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Single and multiple bonds in (strept)avidin-biotin interactions

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Avidin, streptavidin, biotin, single bond, multiple bond, interaction, lifetime, atomic force microscopy (AFM)

Abstract

Thanks to Dynamic Force Spectroscopy (DFS) and developments of massive data analysis tools such as YieldFinder, AFM becomes a powerful method for analyzing long lifetime ligand-receptor interactions. We have chosen the well-known system, (strept)avidin-biotin complex, as an experimental model due to the lack of consensus on interpretations of the rupture force spectrum (Walton *et al.*, 2008). We present a new measurement of force-displacement curves for the (strept)avidin-biotin complex. These data were analyzed using the YieldFinder software based on the Bell-Evans formalism. In addition, the Williams model was adopted to interpret the bonding state of the system. Our results indicate the presence of at least two energy barriers in two loading rate regimes. Combining with structural analysis, the energy barriers can be interpreted in a novel physico-chemical context as one inner barrier for H-bond ruptures ($\gamma < 1\text{\AA}$), and one outer barrier for escaping from the binding pocket blocked by the side chain of a symmetry-related Trp120 in the streptavidin tetramer. In each loading rate regime, the presence of multiple parallel bonds was implied by the Williams model. Interestingly, we found that in literature different terms created for addressing the apparent discrepancies in the results of avidin-biotin interactions can be reconciled by taking into account multiple parallel bonds.

Introduction

Short history of avidin-biotin interactions

Avidin

Avidin was discovered by Esmond E. Snell (Eakin *et al.*, 1940). The road to discovery has been described in details by N. Kresge (Kresge, 2004). It began with the observation on chicks that became deficient in biotin (vitamin B₇ or vitamin H) on a diet of raw egg-white, even though biotin was available in their diet. It was thought that a component in the egg-white was sequestering biotin. Therefore, the protein was tentatively named avidalbumin, yet later it has been revised to avidin due to its high affinity for biotin.

Avidin accumulates in albumen (egg white) and is produced in the oviducts of birds, reptiles and amphibians. In chicken egg white, avidin makes up approximately 0.05% of total proteins (about 1.8 mg per egg). Avidin is glycosylated (10% of the molecular weight) and positively charged; it has a pseudo-catalytic activity (i.e., enhancing alkaline hydrolysis of the ester chemical bond between biotin and a nitrophenyl group), and exhibits a high tendency towards aggregation. Avidin is a tetrameric protein (66-69 kDa in size (Korpela, 1984)) with four identical subunits, each of which can bind to biotin with high affinity and specificity. The apparent dissociation constant (K_d) of avidin is about 10⁻¹⁵ M, one of the strongest non-covalent interaction known (Green, 1963a; Green, 1963b; Green, 1963c; Melamed and Green, 1963).

Streptavidin

Streptavidin is a protein evolutionarily unrelated to avidin, it can be purified from the bacterium *Streptomyces avidinii*. Although the biological function of streptavidin remains

unknown, this molecule shows very similar properties to that of avidin. Streptavidin is a tetrameric protein of 52.8 kDa, it has been widely used in molecular biology because of its extraordinarily strong affinity for biotin (Wilchek and Bayer, 1990), standing together with avidin-biotin as the strongest non-covalent interactions in nature.

The secondary, tertiary and quaternary structures of streptavidin are almost identical to those of avidin despite they share only 30% of sequence identity. Compared to avidin, streptavidin has a slightly lower affinity for biotin ($K_d \sim 10^{-14}M$) with a mildly acidic isoelectric point (pI) of ~5. Taken together with the near-neutral pI and lacking of carbohydrate modification, streptavidin exerts much lower nonspecific binding than avidin to biotin. Nevertheless, deglycosylated avidin (NeutrAvidin) has comparable size, pI and nonspecific binding strength as streptavidin does.

Biotin

On account of Green's description (1975), avidin is only part of the great story of discovery, isolation and synthesis of biotin. Biotin was identified in 1935 by Koegl and Tönnis (Koegl and Toennis, 1936), it is a water-soluble member of B-complex vitamins, usually referred to as vitamin H; for more detailed stories about biotin, see D. Lane (Lane, 2004). The structure of biotin was established by du Vigneaud et al. in the beginning of the 40's (du Vigneaud, 1942). Biotin acts as a CO₂ carrier, and in the pyruvate carboxylase reaction, it is linked to the epsilon-amino group of a lysine residue in the enzyme. Biotin is a necessity for both metabolism and growth in humans, particularly regarding the niacin (vitamin B-3) metabolism and the production of fatty acids, antibodies as well as digestive enzymes.

In the early 60's, Green synthesized [2'-¹⁴C] biotin using ¹⁴CO and measured the rate constants of avidin-biotin association (k_{on}) and dissociation (k_{off}) reactions. He obtained the first

experimental results of k_{on} and k_{off} as $7 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$ and $9 \times 10^{-8} \text{ sec}^{-1}$, respectively. From these data, K_d ($k_{\text{off}}/k_{\text{on}}$) of the complex was estimated at about 10^{-15} M .

Into the three-dimensional space

The crystal structure of streptavidin bound with biotin was first determined in 1989 (Hendrickson *et al.*, 1989). The egg-white avidin in the deglycosylated form and complexed structure with biotin were determined in 1993 (Livnah *et al.*, 1993). Today, more than hundred structures of streptavidin and avidin are deposited in the Protein Data Bank (Berman *et al.*, 2000). Despite differences in the primary structure of the two proteins (~30% for sequence identity and 40% for similarity), both proteins have strikingly similar tertiary topology (β -barrels) and quaternary arrangements (homo-tetramer). In addition, both of them contain four biotin-binding sites wherein the interacting residues are also remarkably similar (**Fig. 1**). Moreover, a critical tryptophan (Trp-110 in avidin and Trp-120 in streptavidin), provided by an adjacent monomer, plays a pivotal role in binding affinity with biotin and in stability of the tetrameric structure (Freitag *et al.*, 1998; Laitinen *et al.*, 1999; Sano *et al.*, 1997). Avidin and streptavidin in their apo forms show a disordered loop L3,4 (i.e. the loop connecting strands β_3 and β_4), an element related to exposure of the biotin-binding site to solvent. Upon the binding of biotin, the lid-like loop adopts an ordered and closed conformation to bury the biotin molecule inside the binding site (Hendrickson *et al.*, 1989; Korndorfer and Skerra, 2002; Livnah *et al.*, 1993; Weber *et al.*, 1989) and render the ligand almost inaccessible to solvent. The binding process involves a highly stabilized network of polar and hydrophobic interactions. By counting the number of each interaction type, the presence of additional hydrophobic and hydrophilic groups in the binding site of avidin, while missing in streptavidin, may explain for its higher affinity constant.

The Avidin-biotin paradigm: How to measure strong interactions

The (strept)avidin-biotin complex allows the formation of multiple bonds due to its tetrameric structure, potentially four binding sites for biotin. The binding avidity could self explain the high affinity observed between the two interacting molecules (Green, 1963a; Green, 1963b; Green, 1963c; Melamed and Green, 1963; Piran and Riordan, 1990). The complex is extremely stable over a wide range of temperature and pH but can be reversibly unbound in water at elevated temperature (Holmberg *et al.*, 2005).

In spite of some recent reports (Butlin and Meares, 2006; Chmura *et al.*, 2001; Howarth *et al.*, 2006; Rao *et al.*, 1998), (strept)avidin-biotin interaction is still considered as having the highest affinity via non-covalent bonding and whose unique properties can be used to offer universal technology and applications. Therefore, the avidin-biotin complex has been used as a model in numerous experimentations for characterization of binding affinity. This has been studied by a large variety of methods and different experimental set-ups (**Fig. 2 and Table I**). Direct characterization of biological reactions with long lifetime (high affinity) is not easy in classical strategies. Most of published results were obtained from estimation or by indirect methods like competition assays, for example. Recently, techniques have been developed to reduce the lifetime of intermolecular binding by an external force, including Biomembrane Force Probe (BFP), micropipettes, Flux chamber and Atomic Force Microscopy (AFM).

However, characterization of the energy landscape of unbinding of (strept)avidin-biotin complex remains challenging. This system has been widely studied in many research groups and the results are not conclusive (**Table II**), indicating lacking for a consensus (Walton *et al.*, 2008). Hence, we attempt to re-characterize the energy landscape of this complex by

explicitly taking into account the presence of multiple parallel interacting bonding resulting in multiple ruptures, which was not considered and addressed clearly in previous studies. Our results indicate that the apparent discrepancies found in the literature may be simply attributed to underestimating the influence of multiple parallel bonding.

Material and Methods

Preparations of gold-coated substrates

A flat gold-coated glass surface was prepared with 11-mercaptoundecanoic acid (MUDA, Sigma-Aldrich) in ethanol (1 mg/mL) for 30 min at room temperature. No particular attention was paid to making well ordered self-assembled monolayers of MUDA. The substrate was rinsed with ethanol and then with water. The substrate was incubated in 1 mL of V/V NHS/EDC (4 mg/mL ethyl-N-[3-diethylaminopropyl] carbodiimide (EDC, PIERCE) and 0.6 mg/mL N-hydroxysuccinimide (NHS, PIERCE) in water for 30 min. Then, 10 μ g/mL streptavidin (Thermo scientific), avidin or BSA (Sigma-Aldrich) in acetate buffer (10 mM, pH 4) was deposited on the substrate surface for 60 min, followed by rinsing the substrate 3 times in the phosphate buffer (10 mM, 50 mM NaCl, pH 7).

Functionalization of gold-coated tips

Gold-coated OBL (Olympus, short tip: $k = 0.03$ to 0.009 N/m, long tip: $k = 0.006$ to 0.002 N/m) or NPG (Veeco, $k = 0.32$ to 0.06 N/m) cantilevers were used. The tip was activated with 1 mM cysteamine (Sigma-Aldrich) in ethanol during 30 min at room temperature. The cantilever was first rinsed once with ethanol and 3 times later with HEPES buffer (10 mM, 50 mM NaCl, pH 8). The coupling of biotin to the tip must be immediately performed using 50 mM of EZ-Link NHS-PEG12-Biotin (PIERCE) stock solution in DMSO that was diluted up to 0.1 μ M with the HEPES buffer for 60 min at room temperature. The cantilever was rinsed one time with 10 mM of HEPES buffer and then rinsed 3 times with phosphate buffer (10 mM, 50 mM NaCl, pH 7).

Experimental setups

A Dimension 3100 AFM microscope with a Nanoscope V controller (Digital Instrument Veeco, Santa Barbara, CA, USA) was used. The measurements of force–displacement curves for the molecular system were carried out using the pico-force mode of Nanoscope software (V7.30). In general, there are 5000 data points in each curve. We independently determined the spring constants of all individual gold-coated tips used (Olympus Biolever, Olympus—NPG Veeco) with the thermal tune module of the Nanoscope software. Calibration of a newly mounted tip was systematically performed. We obtained a wide range of loading rates, which were controlled through either the reverse velocity of the piezo scanner or the spring constant of the cantilever by a linear speed with a closed-loop scanner. The scan rate in this study ranged from 60 nm/s to 3 μ m/s.

Multiple parallel bonding and massive data analysis

Very few experiments have stressed on the study of multiple-bond ruptures (Erdmann *et al.*, 2008; Guo *et al.*, 2008; Hukkanen *et al.*, 2005; Levy and Maaloum, 2005; Odorico *et al.*, 2007b; Sulchek *et al.*, 2005; Teulon *et al.*, 2008; Teulon *et al.*, 2007; Tsukasaki *et al.*, 2007), a likely reason may be due to the difficulty in perceiving the distribution form of data that were collected and analyzed from the force-displacement (FD) curves obtained at different loading rates. The parameter, effective loading rate (r_e), is related to the critical force that ruptures the interaction between the functionalized tip and the molecules on the substrate surface (Odorico *et al.*, 2007b). Detected rupture events were sorted according to their r_e values, and the data from each consecutive interval of 100 r_e were fitted with multiple Gaussian functions.

Analysis of DFS measurements was done using the automatic software, YieldFinder. The treatments of FD records by YieldFinder are twofold: identify the rupture events and extract the force values responsible for the events (Odorico *et al.*, 2007a). From the data processed by YieldFinder, we derived the force constant of the transducer system (made of MUDA, avidin, and PEG-biotin) based on the series-parallel spring model and the effective loading rates (Erdmann, 2005). This information can be further transformed into the energy landscape that characterizes the association/dissociation reaction of the molecular system.

Specific experimental controls

The most common negative controls in the study of the (strept)avidin-biotin system include using non-functionalized tips or substrate surfaces, adding free ligands in the medium for blocking the interaction, and changing pH or salt concentrations in the medium. In this work, two negative controls were used (**Fig. 3**); one is to saturate the (strept)avidin-coated surface with 10 μ l of free Biotin solution (100 nM in phosphate buffer) to reach a final concentration of free Biotin around 5 nM; the other one consists in only coating BSA (Sigma-Adrich) on the substrate at 10 μ g/ml in acetate buffer (10 mM, pH 4) for 60 min without any (strept)avidin.

Results

According to the Bell-Evans model, the dissociation of two reactants catalyzed by an external force can be described by the transition state theory (Bell, 1978; Evans and Ritchie, 1997; Friedsam *et al.*, 2003; Guthold *et al.*, 2001; Strunz *et al.*, 2000; Tinoco and Bustamante, 2002). Provided with a linear force ramping, $F(t)=rt$, and a constant loading rate r , the most probable force to proceed the rupture event, i.e. the maximum of a probability distribution along the force ordinate axis, can be deduced from the classical kinetic theory as follows:

$$F^* = \frac{k_B T}{\gamma} \ln \frac{r_e \gamma}{k_0 k_B T} \quad (\text{eq. 1}),$$

where k_B is the Boltzmann constant, T is the temperature, γ is the energy barrier width, k_0 is the off-rate, and r_e is the effective loading rate. One may obtain the value of γ by plotting F^* against $\ln(r_e)$ and k_0 from the slope of the fitting line.

The experimental setups of the present work can be described as sketch #6 shown in Fig. 2. PEG-12 was chosen as the linker to attach biotin to the tip because it has a much shorter length than most of other linkers. Typical results of force-separation curves from the study system are shown in **Fig. 4A**, where the retract trace of the cantilever away from the substrate is plotted from the right to the left. As previously reported (see references in **Table I**), the forces determined to rupture both avidin-biotin and streptavidin-biotin bonds depend on the loading rate of the applied force. **Fig. 4B** shows the force histogram of rupture events, the black solid lines correspond to the Gaussian fitting results. The values of relevant parameters are co-listed in **Table II** with other published data. To provide a comparison with previous works, we selected rupture forces at a loading rate most often encountered in the Table II (around 1200 pN/s): F^*1 is 75 pN for avidin-biotin binding and 68 pN for streptavidin-biotin

complex. F^*1 indicates the most probable rupture force for a single (strept)avidin-biotin bond. Similarly, F^*2 and F^*3 indicate the most probable rupture forces for double and triple bonds. The Bell-Evans model revealed the presence of multiple segments (**Fig. 5A, B**), which is a characteristic of multiple bond ruptures. The parameters, γ in nm and k_{off} in s^{-1} , that characterize the energy landscape are presented in **Table II**. As observed in most other experiments on (strept)avidin-biotin binding rupture, the presence of two loading rate regimes also appears from our results. At low loading rates (55~1808 pN/s or 4~7.5 in $\ln(r_e)$) four distinct segments for avidin-biotin and only three for streptavidin-biotin were observed. At high loading rates (1808~162,755 pN/s or 7.5~12 in $\ln(r_e)$), five distinct segments were found for avidin-biotin but only four for streptavidin-biotin. Note that the first segments at both loading rates were attributed to non-specific interactions (purple lines in Fig. 5). The need for multi-line fittings in each regime is attributed to the presence of multiple bonds involved in the interactions between (strept)avidin and biotin (Erdmann *et al.*, 2008; Odorico *et al.*, 2007b; Sulchek *et al.*, 2005; Teulon *et al.*, 2007; Williams, 2003). As described in the introduction, (strept)avidin can simultaneously form bonding with up to four biotins, the maximum number of multiple bonds observed from our experiments (**Fig. 5**). However, depending on how (strept)avidin is oriented on the substrate plate, some biotin binding sites may be inaccessible to the biotinylated AFM tip.

Discussion

Caution for analyzing Force-Displacement data

Force-displacement (FD) or force-distance measure represents the raw AFM experimental data for studying the binding-unbinding processes of an intermolecular system. During the approach procedure, or the so-called binding process, some cares need to be taken for parameters such as contact time between the tip and the substrate, the force experienced at the contact point, and the density of receptors distributed over the substrate. For manifesting the specific interactions between a ligand and a receptor, one may choose an experimental condition in which enough contact time (but not too long), low force upon contact, and a flexible linker to attach the ligand and/or the receptor are demanded. On the other hand, how to detect and identify an unbinding event from a FD curve becomes a major issue in the procedure of tip retracting or called as the unbinding process. In particular, the identification of a rupture event corresponding to a specific interaction takes priority over searching for ruptures of single bond in FD measurements. Note that here single bond is specifically to indicate the interaction between one ligand and one receptor; one should not be confused with the definition in Chemistry such as covalent bonds, hydrogen bonds or non-bonded interactions.

Several criteria to identify rupture events of single bond along with the rupture force were set as according to : 1) In the retract trace, no specific rupture event should be detected before the linker molecule pulled at the maximum length; 2) the last rupture event was considered as the preceding event to the cantilever released from the surface; 3) Some small rupture peaks were discarded if their height is within the noisy range of the FD curve; 4) Starting and ending points of an event peak should be located on the baseline. Needless to say, a widely accepted set of rules is important to achieve consensus on research results in the community.

Unfortunately, only rare detail of systematic descriptions on this account is provided in literature. Another bottleneck in interpretations of DFS results on (strep)avidin-biotin interactions comes from the data fitting of force histograms. One may see later that statistically significant data points may play a critical role in accurately elucidating the energy landscape.

To obtain the values of most probable rupture force, Gaussian functions are in general introduced to fit the force histogram. However, the low number (~few hundreds) of collected measurements at a given loading rate may lead the fitting to a low degree of confidence. In addition, single-bond ruptures were usually the main pursuit. Accordingly, only a single Gaussian fit was applied in most of experiments and all the efforts were made to follow this pursuit. Nevertheless, a wide range of rupture force values was found for a single rupture peaks (Guo *et al.*, 2008). In another case where the delineation of multiple Gaussian fits was blurred at different loading rates, only the first population in different rate regimes was often picked up for interpretations (Taranta *et al.*, 2008). This arbitrary choice may mislead our understanding of intermolecular interactions and therefore hamper reliability of DFS results.

Discrepancies in published results

As described above, the data treatments of FD measurements would be enough to derive an apparent discrepancy in characterization of (strept)avidin-biotin interactions (**Table II**). In order to tackle this problem, we have overlaid the previous results (de Odrowaz Piramowicz *et al.*, 2006; Merkel *et al.*, 1999; Thormann *et al.*, 2006; Yuan *et al.*, 2000) based on the Bell-Evans model with the present results and show them in **Fig. 5**.

Most probable rupture forces obtained from Merkel *et al.* (1999) and Thormann *et al.* (2006) match those labeled single events in our study (**Fig. 5**, blue curves) whereas those obtained from Yuan *et al.* (2000) match rupture forces attributed to double or triple events (**Fig. 5**,

green and orange curves). Finally, most probable rupture forces obtained by de Odrowaz Piramowicz et al. (2006) match those obtained for triple or quadruple events (**Fig. 5**, orange and red curves). In fact, these results are easily explained by the experimental operating mode selected by these various authors (**Table II**). Indeed, experimental set-ups using (strept)avidin fixed on the substrate or on the pipette through PEG-biotins (**Fig. 2**, #8 and #10) led to single rupture events likely because (strept)avidin was attached using at least triple bonds leaving therefore only a single binding site for interaction. If it was not the case, singly or doubly biotin-bound (strept)avidin would have been displaced from the substrate ending up on the moving tip. This present work emphasizes that these experiments targeted mostly single rupture events.

An intermediate situation was found for the experimental set-up of Yuan et al. (2000) where the avidin was attached on the tip beforehand coated with biotinylated-BSA (**Fig. 2**, #1). Knowing the structure of BSA, it is unlikely that more than two biotins would be available for attaching (strept)avidin. Consequently, this system is favorable to the probing of double or triple rupture events as evidenced from our analysis (**Fig. 5**). Finally, experimental set-ups that chemically attach (strept)avidin on the substrate or the tip rather than using biotin (de Odrowaz Piramowicz *et al.*, 2006; and this work) allows the probing of all possible interactions ranging from single to quadruple events (**Fig. 5**). Although we cannot exclude the hypothesis that the tip interacts with more than one deposited (strept)avidin, this is rather unlikely since no rupture events greater than 4 were consistently observed in our study. Consequently, the great variation observed in the analysis of the energy landscape of (strept)avidin-biotin is mostly due to operational aspects, a well known trouble in affinity measurement (Azimzadeh, 1992).

Naturally, the discrepancy in the results of **Fig. 5** will directly cause variations on the deduced values of energy landscape parameters: the energy barrier width (0.1~30 Å) and the off-rate

constant ($10^{-9}\sim 13\text{ s}^{-1}$). One should be aware that a chemically linked biotin, e.g., with a PEG through an amide bond, exhibits different interactions from a free biotin, see **Figs. 6A and B**. Consequently, these factors may interfere with the estimated k_{off} , compared to that of free biotins in solution.

Energy landscape

According to the transition state theory, the attempt to escape from the bound state must overcome an energy barrier which is characterized by a width (γ) and a height (ΔG^\ddagger); as known, ΔG^\ddagger is the activation energy and the kinetics dissociation rate $k_{\text{off}} = 1/\tau_0 \exp[-\Delta G^\ddagger/k_{\text{B}}T]$, where τ_0 the reciprocal of the attempt-to-escape frequency, k_{B} the Boltzmann constant and T the temperature. The Bell-Evans model yields a linear relationship between the most probable rupture force and the logarithm of loading rate (see eq. 1). However, most studies on (strept)avidin-biotin interactions have revealed a non-linear tendency. This finding has been reasoned in literature mainly based on four aspects: 1) the presence of multiple energy barriers (Merkel *et al.*, 1999); 2) an asymmetric shape of the energy barrier, implying a relation such as $F^*\sim \ln(V)^{2/3}$ where V is the pulling velocity (Dudko *et al.*, 2003); 3) the effect of multiple parallel bonds (Williams, 2003); 4) artifacts from the data processing (Li *et al.*, 2010). One may not exclude the possibility that the non-linearity can also come from a composite effect of the four factors mentioned above.

Two barriers were postulated in the energy landscape for unbinding the streptavidin-biotin complex (Merkel *et al.*, 1999) in agreement with kinetics of the exchange between unlabelled biotin and [^{14}C]biotin bound to avidin which revealed a biphasic dissociation phase (Green, 1963a). In contrast, some other authors insisted that the assumption of two energy barriers used in characterizing the energy landscape can be potentially misleading (Derenyi *et al.*, 2004; Neuert *et al.*, 2006). This reflects that the simplistic model of two energy barriers may

be insufficient to interpret the underlying dissociation mechanism. Molecular dynamics and some hybrid simulations have also revealed several slips, suggesting a variety of unbinding pathways with multiple energy barriers (Galligan *et al.*, 2001; Izrailev *et al.*, 1997; Zhou *et al.*, 2006).

In this work, the non-linear tendency was observed in our data analysis, see **Fig. 5**. A clear cutoff occurs at 1808 pN/s and 2208 pN/s for avidin-biotin and for streptavidin-biotin unbinding, respectively. Within each regime of loading rate, no severe non-linearity was observed on the Bell-Evans plot. Then we employed the Williams formalism (Williams, 2003) to extract information of multiple parallel bonds from each separate regime of loading rate. Thereby, a curvature found from data treatments of force normalization indicates the characteristic of multiple parallel bonds. The results for streptavidin-biotin are shown in **Figure 5C**. One advantage of applying this formalism is to assign a multiplicity factor to each regression fit that highlights the non-specific interactions (the lowest fitting curves in the plots). Taking together with the Bell-Evans model and molecular structure analysis, we conclude that in the energy landscape two energy barriers are assumed in the unbinding process for the (strept)avidin-biotin complex.

The pathway of dissociation reactions

In the energy landscape of a dissociation reaction, if two energy barriers were found, the inner barrier is usually referred to a binding state close to the bound conformation, while the outer barrier marks the last state before the unbound conformation. The function of an external force on intermolecular interactions is equivalent to a catalyst that reduces the activation energy associated with an energy barrier. Thereby, if the applied force along the reaction coordinate is high enough, some energy barriers may disappear as discussed previously

(Evans and Ritchie, 1997; Merkel *et al.*, 1999). In short, at high loading rate the height of the outermost barrier is strongly reduced and therefore only the inner barrier is probed in the energy landscape.

Identification of unbinding processes has been performed using molecular dynamic simulations by counting, for instance, the hydrogen-bond (H-bond) ruptures (Zhou *et al.*, 2006); however, identification of transition barriers remains challenging. A consensus emerges on multiple studies of (strept)avidin-biotin interactions by DFS experiments: at least two energy barriers exist in the energy profile, one with a very narrow width (≤ 1 Å) and the other with a larger one (≥ 4 Å). The head chemical group of biotin contains limited degree of freedom while the carboxylic tail is extendable into the binding pocket (**Figs. 6 A, B**). Consequently, the narrow inner energy barrier is attributed to the event involving in breaking the H-bonds between biotin and streptavidin as described as follows (**Fig. 6C**). By moving only 1 Å away from the bound conformation, the H-bond distance between the head chemical group of biotin and streptavidin is out of the range, 1.9 ± 0.5 Å, which defines the classical H-bond geometry, thus considered as ruptured. This doesn't imply that biotin totally escape from the binding pocket. On the structural view (**Fig. 6D**), the steric effects exerted from Trp120 (Trp110 in avidin) on biotin during the unbinding process prevent biotin to escape. Consequently, the outer barrier characterized by a width of 4 Å likely underlies the release mechanism of biotin passing over the Trp120 side chain. The visual inspection on the structure detects no further obstacle and biotin sets free from the gate of the binding pocket guarded by the Trp. This hypothesized mechanism can be tested by mutagenesis experiments. Mutating Trp120 to Phe has already been performed (Yuan *et al.*, 2000) and DFS results showed that the mutation not only reduced the outer energy barrier width but also increased the kinetic dissociation rate constant (lower affinity). The first observation is attributed to shrinking the Trp bulky side chain. The second one reflects that Phe raises the ease for biotin

to run away from the binding pocket with smaller activation energy. In addition, the well-known loop L3,4 of (strept)avidin may contribute to the dynamic behavior of biotin through a H-bond network (**Fig. 6A**) in the unbinding process (Merkel *et al.*, 1999; Yuan *et al.*, 2000) although the loop is expected to have a great impact on the association process.

Conclusion

In this paper, we provide an interpretation of discrepancies in previous studies, the so-called (strept)avidin-biotin paradox, by assuming the presence of multiple parallel bonds. The results for this system have been re-interpreted several times, including invoking the force history dependence for the largely scattering data obtained from different groups (Marshall *et al.*, 2005; Pincet and Husson, 2005), the experimental setups (Walton *et al.*, 2008) as well as data treatments (Guo, 2010). From the present DFS results, the dissociation of (strept)avidin-biotin complex undergoes two energy barriers which underlie two stages for the ligand to escape from the trap, i.e., the binding pocket. In addition, the dissociation reaction involves breaking multiple parallel bonds, reflecting the structural character of (strept)avidin tetramer. It is noteworthy that molecular modeling is very helpful to elucidate the unbinding mechanism of ligand-receptor interactions by DFS experiments.

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Legend of Figures

Fig. 1: The tetrameric structure of streptavidin-biotin complex. The quaternary arrangements of the streptavidin tetramer were built based on the crystal structure (PDB code 2IZF, a dimeric form), drawn in blue and cyan (chains B and D, respectively). The other dimer (chains B' and D'), colored in red and orange, was constructed based on the symmetric rule defined in 2IZF. The biotin is drawn in colored CPK: grey for carbons, blue for nitrogens, red for oxygens, yellow for sulfurs, and white for hydrogens. The biotin from chain B is chemically linked to a 12-mer PEG linker. Each biotin binding pocket is closed by a Trp side chain (drawn in sticks) from another chain. Inset: schematic representation of the relative orientation of four biotin binding sites in streptavidin, a global view on accessibility of these binding sites. Figure drawn using VMD (Humphrey *et al.*, 1996).

Fig. 2: Summary of main constructions encountered in literature for measuring avidin-biotin interactions. AFM tips are represented by gray-colored triangles. Substrates made of mica or glasses are drawn as thin rectangles and that of agarose beads are presented as hemispheres. The cross-linkers such as glutaraldehyde are explicitly indicated. Sketch #9 describes laminar flow chamber equipments with a cyan-colored optical camera. Sketch #10 represents Biomembrane Force Probe (BFP) with biotin on the surface, and avidin is held by biotin which is covalently attached to the linkers on the micro-beads at the tip of the pipettes.

Fig. 3: Three setups are presented to measure the rupture events of non-specific interactions. Each setup associated with its results was specified by a different color. The green box and the green histogram correspond to “positive” ruptures events: events attributed to specific interactions between biotin and streptavidin. In red, the design of experiment aims to inhibit

the specific interaction between biotin and streptavidin by adding 0.1 μ M of free biotin in the medium (in blue color) to saturate the binding sites of the receptor. The last negative control is presented in purple, where the streptavidin molecules were replaced by bovine serum albumin (blue star-shaped symbols). 1000~3000 experiments were performed for each setup. The calculated effective loading rates ranged from 3294 pN/s ($\ln(r_e) = 8.1$) down to 3 pN/s ($\ln(r_e) = 1.1$) for purple, to 4.48 pN/s ($\ln(r_e) = 1.5$) for green and to 9 pN/s ($\ln(r_e) = 2.2$) for red.

Fig. 4: (A) Left, set of 15 force-separation curves that were extracted from experiments on the study of streptavidin-biotin interactions using Nanoscope software. On the right, most of rupture events occurred at a distance of 12 ± 8 nm representing 73% of rupture events in a set of 350 randomly chosen events. (B) Distribution of force histograms of rupture events for streptavidin-biotin binding. The results were obtained from the experimental setup #6 (**Fig. 2**) at loading rates 221 pN/s ($\ln(r_e) = 5.4$), 1212 pN/s ($\ln(r_e) = 7.1$), and 13,360 pN/s ($\ln(r_e) = 9.5$), respectively. As indicated at the maximum of the Gaussian functions, the most probable forces (F^*) are determined.

Fig. 5: Plots of the most probable rupture forces F^* against the logarithm of effective loading rates $\ln(r_e)$. (A) Streptavidin-biotin interactions. (B) Avidin-Biotin interactions. The main plots for each system were made according to the Bell-Evans model. The most probable rupture forces are obtained within the 95% confidence intervals. Horizontal bars in B) correspond to the dispersion of the loading rates from the distribution analysis. In both plots, two loading rate regimes are distinguishable. The outer energy barrier of dissociations for the two systems can be probed in the low loading rate regime whereas the inner barrier can be detected in the high loading rate regime. (C) Application of the Williams' formalism that characterizes the presence of multiple bonds by non-linear fitting curves for the streptavidin-

biotin complex at low loading rate (left) and high loading rate (right). The fitting lines are enumerated from the bottom to the top and kinetic parameters (k_{off} and γ) used to fit these lines are indicated on the top. It shows that the first lines in purple remained systematically unfit and therefore were attributed to non-specific rupture events. The remaining fits in blue, green, orange and red correspond to single, double, triple and quadruple parallel bonds. Black solid lines represent the fittings from the previously published results using the Bell-Evans model. ▼ (de Odrowaz Piramowicz *et al.*, 2006); ■ (Yuan *et al.*, 2000); ● (Thormann *et al.*, 2006); * (Merkel *et al.*, 1999).

Fig. 6: Structural analysis on streptavidin-biotin interactions. The PDB code for the crystal structure of streptavidin presented is 2IZF. Secondary structures are specified by arrows (β -strand); and turns/coils by tubes. Monomer D is drawn in cyan and Trp120 from the neighboring monomer is in purple. The ligand, biotin, is drawn as balls (colored atoms) and sticks (in orange). Side chains of monomer D are displayed by balls (colored atoms) and sticks (in cyan). (A) The structure of the biotin binding pocket in monomer D of streptavidin. The H-bond interactions are drawn in green dots. The number in the rectangles indicates the H-bond distance between streptavidin and biotin. The acceptable range of H-bond distance between the acceptor and hydrogen is within 2.5 Å. (B) Same as A except that the formation of amide bond between biotin and the PEG linker withdraws one native H-bond with streptavidin. For orientation purpose, the top and the bottom of the binding pocket is indicated. (C) Same as B but biotin was moved outwardly from the bottom of the binding pocket by about 1Å, leading to ruptures of all the H-bond interactions between biotin and the bottom of the binding pocket. However, biotin is still blocked by the side chain of Trp120 from a neighboring monomer. For comparison, the trace of the original position of biotin is illustrated in thin orange color. (D) Same as B but biotin was moved farther, about 4Å away

from the bottom of the pocket. No H-bonding was found and biotin overcame the blockage of Trp120 from a neighboring monomer. Figure drawn using Molscript (Kraulis, 1991) and rendered using Raster3D (Merritt and Bacon, 1997).

Figure 1

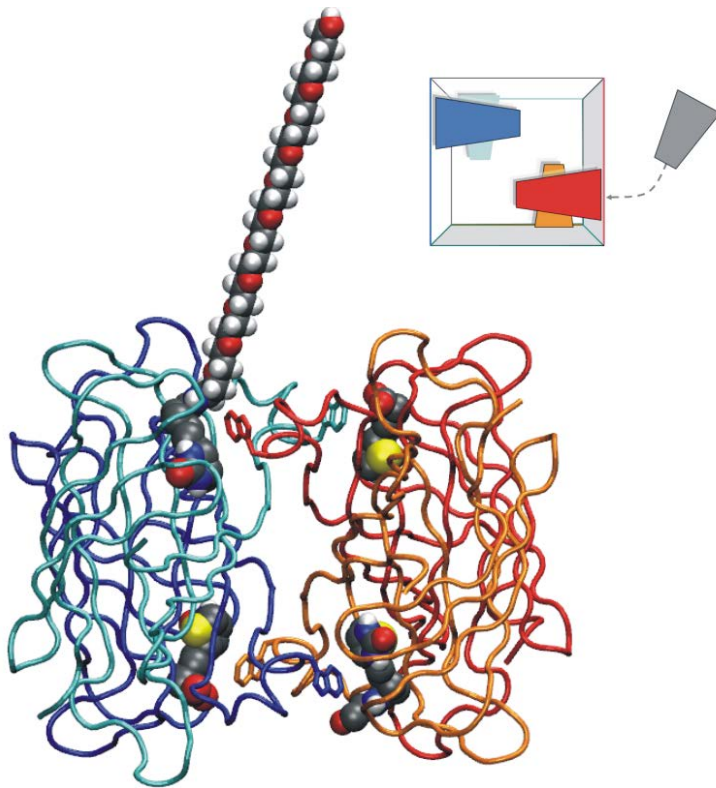


Figure 2

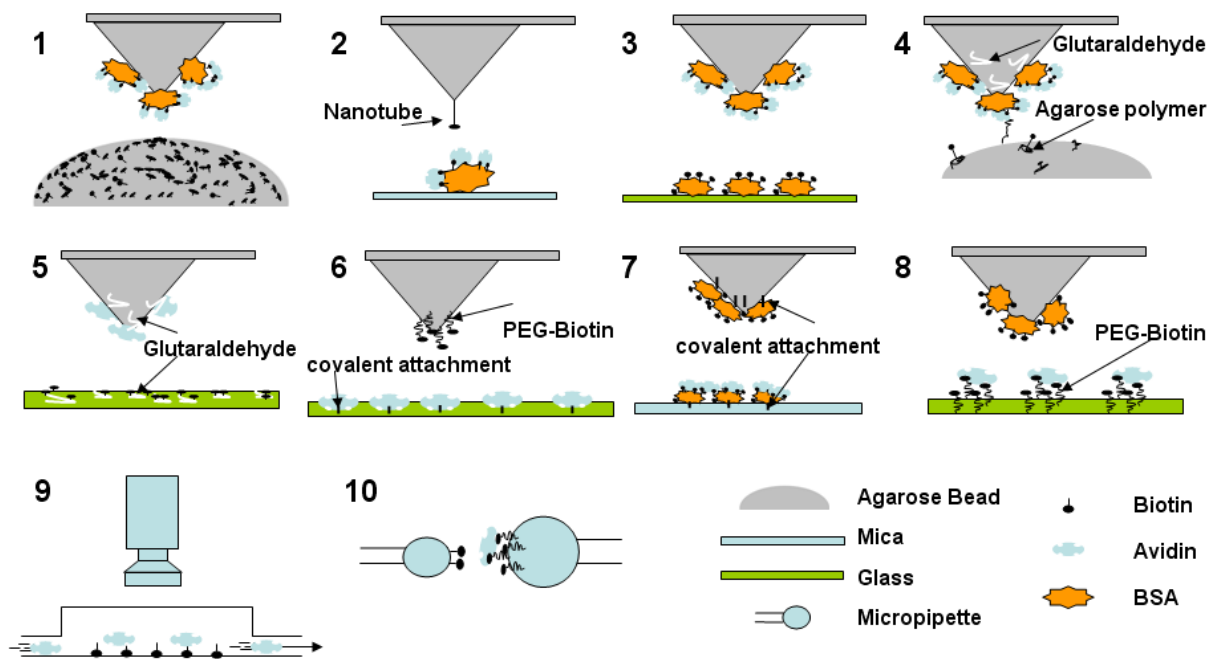


Figure 3

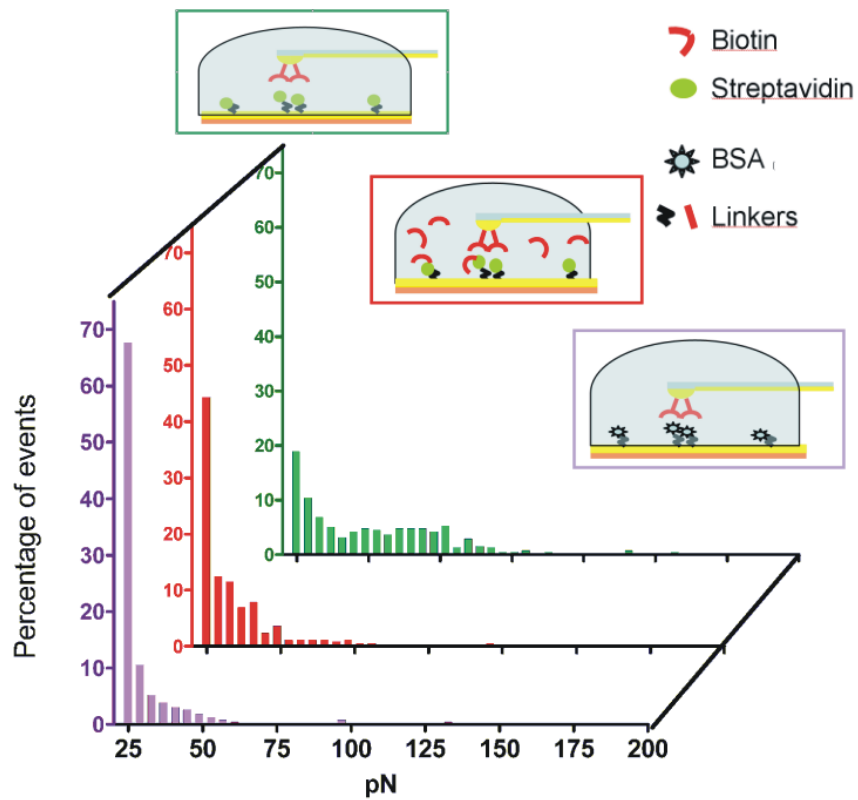


Figure 4

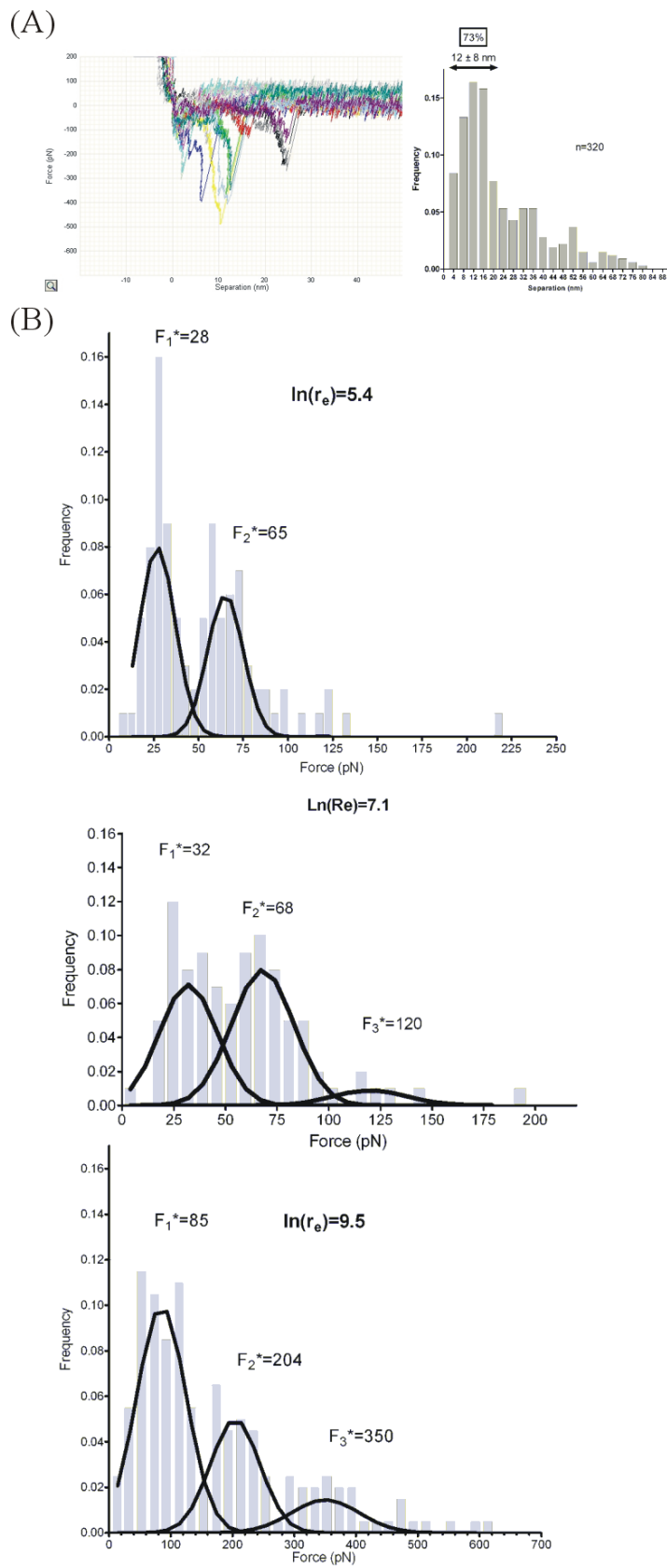


Figure 5

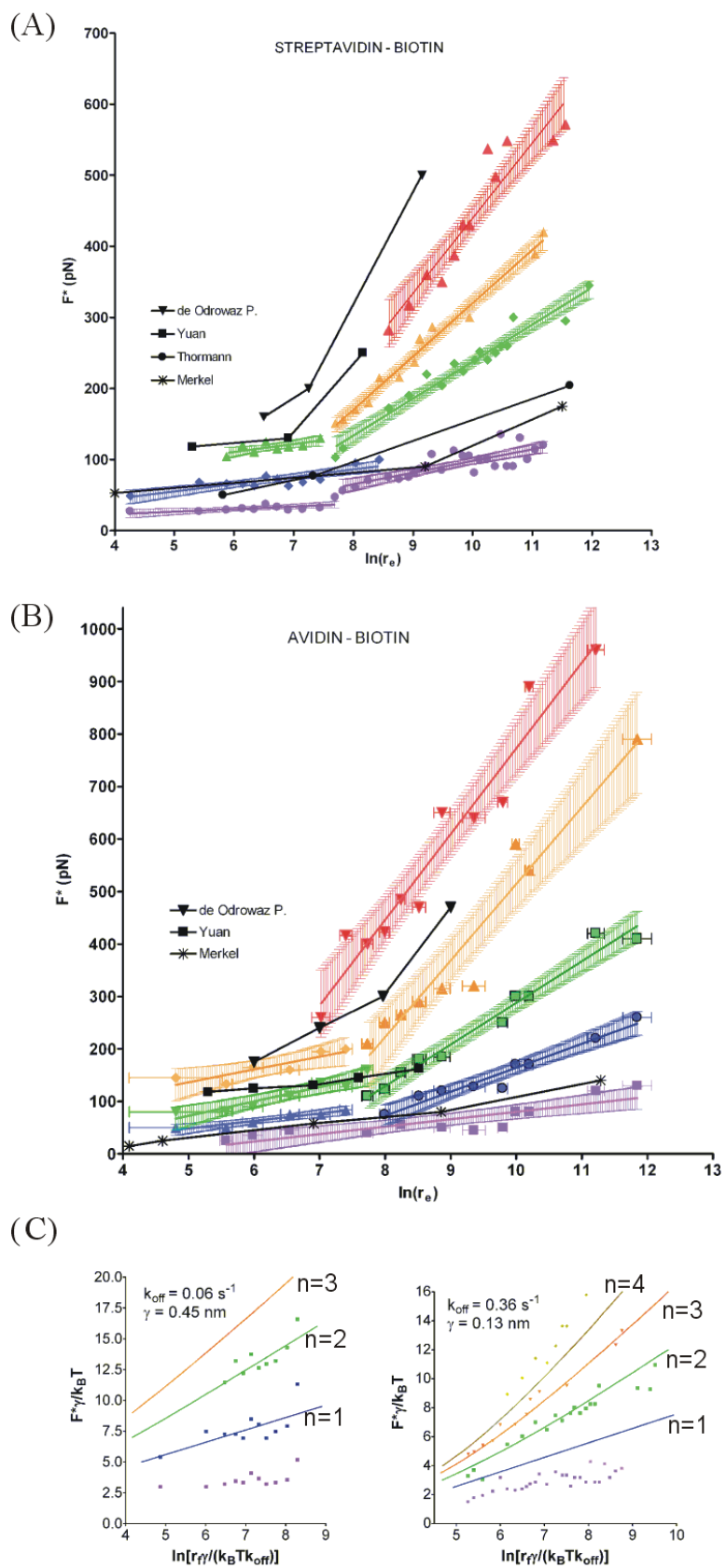


Figure 6

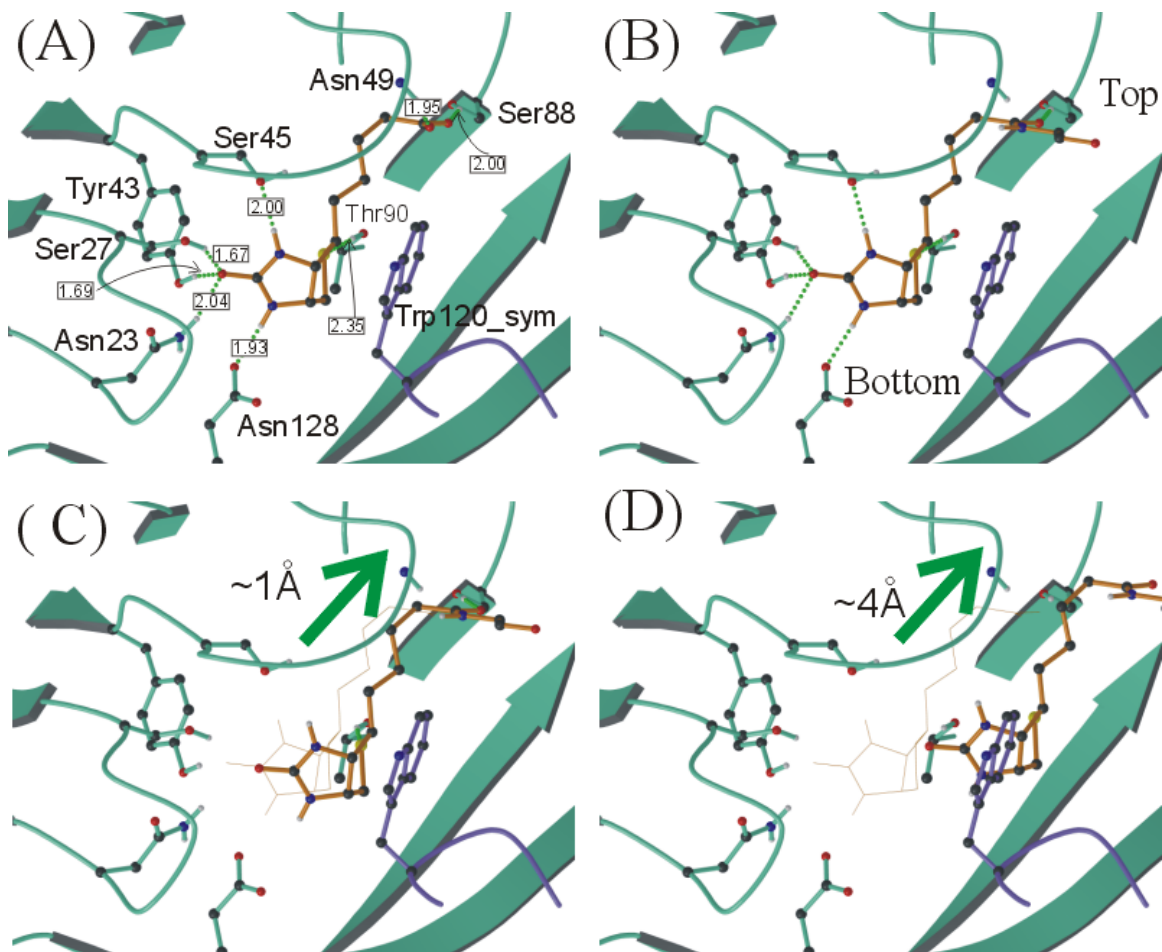


Table I: Detail of the various experimental set-ups used to measure (strept)avidinbiotin interactions using AFM

Reference	Tip				Substrate			Controls	Buffer		Scheme # (Fig. 2)
	type	k (pN/nm)	linker	Object	Type	Linker	Object		pH	Conc	
(Florin <i>et al.</i> , 1994)	Si ₃ N ₄	61±10	BBSA ^a	Avidin	Agarose		Biotin	Saturation with biotin and avidin control BSA	7.4	140 mM NaCl	1
(Moy <i>et al.</i> , 1994a)	Si ₃ N ₄	?	BBSA ^a	Avidin	Agarose		Biotin		4 - 7 -10	140 mM NaCl	1
(Wong <i>et al.</i> , 1998)	Nanotube	500–5000		5-(biotinamido)pentylamine	Mica	Adsorption	Streptavidin	Unfunctionalized nanotube	7	PBS ^c	2
(Yuan <i>et al.</i> , 2000)	Si ₃ N ₄	10-50	BBSA ^a	Streptavidin	Agarose		Biotin	Avidin free -biotin free	7.2	PBS	1
(Lo <i>et al.</i> , 2001)	Si ₃ N ₄	39±3	BBSA ^a	Streptavidin	Glass	BBSA ^a		Biotin-biotin bsa-bsa	7		3
(Wong <i>et al.</i> , 1999)	Si ₃ N ₄	60	BBSA ^a Glutaraldehyde	Avidin	Agarose	Agarose (100nm)	Biotin	Saturation with biotin and avidin Stability of the measure	7.2		4
(Lo <i>et al.</i> , 1999)	Si ₃ N ₄	39; 150	BBSA ^a	(strept)avidin	Glass	BBSA ^a		Free avidin-streptavidin		PBS	3
(Zhang and Moy, 2003)	Si ₃ N ₄	~10	BBSA ^a	(strept)avidin	Agarose		Biotin		10-6		1
(de Odrowaz Piramowicz <i>et al.</i> , 2006)	Si ₃ N ₄	10; 400	Glutaraldehyde	(strept)avidin	Glass	Glutaraldehyde	Biotin		7.2	150 mM NaCl	5
(Rico and Moy, 2007)	Si ₃ N ₄	10	BBSA ^a	Streptavidin	Agarose		Biotin			PBS	1
(Guo <i>et al.</i> , 2008)	Si ₃ N ₄	155; 70; 260; 270	PEG ^b	Biotin	Glass	Ethanolamine	Streptavidin		7	PBS	6
(Walton <i>et al.</i> , 2008)	Si ₃ N ₄	11; 35; 58; 121	BBSA ^a		Mica	BBSA ^a	Streptavidin			PBS	7
(Thormann <i>et al.</i> , 2006)	Si ₃ N ₄	10-100	BBSA ^a		Glass	PEG ^b -biotin BBSA ^a	Streptavidin			PBS	8 3

^aBiotinylated bovine serum albumin (BBSA)

^bpolyethylene glycol (PEG)

^cPBS: 8 g/l NaCl; 0.2 g/l KCl; 1.44 g/l Na₂HPO₄; 0.24 g/l KH₂PO₄; pH 7.4

Table II: Published data on (strept)avidin-biotin interactions studied by AFM or related techniques

Molecular partners Substrate - AFM tip	Scheme (Fig.2)	Average forces (pN)	Loading rate (pN/s)	γ (nm) ^a	k_0 (s ⁻¹) ^b	ΔG^\ddagger (kJ·mol ⁻¹) ^c	Ref.
Avidin - biotin	4	160 ± 20 125 ± 25 85 ± 15		0.9			(Florin <i>et al.</i> , 1994)
Streptavidin /Biotinylated BSA	3	340 ± 120					(Lee <i>et al.</i> , 1994)
Avidin - biotin	1	175 pN				88	(Moy <i>et al.</i> , 1994b)
Avidin - biotin (1) Iminobiotin (2) Streptavidin - biotin (3) Avidin/desthiobiotin (4) Streptavidin - biotin (5)	8	1) 160 ± 20 2) 85 ± 10 3) 257 ± 25 4) 94 ± 10 5) 257 ± 25					(Moy <i>et al.</i> , 1994a)
Streptavidin - Biotinylated BSA	3	253 <input type="checkbox"/> 393 <input type="checkbox"/>				76.49	(Chilkoti <i>et al.</i> , 1995)
Streptavidin - biotin	2	~200 (single)					(Wong <i>et al.</i> , 1998)
Streptavidin - biotinylated BSA	10	Bond strength ↑ 5pN to 170pN	0.05-10000 10000-60000	0.5 0.12	6.14·10 ⁻⁵ 2.9		(Merkel <i>et al.</i> , 1999)
Avidin - biotin	10		0.05-30 30-10000 10000-60000	3.0 0.3 to 0.5 0.12	0.74 2.9		(Merkel <i>et al.</i> , 1999)
Avidin - biotin Biotin - streptavidin	7	173 ± 19 326 ± 33	2·10 ⁵ - 8·10 ⁵				(Lo <i>et al.</i> , 1999)
Streptavidin - biotin	1	126 ± 2.3 207 ± 5.8	198 2300 100-1000 1000-5000	0.49 (outer) 0.05 (inner)	1.67·10 ⁻⁵ 2.09		(Yuan <i>et al.</i> , 2000)
Avidin - biotin	1		100-1000 1000-5000	0.53 (outer) 0.2 (inner)	6.45·10 ⁻⁶ 0.08		(Yuan <i>et al.</i> , 2000)
Avidin - biotin	6/7	44 ± 3	1600	2 0.3	10 ⁻³ 10		(DeParis <i>et al.</i> , 2000)
Streptavidin - Biotin	N/A ^d		2·10 ⁸ to 55·10 ⁹	0.56 0.48 0.23 0.12		85.69 ^f 77.33 36.37 7.11	(Galligan <i>et al.</i> , 2001)
Streptavidin - Biotinylated BSA	7	167 ± 20 236 ± 26 289 ± 13 350 ± 30 442 ± 17	39,000 390,000 1,950,000 3,740,000 6,280,000				(Lo <i>et al.</i> , 2001)
Streptavidin - biotin	4	150 to 500 pN	10 ³ to 10 ⁴				(Zhang and Moy, 2003)
Streptavidin - biotin	5	200	1267 300-1700 1700-9600	0.081 ± 0.002 0.024 ± 0.003	0.56 ± 0.46 2.98 ± 2.61		(de Odrowaz Piramowicz <i>et al.</i> , 2006)
Avidin - biotin	5	236	1382 300-1700 1700-9600	0.073±0.002 0.024±0.002	0.25 ± 0.18 2.97 ± 2.71		(de Odrowaz Piramowicz <i>et al.</i> , 2006)
Streptavidin - biotin	N/A ^d	263 ± 36 378 ± 42 494 ± 39	3,900 39,000 1,950,000				(Zhou <i>et al.</i> , 2006)
Streptavidin - biotin	8		~200- 113,000	0.64 0.072 (inner) 0.54	4·10 ⁻⁴ 13.46 0.11		(Thormann <i>et al.</i> , 2006)
Streptavidin - biotin	4	17°C 72 ± 2 95 ± 3 177 ± 4 37°C 27 ± 1 66 ± 2	338 ± 7 5977 ± 65 65,602 ± 758 387 ± 9 6997 ± 94 69,457 ± 1055	17°C 0.35 ± 0.27 (outer) 17°C 0.09 ± 0.02 (inner) 24°C 0.38 ± 0.21 (outer) 24°C 0.09 ± 0.03 (inner) 37°C 0.31 ± 0.07 (outer) 37°C 0.15 ± 0.05 (inner)	0.02 ± 0.13 12 ± 9 0.10 ± 0.33 23 ± 16 1.22 ± 0.93 15 ± 1 7		(Rico and Moy, 2007)

		121 ± 4					
Streptavidin - biotin	6			0.40 ± 0.05 0.44 ± 0.06	0.23 0.27	66.4 ± 1.6^e 66.0 ± 1.6	(Guo <i>et al.</i> , 2008)
Streptavidin - biotin	N/A ^d	46	301	0.05 (MD) 0.11 (AFM)	$5.1 \cdot 10^{-9}$ to $2.1 \cdot 10^{-7}$		(Walton <i>et al.</i> , 2008)
Avidin - biotin	6	75 (F*1) 135 (F*2) 195 (F*3) 260 (F*4)	1212 122-1808 122-1808 122-1808 1808- 133,252 1808- 133,252 1808- 133,252 1808- 133,252	 0.29 \pm 0.05 (F*1 outer) 0.15 \pm 0.02 (F*2 outer) 0.17 \pm 0.05 (F*3 outer) 0.09 \pm 0.01 (F*1 inner) 0.05 \pm 0.003 (F*2 inner) 0.03 \pm 0.003 (F*3 inner) 0.03 \pm 0.002 (F*4 inner)	 0.35 \pm 0.35 ^c 0.38 \pm 0.40 0.02 \pm 0.04 13.07 \pm 11.19 7.34 \pm 3.99 4.43 \pm 3.94 1.19 \pm 0.86		This work
Streptavidin - biotin	6	68 (F*1) 120 (F*2) 260 (F*3)	1153 67-2208 67-2208 2208- 162,755 2208- 162,755 2208- 162,755	 0.40 \pm 0.08 (F*1 outer) 0.37 \pm 0.14 (F*2 outer) 0.08 \pm 0.004 (F*1 inner) 0.05 \pm 0.002 (F*2 inner) 0.04 \pm 0.004 (F*3 inner)	 0.07 \pm 0.09 0.002 \pm 0.005 4.38 \pm 2.47 4.09 \pm 1.61 3.26 \pm 3.54		This work

^a Energy barrier width in nm according to the Bell-Evans formalism

^b Kinetic dissociation rate constant in s⁻¹ which is related to the energy barrier height

^c Kinetic activation energy in kJ/mol.

^d Molecular dynamic simulations

^e σ_{γ} and $\sigma_{k_{off}}$ are respectively the standard deviation of the Bell distance and of the constant of dissociation calculated according to Björnham et al (2009).

^f To calculate the activation energy from k_0 , the reciprocal of the attempt-to-escape frequency ($1/t_0$) was 10^9 s⁻¹

^g To calculate the activation energy from k_0 , the reciprocal of the attempt-to-escape frequency ($1/t_0$) was 10^{11} s⁻¹