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Single molecule binding dynamics measured with atomic force microscopy

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ABSTRACT

We present a new method to analyse simultaneous Topography and RECognition Atomic Force Microscopy data such that it becomes possible to measure single molecule binding rates of surface bound proteins. We have validated this method on a model system comprising a S-layer surface modified with *Strep-tagII* for binding sites and strep-tactin bound to an Atomic Force Microscope tip through a flexible Poly-Ethylene-Glycol linker. At larger distances, the binding rate is limited by the linker, which limits the diffusion of the strep-tactin molecule, but at lateral distances below 3 nm, the binding rate is solely determined by the intrinsic molecular characteristics and the surface geometry and chemistry of the system. In this regime, K_{on} as determined from single molecule TREC data is in agreement with K_{on} determined using traditional biochemical methods.

1. Introduction

Binding processes between molecules such as proteins have not been researched extensively on a single molecule level, in contrast to unbinding processes, for which the single molecule approach has been very fruitful. This is because the binding process is physically more complex and because binding is experimentally more difficult to address [1].

Firstly, *unbinding* of proteins is mostly governed by the properties of the small contact area between both molecules, while *binding* is also governed by their chemical and geometrical surroundings as well as linkage properties such as rotational and translational freedom and stiffness [2].

Secondly, unbinding can be easily tested by pulling on molecules that have been allowed to bind and measuring the rupture force or time as the two supporting surfaces are separated. On the other hand, detecting the binding between molecules requires multiple close contacts between the molecules followed by testing of the bond.

There are many examples where single molecule unbinding studies have been important to understand molecular pathways and mechanisms. This includes the mapping of the energy landscape of a bond [3] and understanding the effect of cooperative interactions on bond strength [4]. We envision that binding processes will also have to be studied at this level in detail to understand them. It has recently been suggested for example that cooperative effects may be important for binding processes too [5].

So far, very few experimental studies have been concerned with single molecule binding rates. Pierres et al. [6,7] used flow chambers to study distance dependent binding rates. With this technique, particles can quickly search an extensive contact area while the frequency of arrest may be used to measure bond formation kinetics. The dependence of the binding rate on the distance between the two anchoring sites is a good candidate to characterize the binding process on a single molecule level from an experimental point of view. When the binding rate is independent of distance, it is reasonable to argue that it is set by the molecular characteristics of the ligands; otherwise the binding rate is determined at least partly by the properties of the linkers and the surface(s). If the surfaces are not homogeneous and isotropic, there might additionally be an orientational dependence of the binding rate.

There have also been a number of studies concerned with single molecule binding using Atomic Force Microscopy (AFM) techniques, but these have not gone to the same functional depth

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as the studies by Pierres et al. Specifically, there are some early attempts to estimate bulk K_{on} for dissolved molecules using AFM by Hinterdorfer et al. and Baumgartner et al. [8,4]. Also, recently, Kaur et al. [9] analysed simultaneous Topography and RECognition (TREC) [10] images for binding/unbinding stochastics in a somewhat similar manner as what we present in this paper, but without analysing the distance dependence of the binding rate. Furthermore, Favre et al. investigated single molecule binding between biotin and streptavidin using an AFM force clamp technique [11]. They necessarily studied the association under relatively high applied forces leading to very low binding rates.

In the present study, we analyse TREC images to determine the distance dependent binding and unbinding rates of single molecules, simultaneously providing an image of the surroundings of the molecules. TREC imaging allows us to locate binding sites on a surface using a ligand tethered to the AFM tip. The tether allows us to separate unspecific interactions between the tip and sample, which occur in the downswing when the tip touches the sample, from specific interactions between ligands which the tip feels when it is in the upswing, stretching the tether [10]. Special AFM electronics (N9630A PicoTREC, Agilent Technologies) extract the amplitude on the downswing for feedback and the amplitude on the upswing as the TREC signal. On performing high resolution TREC imaging, we noticed that recognition spots are 'noisy', and display in effect multiple unbinding and binding events which are stochastically distributed, even within a single scan-line. The oscillation of the AFM tip allows the ligand to repeatedly attempt binding at a low force, followed by a test for the success of binding at a high force and loading rate. This is repeated many times for one specific pair of molecules at different distances. The TREC imaging mode can therefore be used to probe binding and unbinding rates as a function of distance between the anchoring point of the ligand on the tip and the binding site on the surface by analysing this apparent switching 'noise'.

We describe in this paper how we performed the analysis of the distance dependent binding process for a model system and we discuss what the influence of the different components of the system is on the measured binding rate. We also discuss limiting factors in the experiment and how it may be further optimized. We expect that this technique will be of broad interest as a new tool for biophysicists.

2. Materials and methods

2.1. Atomic force microscopy

The AFM cantilever is modified according to the protocol developed in [12], attaching a strep-tactin molecule covalently to the AFM tip through a flexible Poly-Ethylene-Glycol (PEG) linker with a length of 8 nm. See Barattin et al. [13] (esp. Section 4.1) for an in-depth discussion of the advantages of this functionalization method for single molecule experiments.

We use Agilent MAC mode Type IV cantilevers E with a nominal spring constant of 0.1 N m^{-1} and resonance frequency in liquid of $\sim$10 kHz. The AFM is an Agilent 5500 AFM (Agilent Technologies, 5301 Stevens Creek Blvd, Santa Clara, CA 5051, United States) equipped with TREC electronics N9630A PicoTREC. All measurements are done in Magnetic AC (MAC) mode, with the drive frequency set at about 75% of the free cantilever resonance frequency in liquid far away from the surface. TREC signals are recorded in full amplitude mode using Agilent electronics. Measurements are performed with a setpoint amplitude close to the free amplitude and about 8 nm peak to peak as prescribed by the linker length. Both left-to-right and right-to-left (trace and retrace) of the topography, amplitude and recognition signals are recorded.

2.2. Surface preparation

For the surface, we use S-layer protein SbpA of *Bacillus sphaericus* CCM 2177. One in seven of the proteins is genetically modified to expose a *Strep-tagII* peptide, which binds with high affinity to strep-tactin [14]. This ensures a regular, well-defined surface with a low corrugation of only a few nm over large distances [15] and many well-spaced binding sites.

The activity of the fused SbpA/*Strep-tagII* construct is tested by force spectroscopy measurements on a lattice fully consisting of modified proteins, as described in [15,16]. The probability of binding event in the force curves, and more importantly the reduction of this probability after biochemically blocking the interaction, is a good test for functionality of the surface and tip. We find a binding probability of 13%, which reduces to 3% after adding free *Strep-tagII* to the solution (data not shown).

Moreover, the rupture force distribution measured from force–distance curves indicates that [13] a single molecule on the tip can reach the surface and bind, i.e. we do not observe double rupture events in force–distance curves or multiple peaks in the rupture force distribution (data not shown).

2.3. Localization of binding site and events

The AFM images used for analysis in this paper suffer from considerable lateral drift, both from thermal expansion and from piezo-creep. As accurate determination of distances across several scanlines is important for the analysis discussed in this paper, we use the known square lattice of S-layer to correct for drift in our analysis. In short, we compare the 2D FFT image of the topography with what we expect for a square lattice, and from the difference we compute a drift vector. All measured positions are corrected using this drift vector.

To locate TREC spots, we perform thresholding on the recognition image, with the threshold set by Otsu's method, which, assuming that there are two possibly overlapping distributions of values in an image, chooses the 'best' threshold in between the distributions [17]. A total of 11 recognition spots from one image are used in the analysis here. These spots are selected on the basis that they do not overlap in the combined trace and retrace images. Next, the actual location of the binding sites is taken as the centre of each recognition spot as determined from the centre of mass of all pixels above the threshold from the combined trace and retrace images in that particular recognition spot.

Next, we locate all binding and unbinding events in a recognition spot from the thresholded images. The distances between events and the binding site are recorded, and corrected for drift as described above.

2.4. Calculation of distance dependent binding and unbinding rates

From the thresholded images, we first calculate the probabilities for the ligands to be in the (un)bound state $P_{(un)bound}$ and the probability of (un)binding events $P_{(un)binding}$ as

$$P_{(un)bound}(d) = n_{(un)bound}(d, d+\delta d)/n_{pixels}(d, d+\delta d) \quad (1)$$

$$P_{(un)binding}(d) = n_{(un)bindingevents}(d, d+\delta d)/n_{pixels}(d, d+\delta d) \quad (2)$$

respectively where n_{pixels} is the total number of pixels at a distance between d and $d+\delta d$ from the centre of the recognition spot, $n_{(un)bound}$ is the number of pixels where the ligand is in either the bound or the unbound state, and $n_{(un)binding}$ is the number of pixels with a transition from unbound to bound state or vice versa.