Single molecule binding dynamics measured with atomic force microscopy

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ABSTRACT

We present a new method to analyse simultaneous Topography and RE cognition 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.
as the studies by Pierres et al. Specifically, there are some early attempts to estimate bulk $k_B^\text{m}$ 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 stochastic 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 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 $P_{\text{bound}}$ and the probability of (un)binding events $P_{\text{unbinding}}$ as

$$P_{\text{bound}}(d) = \frac{n_{\text{pixels}}(d)}{n_{\text{pixels}}(d + \delta d)}$$

$$P_{\text{unbinding}}(d) = \frac{n_{\text{pixels}}(d + \delta d) - n_{\text{pixels}}(d)}{n_{\text{pixels}}(d + \delta d)}$$

respectively where $n_{\text{pixels}}$ is the total number of pixels at a distance between $d$ and $d + \delta d$ from the centre of the recognition spot, $n_{\text{bound}}$ is the number of pixels where the ligand is in either the bound or the unbound state, and $n_{\text{unbinding}}$ is the number of pixels with a transition from unbound to bound state or vice versa.
The (un)binding rates $\Gamma_{\text{binding}}$ and $\Gamma_{\text{unbinding}}$ as a function of $d$ can then be calculated as

$$\Gamma_{\text{binding}}(d) = \frac{p_{\text{binding}}(d)}{(p_{\text{unbound}}(d) \cdot T_{\text{pixel}})}$$

$$\Gamma_{\text{unbinding}}(d) = \frac{p_{\text{unbinding}}(d)}{(p_{\text{bound}}(d) \cdot T_{\text{pixel}})}$$

with $T_{\text{pixel}}$ being the sampling time for a pixel.

3. Results and discussion

The topography and recognition images that are used for analysis throughout this paper are shown in Fig. 1. The regular lattice of the S-layer is visible in the topography image. The recognition image shows clear recognition spots. Here, the setpoint amplitude was adjusted for maximum contrast in the recognition image. As should be expected in recognition imaging [18], with slightly larger amplitudes we saw no recognition spots, as the ligand got pulled away from the binding site every oscillation cycle, while with smaller amplitudes, contrast quickly diminished as the force on the linker/cantilever became smaller. Additionally, at the end of the experiment and according to the standard protocol for single molecule force measurements [19], free Strep-tagII was added to check that the recognition spots disappeared as the ligand on the tip got blocked.

The recognition spots appear more noisy than their surroundings in the recognition image, which is unexpected, as the stretching linker effectively makes the cantilever stiffer and should therefore reduce the thermal amplitude noise. On closer examination, the signal in the recognition spots switches between two levels, one similar to the surroundings and one where the amplitude is reduced. This is due to the fact that the ligand is binding and unbinding multiple times while passing the binding site. We find this to be generally the case when the setpoint amplitude is set close to the linker length.

We subsequently use Otsu’s method [17] to threshold the images and thereby locate all binding and unbinding events. Fig. 2 shows two views of the thresholded recognition image used throughout the analysis in this paper. Fig. 2a shows a combination of trace and retrace of the recognition image after thresholding, with the dark grey representing the recognition spots from the left to the right image, middle grey from the right to the left image, and light grey where both overlap. The overlap is relatively small because of piezo-creep and drift. In Fig. 2b, the image is first transformed to remove piezo creep and drift, and then thresholded with the same threshold as in Fig. 2a. Because of the transformation, pixel-level details have been smeared out and are lost in this image. However, Fig. 2b shows the shape of the spots better and how well they are separated. For the analysis we pick the 11 indicated spots based on their shape (symmetric about the x-axis of the spot), size, and how well they are separated from neighbouring spots. We then locate these spots in the original, untransformed image, and extract all data for analysis from there.

From the thresholded data we can calculate $p_{\text{bound}}(d)$ according to Eq. (1), shown in Fig. 3a. $p_{\text{bound}}$ is shifted with respect to the location of the binding site ($x=0$): The ligand is more likely to be bound when leaving the binding site as compared to approaching the binding site. This is not because it takes time to bind, as we see binding, unbinding and rebinding even before the tip is right above the binding site, but because leaving the binding site, the ligand is likely to be bound, and the linker needs to be stretched quite far to induce unbinding. On approaching, however, the linker has to use thermal energy to stretch to the binding site, which it is not likely to stretch to as large a distance.

Fig. 3b shows the distance dependent binding and unbinding rates that we calculate from the images. We note that, although $p_{\text{bound}}$ is not symmetrical around $d=0$, the binding and unbinding rates are, as expected. The binding rate shows a plateau about 3 nm to each side of the binding site ($d=0$ nm) at about 100–200 s$^{-1}$, after which it drops quickly by 3 orders of magnitude out to 12 nm distance, beyond which no events are observed anymore. Our explanation is as follows. Diffusion of the Strep-tagII molecule is limited by the linker dynamics, and as such is dependent on its distance to the linker anchorage site. At less than 3 nm lateral distance, diffusion is quick enough that the association between Strep-tagII and strep-tactin is given by molecular or surface characteristics. At larger distances, diffusion is slower and limiting the binding rate down to where the Strep-tagII cannot reach the strep-tactin binding site anymore at 12 nm distance. For comparison, we also estimate the bulk solution on-rate $K_{\text{on}}$ (M$^{-1}$ s$^{-1}$) that would correspond to a binding rate of 200 s$^{-1}$. The single molecule of strep-tactin represents an effective concentration determined by the volume it can explore. For this volume we take a lateral distance of 4 nm, set by the distance the strep-tactin can easily explore as determined from our measurements, and a vertical distance of 7 nm, set by the tip motion. This gives the molecule 350 nm$^2$ to move in or an effective concentration of 4.7 mM. Together with the measured on-rate this yields an effective $K_{\text{on}} = 4.2 \times 10^4$ M$^{-1}$ s$^{-1}$. According to personal communication with Iba Life Sciences, from whom we bought Strep-tagII and strep-tactin, $K_{\text{on}} = 1 \times 10^5$ M$^{-1}$ s$^{-1}$ for binding in solution for this system.

As unbinding of proteins has been extensively studied in the last decade and the specific pair of Strep-tagII and strep-tactin has...
been well characterized, we looked into modeling unbinding in our system numerically to compare our experimental unbinding rates with theory. However, we found that there are too many poorly understood factors at present to be able to realistically model this system and calculate a representative unbinding rate. Many of these factors are actually to do with our poor understanding of surface bound association and are not merely technical difficulties. For a start, it is actually ill-defined when a protein is bound to another protein [20]. The energy landscape for association shows many peaks and valleys, and a complex of proteins will not immediately end up in the lowest-lying valley. If we start pulling again before the complex has had time to relax to the ground state, the unbinding rate will be markedly larger than for a complex that has had enough time to relax completely. In fact, this effect has been shown already in experiments on the refolding of DNA [21] and RNA–protein interaction [22].

A better understanding of the mechanical characteristics of the individual components is also important for a precise quantitative understanding of binding and unbinding in TREC imaging. Especially important in this respect are compliance and rotational freedom in the linker/Strep-tagII system, which may also be dependent on direction. Furthermore, the linker is well described as a worm-like-chain or entropic spring, but its ability to diffuse through space is severely limited by the presence of the AFM tip and the surface, as well as the large strep-tactin molecule on its end. We believe that a more detailed understanding of molecular dynamics in TREC imaging is required to fully realize its potential for analyzing the distance dependence of binding and unbinding rates; however, we would like to stress here that these effects are not only measurement artifacts, but that these effects also occur in binding processes in real-life systems.

Some modifications could open up the experiment to easier in-depth interpretation. A better defined anchoring point for the linker, truly at the end of the AFM tip, would be of benefit. This would allow better defined numerical simulation, but it would also allow to more easily vary linker length to investigate the influence of the linker on the binding rates. If a nanotube tip were to be used, it would also exclude significantly less volume that the linker can explore compared to a standard pyramidal tip, further improving the applicability of simulations. Another possible improvement is that with more data, it will become possible to analyze binding rates not only as a function of distance, but also as a function of direction.

Fig. 2. (a) Superposition of left–right (light grey) and right–left (dark grey) thresholded recognition images. Where both images are below the threshold, the colour is white. (b) Same data, but a transformation was applied to revert distortion caused by piezo creep and thermal drift before the thresholding. Note that this procedure requires interpolation between data points and thus removes small details from the image. Spots selected for further analysis are indicated here with a white rectangle.

Fig. 3. $P_{\text{bound}}$ (a) and $\Gamma_{\text{unbinding}}$ (b) as a function of distance between AFM tip and binding site, calculated directly from the data according to Eqs. (1) and (2). Negative distances denote the tip approaching the binding site, and positive distances denote the tip leaving the binding site.
It would be interesting to see if the approach direction has an influence on the binding rate in natural systems.

4. Conclusion

We have presented a new way to look at AFM TREC data. We have shown that high resolution TREC images contain information on binding and unbinding rates for surface bound molecules, and we have presented a method to analyse the TREC images to extract these rates as a function of distance between the AFM tip and the binding site. We find that for our model system, the binding rate is determined by linker dynamics for distances larger 3 nm, but for shorter distances we find a binding rate of 100–200 s⁻¹ which is set by the intrinsic molecular characteristics of the Strep-tagII–streptactin system and the S-layer support surface. From this rate and the effective concentration of the ligand, we can determine $K_{on} = 4.2 \times 10^9$ M⁻¹ s⁻¹, which is in good agreement with $K_{on}$ determined using traditional biochemical methods. Thus high resolution TREC imaging is a new method to determine $K_{on}$ at the single molecule level. We have also discussed the importance of binding rates for surface bound molecules, and hope that our new technique will provoke a renewed interest in improving the clarity of data and theory on this aspect of molecular interactions.

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