer (PBS), this lead to a drastic decrease of the unbinding events. However there might still occur some adhesion events due to unspecific tip sample interaction. In figure 2 the result of such measurements are shown as force histograms where the distribution of the last unbinding forces is plotted. This figure demonstrates that the observed unbinding forces were due to the specific interaction of the specific avidin-biotin complexes (grey bars). The distribution was markedly different for the specific and the blocked sample (hatched bars), especially there are only a few unbinding events in the blocked sample that showed the non-linear elasticity of the poly(ethylene glycol) spacer. The most probable unbinding force was determined by fitting a Gauss distribution to the histogram data. The statistical error of the maximum was estimated as the width of the distribution divided by the square root of the number of unbinding events in the histogram. From the histogram of figure 2, measured at a retract velocity of 750 nm/s, we determined an unbinding force of 44 ± 3 pN. Because the over all probability to find a unbinding event was greater than ~50 % the unbinding events with forces greater approximately 100 pN may be due to multiple unbinding events. However, the non-linear elasticity of the linker and the tip radius of 50 nm reduced the probability that the unbinding events of two or more biotin-avidin pairs could not be distinguished as two separate discontinuities in the force curves.

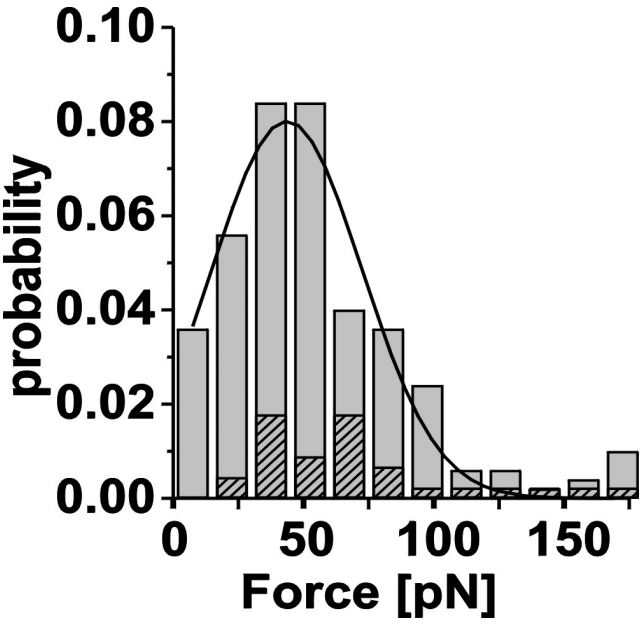

Fig. 2. *Probability distribution of the unbinding force, the last unbinding events of a single avidin-biotin complex. Histogram from ≈500 approach/retract cycles of specific complex for a retract velocity of 750 nm/s corresponding to an effective loading rate of ≈1600 pN/s. Grey bars show the specific interaction of one complex and hatched bars the unspecific tip-surface interaction. In the histogram the most probable unbinding force is around 50 pN. The maximum of the distribution is found by a Gauss fit to be 44±3 pN.*

As mentioned above we have measured unbinding events at different retract velocities ranging from 10 nm/s to 5000 nm/s keeping the approach velocity constant to 100 nm/s.

The effective loading rate in our experiment was determined by least square fits of the slopes of the force versus time curves before the last unbinding events giving the effective loading rate for each retract velocity. The loading rate dependence of the (most probable) unbinding forces is shown in figure 3. On the logarithmic loading rate scale two regimes can be distinguished (i.e. 20 - 40 pN and 40 - 80 pN) where the unbinding forces scale linear as predicted by eq. 3. These results are in close agreement with the measurements of Merkel et al. [10] where a different force probe was used.

The observation of the two regimes (in the data of Merkel et al. a third regime with an even steeper slope appears at higher loading rates) is attributed to the internal structure of the binding pocket: If the dissociation proceeds via an intermediate state (which is not unlikely for the biotin-avidin system where a flap closes behind biotin in the binding pocket [28]) the dynamics of the ligand in the binding pocket is described by the transition rate from the ground to the intermediate state, the backward rate from the intermediate to the ground state and the transition rate from the intermediate to the unbound state. The thermal off-rate is determined by all three transitions [12].

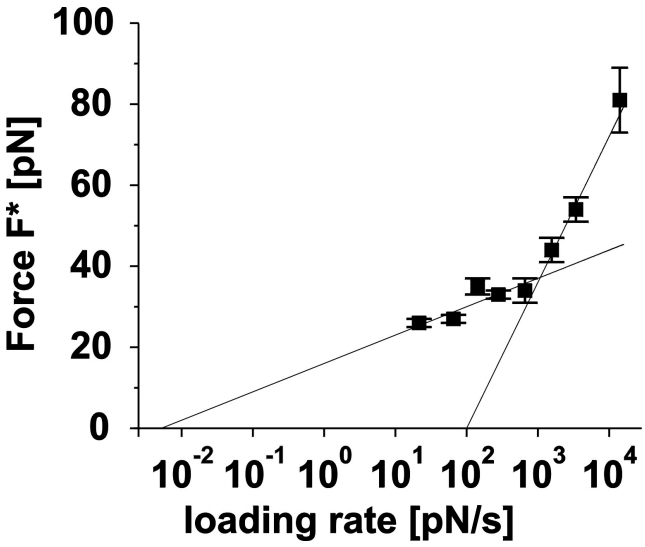

Fig. 3. *Loading rate dependence of the unbinding forces of the avidin-biotin system under physiological conditions. Two regimes can be distinguished: The unbinding force increases slowly from ≈ 20 pN to ≈ 40 pN. A linear fit according to equation (3) results in an estimate of the thermal off-rate. The second regime is governed by a steeper increase of the force from ≈ 40 pN to ≈ 80 pN with the loading rate. This regime is attributed to an intermediate transition in the dissociation process. The rate of the intermediate transition can again be estimated by a linear fit with equation (3) as indicated (see text).*

If the unbinding at low loading rates is also determined by all three transitions a linear fit of the unbinding force versus the logarithm of the loading rate would give the thermal off-rate k_{off} according to equation (3). We find k_{off} ~$10^{-3\pm1}$ s^{-1} compared to ~10^{-4} s^{-1} of Merkel et al. [10] who also give an off-rate of the PEG-Biotin-Avidin complex in solution of ~10^{-5} s^{-1} which is reasonable close to the estimates based on the force measurements (note that the off-rate of the unmodified Biotin-Avidin complex is ~10^{-8} s^{-1} [29]). The slope F_0 in the low force regime corresponds to a length scale Δx ~ 2 nm which differs from the value of ~ 3 nm found by Merkel et al. [10]. This difference in the observed slope of the force versus log (loading rate) curve may be attributed to the much smaller spring stiffness of the BMF used at low loading rates compared to the SFM force probe [30]: Because the energy landscape tested in the measurements is generally the sum of the molecular energy landscape and the energy of the spring, a harder spring will tend to decrease the distance between the bound and the transition state of the total energy landscape.

In the intermediate state picture a change in the dissociation process occurs at higher forces when the backward transition rate from the intermediate to the ground state becomes negligible. At high enough forces only transitions in the direction of the applied force occur and either the transition from the ground to the intermediate state or from the intermediate state to the unbound state become rate determining. On the bases of these force-spectroscopy measurements alone it is not possible to determine which transition is being measured [12].

Fitting again (2) to the second regime in figure 3 we obtain a transition rate $k_{off\_int} \sim 10^1$ s^{-1}. The much shorter length scale $\Delta x \sim 0.3$ nm (~0.3 nm, [10]) indicated by the steep slope in figure 3 is also indicative for an intermediate transition inside the binding pocket.

Conclusion

We have used dynamic force microscopy to probe the mechanics of molecular recognition of avidin-biotin. Our results are in close agreement with the data obtained by Merkel et al. with a different force probe technique, which demonstrates the reproducibility of data from single molecule experiments with different techniques. These experiments demonstrate that for small unbinding forces the thermal off-rate can be determined from loading rate dependent force measurements. For Avidin-Biotin as well as other investigated ligand-receptor bonds the SFM based method allows small enough loading rates to reach this regime. Therefore it is possible to estimate the thermal off-rate by measurements at finite forces where the dissociation is several orders of magnitude faster. Using this approach it is possible to have access to the thermal off-rate of any system as long as the interacting molecules can be bound to the surfaces in a site-specific, oriented and native way. SFM has the potential to combine the technique with the high spatial resolution. A speed up of this newly developed measurement technique is possible with a parallel approach of detection, e.g. with arrays of cantilevers [31].

At high loading rates the internal structure of the binding pocket becomes important. The rate of internal transitions can again be estimated from the loading rate dependent force measurements. Therefore the high loading rate regime is of importance for fundamental studies of ligand receptor interaction as well as the unfolding of proteins. SFM has the potential to explore this regime as smaller cantilevers for force measurements are being developed [32].

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