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Biophysical Chemistry 117 (2005) 233 – 237

Biophysical Chemistry

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Specific molecular interactions by force spectroscopy: From single bonds to collective properties

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Received 11 January 2005; accepted 23 March 2005
Available online 15 June 2005

Abstract

Interactions involving multiple bonds occur throughout biology, and have distinct properties that are fundamentally different from those present in single bond systems. We have developed a new method to analyse the AFM force measurements in order to extract relevant information and to characterise the interactions involving from single to multiple bonds. Our study reveals a surprising behaviour in the presence of multiple bonds with a high rebinding probability: the mean binding forces increase with decreasing pulling velocity. Such behaviour is different from the force dependence on the loading rate for single bond rupture or existing models for multiple bonds rupture.

Keywords: Biophysics; Binding strength; Force spectroscopy; Molecular recognition; Single molecule studies

As emphasized by Jacques Monod in *Le hasard et la necessite* [1], non-covalent interactions are of crucial importance for our understanding of all living beings. The efficiency of proteins relies on the formation of stereo-specific complexes. The fact that these complexes are stabilised by non-covalent interactions is also essential: the complexes must be able to form and dissociate without excessive consumption of energy. Our understanding of the cells therefore largely depends on our capacity to demonstrate these complexes, their respective roles, their formation and their dissociation.

Since the beginning of the 1990s, the development of the AFM and other techniques (optical tweezers, optical deformation of membranes) has made possible the measurement of forces between molecules. More than 100 articles have been published that describe force measurement experiments between biological molecules using AFM, in particular between ligands and receptors or antigens and antibody [2–4].

However, interactions involving multiple bonds have become a new focus of study [5]. The ability to understand these interactions is important and relevant because multiple bonds have distinct properties and can provide the basis for mechanisms of biological interactions that are fundamentally different from those present in single bond systems.

Unfortunately, the evolution from single molecules to collective properties is a very difficult step. The main limitation of the force spectroscopy approach comes from the difficulty in retrieving relevant information from complicated force profiles. The energies implicated are of the order of the thermal energy and therefore each force profile is different. Each force curve can be seen as a path in a complex energy landscape [6]. Even for simple systems with only two interacting partners, a careful analysis of the experimental results is needed. In order to obtain significant results, it is necessary to perform a large number of approach–retraction cycles, followed by the evaluation of the position and the amplitude of the ruptures.

To date, several questions remain to be answered. How should this huge amount of data be represented? How can the relevant information be extracted from it in order to characterise the phenomena? How can physical phenomena be observed by a simple analysis of the force curve profiles?

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doi:10.1016/j.bpc.2005.03.013

In this study we were interested in a specific molecular interaction between nickel and the histidine amino acid. We address the questions cited above by making force measurements ranging from a single bond to a system involving several bonds and by introducing new representations of the experimental results.

1. Experimental section

We have used a home-made AFM to measure the interaction forces between polyhistidine and nitrilotriacetic acid (NTA) loaded with a metal ion, nickel (Ni^{2+}). Histidine is an amino acid capable of binding to an ionic nickel. Like biotin–streptavidin or antibodies, this interaction is a classical tool to manipulate proteins. Its main use in molecular biology is in immobilised-metal affinity chromatography (IMAC) to purify proteins that have previously been genetically modified by the addition of several histidine amino acids [7,8]. In IMAC, elution of the target protein is achieved either by protonation using buffers with lower pH, or with imidazole, at nearly neutral pH, in order to block the Ni-NTA binding site. Numerous interesting proteins from a biological point of view are thus available possessing an histidine label.

The unbinding force between histidine and Ni-NTA has been evaluated by three different groups with significantly different result (38 pN, 180 pN and 500 pN) [9–11].

The principle of a force measurement by AFM is simple: a surface is repeatedly extended towards and retracted away from a sharp tip (fixed to a cantilever) and the position of the tip is recorded as a function of the surface displacement. To achieve accurate quantitative results, it is imperative to measure as precisely as possible the spring constant of the cantilever. The thermal fluctuation method has been shown to be a good method when the shape of the mode and the repartition of the energy between the vibration modes are taken into account [12]. The spring constant of the cantilever used in our experiments is equal to 32±3 mN/m.

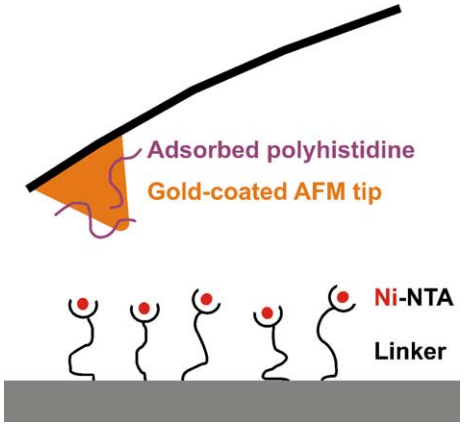

Fig. 1. Schematic of our histidine Ni-NTA force measurements.

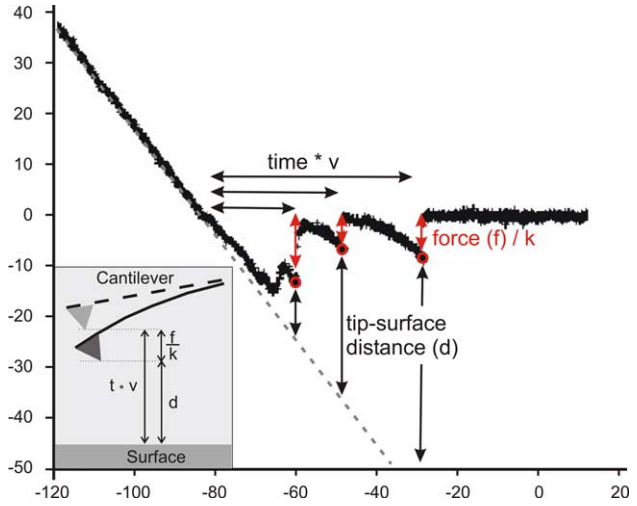

Fig. 2. A typical AFM force curve between polyhistidine and Ni-NTA with three rupture events. The force curve is recorded at pH 7.5 with a retraction speed of 0.5 μm/s. The open circles are the rupture events detected by our program. The first minimum in the force curve is not identified as a rupture since there is no sudden relaxation but a continuous process. Arrows show the rupture force, tip-surface and time. The time in seconds is obtained by dividing by the retraction speed. The force in pN is obtained by multiplying by the spring constant k of the cantilever (32 mN/m).

In order to obtain significant results, we have performed a large number of approach–retraction cycles, followed by statistical evaluation of the position and the amplitude of the ruptures. We have developed a program which allows us to automatically detect these ruptures [13]. The algorithm is based on an analysis of the standard deviation computed in a sliding window (section of the force curve) along the length of the force curve. Ruptures are defined as sudden variations of the force, and thus give rise to standard deviation maxima.

We have adsorbed polyhistidine (Sigma, of molecular weight 5000, on a gold-coated tip) and then measured the interaction forces with a functionalised surface (Ni-NTA HisSorb Plates, Qiagen). Ni-NTA HisSorb plates are normally used in protein assay with histidine tagged proteins. These plates are optimised to avoid unspecific binding. The bottom surface of the well was removed and used as our Ni-NTA functionalised surface (see Fig. 1). The strong affinity of polyhistidine to the gold surface has been demonstrated using the quartz crystal microbalance and AFM force measurements. We have demonstrated that the adsorption of polyhistidine on the gold surface is irreversible and also that the adsorption is not disturbed by the imidazole. Hence we can use imidazole as a blocking agent to demonstrate that the ruptures in our force curves are due to the Ni-NTA/histidine interaction.

2. Results and discussion

Fig. 2 shows a characteristic shape of the force versus extension unbinding traces associated with the rupture of

the coordination bond between polyhistidine and Ni-NTA in citrate phosphate buffer solution at pH 7.5. If the contact involves the formation of a bridge between the tip and the surface, the retraction of this latter exerts a force that involves an increasing deflexion of the cantilever, up to a rupture point. Events were recognized by unbinding traces with single or multiple saw tooth patterns. Each tooth consists of a part with a rising deflection followed by a sudden jump. The rising part corresponds to the stretching of the molecular bridge; the jump reflects the break bond and provides an estimate of the bond strength. To validate the biological specificity of the Ni-NTA/histidine interaction, imidazole was added to the solution to block the binding site of the Ni-NTA on the surface. After addition of imidazole the rupture probability decreased strongly (Fig. 3). These results show clearly that the bond ruptures observed in Fig. 3 can be assigned to the Ni-NTA/histidine.

In most of the previously published papers, the authors focused on the histogram of the last rupture forces. In some papers, an improved analysis of these same ruptures is based on a probability density function introduced by Baumgartner et al. [14]. We propose to address the full complexity of the force curves using an automated detection followed by the visualisation of the results as a two-dimensional density function (referred to as a density map below).

A rupture is characterised by the rupture force, the time needed to break the bond and the distance between the tip and the surface. By the expression "time needed to break the bond", we mean the time between the beginning of the applied load and the rupture. In the paper, "time" will refer to this value. Two of these three values are enough to fully characterise the rupture since

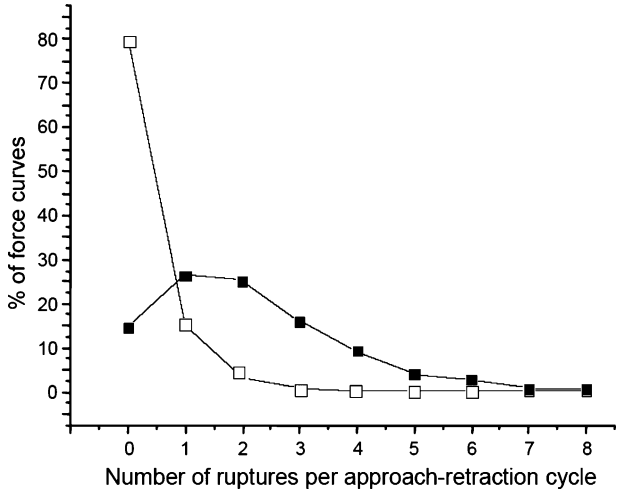

Fig. 3. Effect of imidazole on rupture distribution. The percentage of force curves presenting a given number of ruptures is plotted as a function of this number of ruptures. Black squares represent the distribution in phosphate buffer, hollow squares correspond to the distribution after adding imidazole up to an approximate concentration of 200 mM.

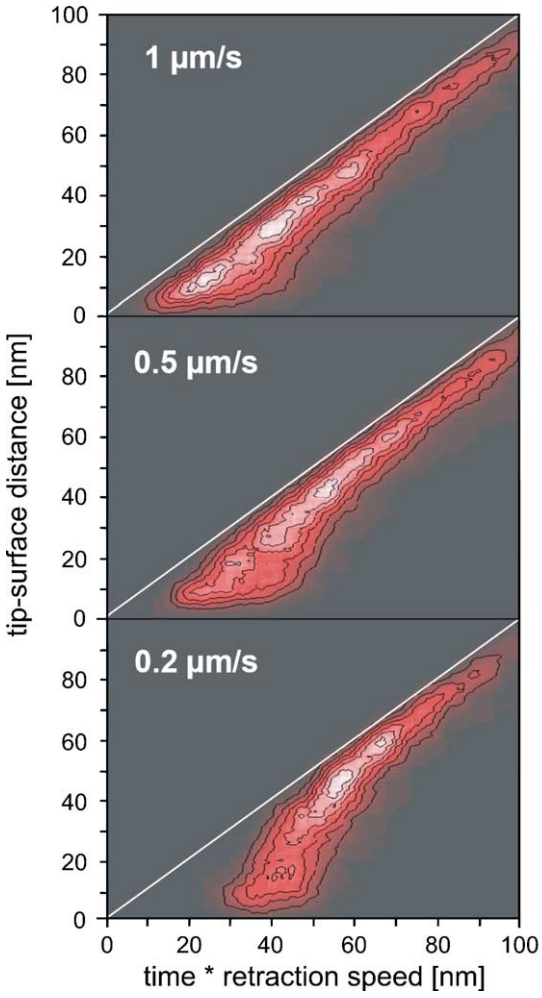

Fig. 4. Density maps presenting all the rupture events for each retraction speed. For each point in the plane (time*speed)/tip-surface, the density is calculated from the number of ruptures occurring within a 10 nm*10 nm square. Each figure is obtained from analysis of 800 approach/retraction cycles presenting more than 2000 rupture events. The deflexion of the cantilever (force/k) is the difference between the diagonal and the data.

there is a geometrical relation between these three values (Fig. 2):

$$\text{time*retraction speed} = \text{force}/k + \text{tip} - \text{sample distance}.$$

One rupture is described by a point (x,y) in one of the following planes (force, time), (force, tip-surface distance) or (tip-surface distance, time). It follows that the best representation of an experiment resulting in hundreds of ruptures is a density map $f(x,y)$ measuring the number of ruptures per unit of area in one of these planes. It is important to note that the specification of the force does not give a complete description of the event. For a same cantilever and retraction speed, depending on the elasticity of the link between the tip and the surface, a rupture occurring at a given force can correspond to two different loading rates. It is actually difficult to use the loading rate as a parameter in experiments where multiple bonds are present. This can be seen directly in Fig. 2; the loading

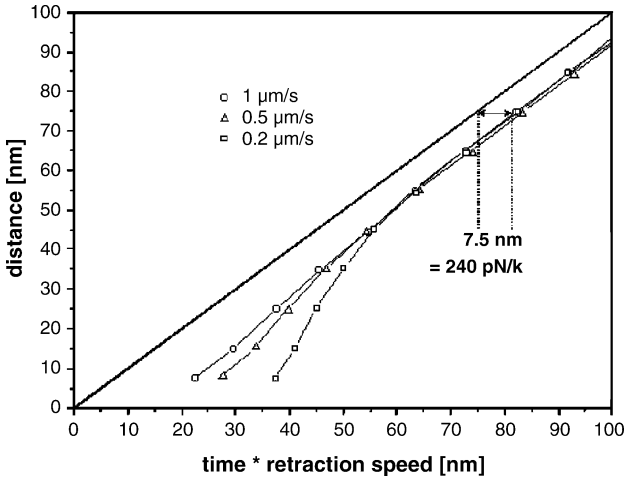

Fig. 5. The variation of the mean distribution events with pulling velocity. Triangles, circles and squares correspond respectively to 0.2, 0.5 and 1 µm/s. As shown in the figure, the mean binding force at high tip-surfaces can be deduced by taking the difference between the diagonal line and the data (k = 32 mN/m).

rate before the first rupture (slope of the force curve) is much higher than before the second and third ruptures.

The strength of a single bond with a thermally activated barrier depends on the loading rate. As stated before, in the case of multiple bonds occurring at different tip-surface distances the loading rate is not an operational parameter. However kinetic effects certainly do take place. The representation of the force distribution in the plan (time * velocity, tip-surface distance) allows a direct visualisation of kinetic effects. Fig. 4 shows three density maps in the plane (time * velocity, distance) reflecting rupture bonds detected in force curves at three different retraction velocities (0.2, 0.5 and 1 µm/s). The surface is approached to the tip at the same velocity to that of the retraction. The diagonal line corresponds to the ruptures that would be produced at zero force. The area above the diagonal corresponds to positive force. As can be deduced from the density map no quantization in force is observed. A quantization in force would correspond to periodicities parallel to the diagonal, which is not the case here. As we normalised the time scale by the retraction velocity, if no kinetic effect were to take place, the three distributions should superimpose.

The three rupture distributions are identical beyond 45 nm in tip-surface distance (60 nm in time * velocity) in the range of 0.2–1 µm/s of retraction velocity. In contrast, a strong dependence on the speed of the retraction is observed below this tip-surface distance (45 nm). As can be deduced from Fig. 5, beyond 45 nm in tip-surface distance, the mean binding force in the Ni-NTA/histidine system is independent of the retraction speed and has a value equal to 240 ± 25 pN. The probability of rebinding, beyond this tip-surface, is very small. This force is attributed to the interaction Ni-NTA/histidine at the single molecule level. Compared with other AFM experiments, 240 pN is of the same order of magnitude as the measured value in the paper [11]. The mean force associated with this rupture is independent of the speed retraction in the range of 0.2–1 µm/s. In this range of retraction speed, a small variation of the force as a function of speed pulling is expected for single molecule experiments. In the case of cadherin [15] and biotin/avidin [16] interactions probed by AFM, a variation of the order of 20 pN, in the range of 0.1–1 µm/s, was obtained, which is within the bar of error of our force measurements.

Note that we can imagine other experiments by controlling the dwelling time, i.e. the time the cantilever remains close to the sample at constant force before retraction occurs, as we have done using the adsorption of fibrinogen on glass surfaces [17] or recently by other group [18] in the case of mucin protein interaction with mica. The control of dwelling time is important parameter, which would be studied elsewhere.

The most distinctive regime is the behaviour of the rupture distributions for tip-surface distances below 45 nm. The mean binding forces increases with decreasing pulling velocity. The behaviour of these multiple parallel bonds is different from the dependence of the loading rate for single rupture bonds or existing models for multiple bonds [19–26]. This could be also described as a retardation effect. The first rupture occurs later (even in our normalised scale) at slower retraction rate. The complex link between the tip and the surface resists more and longer at slow retraction rate. This unexpected phenomenon is very likely to be due to a rebinding effect. Rebinding is highly favoured in this system since each accessible monomer of the adsorbed polyhistidine is a possible binding site. The 45 nm distance is likely to correspond to the Qiagen NTA-linker on the surface. The rebinding is also favoured at small separation when the linker relaxation and diffusion allows new binding sites to be found. Then a possible qualitative explanation of the unexpected dependence on retraction velocity would be that at slow velocity, more time is available for this diffusion process to take place.

The most important conclusions that we reach through this set of studies are: (i) We have developed a new way to analyse the AFM force measurements in order to extract relevant information and to characterise the interactions involving multiple bonds. Presentation of force measurements in 2D maps allows a more accurate and complete understanding than simple histograms. (ii) Our study reveals that in the presence of multiple parallel bonds with high rebinding probability, the force increases with decreasing speed velocity. We propose that the rebinding via the fluctuation of the linker is responsible for the observed behaviour. A complete understanding of the rebinding effect would require additional experiments by varying length and rigidity of the linkers.

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