

Dynamic force measurements of avidin–biotin and streptavidin–biotin interactions using AFM

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Using atomic force microscopy (AFM) we performed dynamic force measurements of the adhesive forces in two model systems: avidin–biotin and streptavidin–biotin. In our experiments we used glutaraldehyde for immobilization of (strept)avidin on the tip and biotin on the sample surface. Such interface layers are more rigid than those usually reported in the literature for AFM studies, when (strept)avidin is coupled with biotinylated bovine albumin and biotin with agarose polymers. We determined the dependence of the rupture forces of avidin–biotin and streptavidin–biotin bonds in the range 300–9600 pN/s. The slope of a semilogarithmic plot of this relation changes at about 1700 pN/s. The existence of two different regimes indicates the presence of two activation barriers of these complexes during the dissociation process. The dissociation rates and activation energy barriers, calculated from the Bell model, for the avidin–biotin and streptavidin–biotin interactions are similar to each other for loading rates > 1700 pN/s but they are different from each other for loading rates < 1700 pN/s. In the latter case, the dissociation rates show a higher stability of the avidin–biotin complex than the streptavidin–biotin complex due to a larger outer activation barrier of $0.8 k_B T$. The bond-rupture force is about 20 pN higher for the avidin–biotin pair than for the streptavidin–biotin pair for loading rates < 1700 pN/s. These two experimental observations are in agreement with the known structural differences between the biotin binding pocket of avidin and of streptavidin.

Keywords: avidin, streptavidin, biotin, atomic force microscopy, rupture force, dissociation rate, loading rate

Living organisms are complex highly ordered systems. Physico-chemical interactions between their arranged components regulate the living processes on a molecular level. The equilibrium between the repulsive and attractive forces determines the minimum of the potential net energy for a given complex of molecules. It characterizes the stability of such a ligand–receptor system at certain environmental conditions. Attractive forces, which bring the molecules together, are divided into strong and weak interactions. Strong ionic and covalent bonds have fixed angles and lengths and often store chemical energy.

Energies of hetero- and monopolar interactions are of the order of 100–1000 kJ/mol. Energies of weak attractions (van der Waals forces, ion-dipole forces, hydrogen bonds) are an order of magnitude smaller. Although these interactions are relatively weak, they are important because of their numbers.

Weak bonds are responsible for intermolecular and intramolecular attractions of biological molecules and therefore they are of great importance (Fersht, 1985; Dammer *et al.*, 1996; Bongrand, 1999). For example, recognition of substrate by enzyme, antigen by antibody or neurotransmitter by neurore-

Abbreviations: AFM, atomic force microscopy; APTES-(3-aminopropyl)triethoxysilane; BFP, biomembrane force probe; F_y , bond rupture force; k , dissociation rate in the presence of an external force; k_B , Boltzmann constant; k_c , spring constant of cantilever; k_{eff} , effective spring constant; k_s , spring constant of elastic substrates and covers; k_0 , dissociation rate in the absence of an external force; L_v , force loading rate; ML, microlever; OT, optical tweezers; PBS, phosphate-buffered saline; r_0 , width of potential energy well; T , absolute temperature; UL, ultralever.

ceptor rely on weak, non-covalent binding forces. The architecture of the participants and their flexibility are important factors in this process. Investigations of the reversibility and specificity of ligand–receptor interactions are essential to fully understand the formation and dissociation of individual bonds in more complex biosystems.

There are several biophysical methods allowing direct measurements of ligand–receptor interactions, for example: optical tweezers (OT) (Askin & Dziejic, 1987), biomembrane force probe (BFP) (Evans *et al.*, 1995), magnetic torsion device (Guttenberg *et al.*, 2000) or atomic force microscopy (AFM) (Binning *et al.*, 1986). Using AFM equipped with a liquid-cell one can measure short range forces in biological systems under physiological conditions. Typical detected forces are in the range from a few pN to several μ N. Moreover, this method allows one to measure molecular binding forces between a small number of various ligand and receptor molecules because of the size of the AFM cantilever. One of the interacting particles is immobilized on the cantilever and the other on a surface. The adhesion is probed with force–distance curves. The rupture (unbinding) force, being a measure of the interaction between the ligand and the receptor, is detected upon withdrawal of the cantilever from the surface.

In this paper we present dynamic studies of the unbinding force in two model systems for protein–ligand interaction systems: avidin–biotin and streptavidin–biotin. Chicken avidin and bacterial streptavidin proteins are tetrameric molecules having a molecular mass of about 60 kDa and 66 kDa, respectively. Both proteins contain four identical subunits, each with one binding site for biotin. These proteins are so interesting because of their extremely high binding affinity for biotin (vitamin H) (Green, 1975; Lindqvist & Schneider, 1996). In the avidin–biotin and streptavidin–biotin complexes the dissociation constants of the range of 10^{-15} M⁻¹ are much higher than the affinities detected for very stable antigen–antibody complexes (10^{-6} – 10^{-9} M⁻¹). Van der Waals forces between biotin and the aromatic side chains of the binding residues of the proteins, an extreme network of hydrogen bonds between the protein pockets and the ligand, and the rearrangement of L3,4 loops of the proteins upon biotin binding are the recognized interactions mainly contributing to the extremely tight biotin bonds with avidin and streptavidin (Freitag *et al.*, 1997; Livnah *et al.*, 1993; Grubmüller *et al.*, 1996). These specific interactions have been utilized in different applications in medicine and biotechnology (Bayer & Wichel, 1990; Shettlers, 1996; Wilchek & Bayer, 1999; Akahara & Soga, 1999).

Measurements of the unbinding forces between (strept)avidin and biotin (or modified biotin

forms) using AFM dynamic force spectroscopy have already been performed by several groups (Florin *et al.*, 1994; Allen *et al.*, 1996; Merkel *et al.*, 1999; Wong *et al.*, 1999; Yuan *et al.*, 2000; Lo *et al.*, 2001; Zhang & Moy, 2003). However, there are some discrepancies in the observed relations between the measured bond rupture forces and the force loading rates (retraction rates) for the analyzed complexes. One suspects that experimental conditions, namely the multiple links between the adsorption surface and the proteins as well as the flexibility of the intermediate layers may have influenced the observed ligand–receptor unbinding forces. In order to verify this hypothesis we studied the avidin–biotin and streptavidin–biotin interactions in a modified experimental protocol applying AFM.

We used the Bell model for describing the relation between the rupture forces of these two complexes and the applied force loading rates. Our results are in agreement with Evans *et al.* (1995) predictions that one should observe multiple sections on the graphs of rupture forces *versus* the logarithm of loading rates for a (strept)avidin–biotin complex. The slope of each section corresponds to a different energy state of the system. The observed relations between the unbinding forces of the avidin–biotin and streptavidin–biotin complexes are consistent with our knowledge regarding the structural differences between their biotin-binding pockets.

MATERIALS AND METHODS

AFM force measurements. We applied an atomic force spectrometer AFM (Park Scientific Instruments) equipped with a liquid cell for adhesive force measurements. One of AFM components is a cantilever tip mounted on a small arm and deflected by external forces. Deflection of the cantilever is monitored by a laser beam reflected from an upper surface of the cantilever and detected in a 4-segment photodetector. The difference between the photocurrent of the top and bottom segments gives information about forces acting between the tip and a sample.

Two kinds of cantilevers were used to obtain a wide range of loading rates. Silicon nitride unsharpened cantilevers, microlevers, with a spring constant $k_c = 0.01$ N/m (ML06C type, MLCT-NONM, ThermoMicroscopes, Sunnyvale, CA, USA) and silicon cantilevers, ultralevers, with a spring constant $k_c = 0.4$ N/m (UL06B type, ULCT-AUNM, ThermoMicroscopes, Sunnyvale, CA, USA) were applied for loading rates from 300 to 4000 pN/s and from 4000 to 9600 pN/s, respectively. We independently checked the spring constants of the cantilevers using Sader method (Sader *et al.*, 1999) based on the meas-

urements of the resonant frequency. The estimated spring constants agreed with the nominal values within 3–8%. All force scan measurements were performed in phosphate-buffered saline (PBS: 20 mM PO_4^{3-} , 150 mM NaCl, pH=7.2) at room temperature.

Preparation of biosensors and sample surface. Cantilever tips coated with avidin or streptavidin are called biosensors. Such coated tips were then brought into contact with a substrate covered with immobilized biotin. The cantilevers and substrates were functionalized with (strept)avidin and biotin, respectively, according to the method described by Lukham and Smith (1998). The procedure is shown in Fig. 1.

A tip, after washing in acetone, was incubated in 10% APTES (3-amino-propyltriethoxysilane) for two hours in order to create amino groups ($-\text{NH}_2$) on its surface. Then, the tip was kept in 2.5% glutaraldehyde for 20 min. Finally, it was coupled with avidin (avidin from egg white, 0.17 mg protein/ml PBS, Sigma-Aldrich) or with streptavidin (Streptavidin-C-Phycocyanin, 0.17 mg protein/ml PBS, Fluka, Sigma-Aldrich) during a 10-min incubation. Glutaraldehyde mediated (strept)avidin binding *via* its aldehyde groups. Functionalized tips were stored at 4°C until needed.

The sample surface was coated with biotin (D-Biotin, 1 $\mu\text{g}/1$ ml PBS, Sigma-Aldrich,) in a similar way as described for the tips. We could omit the first stage of the procedure presented in Fig. 1 because we used glass plates covered with poly-L-lysine, which provided exposed amino groups. The substrate surface was incubated in 2.5% glutaraldehyde and then biotin was immobilized on it.

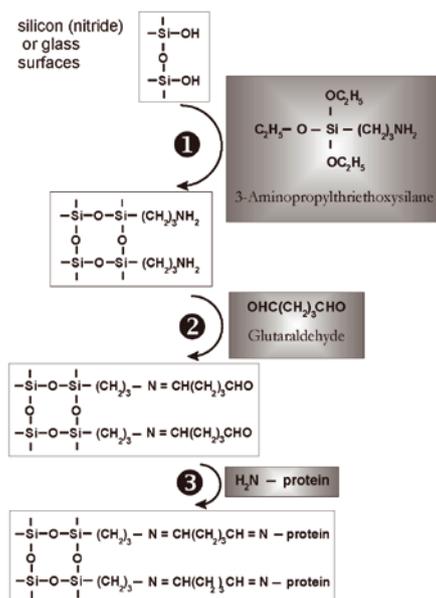


Figure 1. Scheme of (strept)avidin-functionalized AFM tip preparation.

Substrate silanization with APTES (1), glutaraldehyde as a cross-linking mediator (2), protein immobilization (3).

RESULTS

Molecular binding forces between biotin and avidin/streptavidin molecules were studied by AFM. The force measurements were carried out with a substrate surface coated with biotin moving towards the (strept)avidin functionalized tip. During the approach-retract cycles one can observe unbinding events as sharp jumps of the cantilever. The length of the jump is taken as a measure of the interaction between the ligand and the acceptor. An example of a force-displacement curve for the streptavidin–biotin system is shown in Fig. 2. The upper curve corresponds to the approach and the lower one to the retraction of the cantilever tip. There are three visible transitions in the force measurement presented in Fig. 2. The rupture forces assigned as F_1 , F_2 and F_3 correspond to the applied force needed to break one, three or two bonds in a streptavidin–biotin complex, assuming that the F_1 force is the unbinding force of a single streptavidin–biotin pair for the force loading rate of 3900 pN/s under our experimental conditions. One can find that $F_1 \approx 255$ pN, $F_2 \approx 3 \times F_1 \approx 765$ pN and $F_3 \approx 2 \times F_1 \approx 510$ pN. We checked the specificity of the interactions adding free biotin, which abolished the adhesion forces. In about 95% of cases for which the adhesion has been detected (about 60% of all trials), the retract traces of the force measurements exhibited from one to several transitions, whose magnitudes corresponded to a single rupture force of the avidin/streptavidin–biotin pair. This means that under our experimental conditions with a rigid interface layer (APTES + glutaraldehyde) connecting the ligand (receptor) to the tip (substrate), we observed mainly independent molecule adhesion due to sequential breakage of bonds in the (strept)avidin–biotin pairs. Elastic and flexible

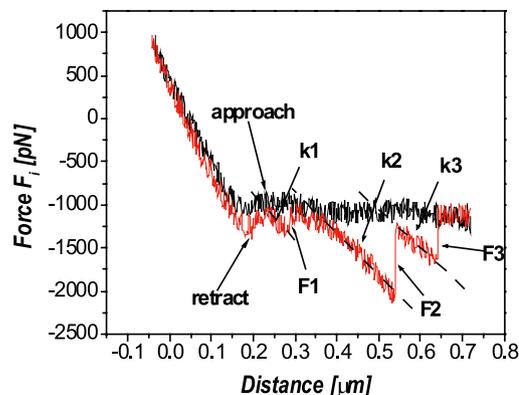


Figure 2. Force-displacement curves of the streptavidin–biotin interaction at the loading rate of 3900 pN/s.

Approach and retraction traces are indicated. F_1 , F_2 and F_3 are rupture forces corresponding to a single, triple and double bond of the streptavidin–biotin complex, respectively. Effective spring constant k_{eff} of the streptavidin-functionalized tip can be estimated from the slope of the force *vs.* displacement curve as shown in this figure (dotted lines k_1 , k_2 , k_3).

intermediate cross linkers, such as agarose beads binding biotin and biotinylated bovine serum albumin attaching (strept)avidin, result in a higher probability of cooperative adhesion (Wong *et al.*, 1999). However, the probability of multiple bond ruptures also increases with an increasing force loading rate (Fig. 2). Representative histograms of rupture forces derived from about 100 force measurements of avidin–biotin and streptavidin–biotin interactions at similar rates of cantilever retractions are shown in Figs. 3A and 3B, respectively. The average unbinding force of a single biotin–streptavidin or biotin–avidin pair was determined from such a force histogram at a given cantilever retraction velocity. A single Gaussian function was fitted to the force histograms (Fig. 3). We carried out adhesion strength measurements of the chosen ligand–protein systems under various scan rates using cantilevers of different spring constants. In this way we achieved variation in the force loading rate L_r . It is well known that the measured disrupting force increases with increasing loading rate (Evans & Ritchie, 1997; Merkel *et al.*, 1999). Moreover, chemical fixation of the ligand and of the acceptor influences the system compliance (Lo *et al.*, 2001). This means that the effective spring constant k_{eff} which should be considered in the calculations of the force loading rate, differs from that of the cantilever applied, k_c . Taking into account the spring constant of the elastic background and the

attached probes, k_s , one can calculate the effective spring constant from the relation: $1/k_{eff} = 1/k_s + 1/k_c$. The elasticity of the substrates may cause a decrease of the system spring constant and in addition an increase of the rupture force. Therefore, it is important to estimate the effective spring constant. Usually it is not possible to calculate it directly because of the incomplete knowledge on the molecular linkages contributing to k_s . The spring constant can be, however, obtained from the average slope of the force *vs.* displacement of the retraction traces (see Fig. 2). The measured effective spring constants in our experiments were in the range of 2–12 mN/m. The curves representing the dependence of force measurements on the loading rates in the range from 300 pN/s to 9600 pN/s for the avidin–biotin and streptavidin–biotin complexes are shown in Fig. 4.

DISCUSSION

Dynamic force measurements of unbinding forces in two investigated systems avidin–biotin and streptavidin–biotin, are summarized in Fig. 4. One clearly sees two regimes of the bond rupture dependence on the logarithm of the force loading rate ($\ln L_r$) in the range from 300 to 9600 pN/s. This observation is consistent with theoretical predictions. Bell's theory (Bell, 1978) predicts that the ligand–acceptor dissociation rate constant k depends exponentially on the externally applied force F :

$$k(F) = k_0(0) \exp\left(\frac{F r_0}{k_B T}\right) \quad (1)$$

where k_B is the Boltzmann constant, T is absolute temperature, r_0 is the distance between the bound and the transition state projected along the direction of applied force, k_0 is the dissociation rate constant

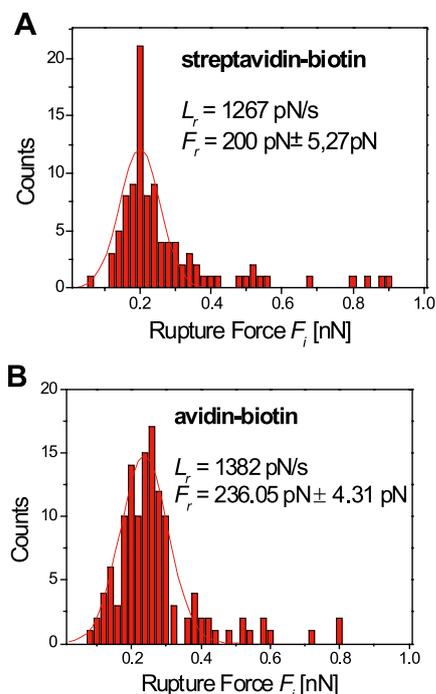


Figure 3. Force histograms of about 100 measurements for streptavidin–biotin (A) and avidin–biotin (B) interactions with fitted Gaussian functions.

The average unbinding force for streptavidin–biotin is 200 pN at the force loading rate of 1267 pN/s (A) and for avidin–biotin it is 236 pN at the force loading rate of 1382 pN/s (B).

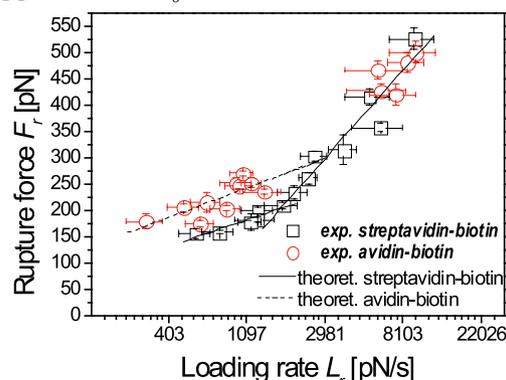


Figure 4. Loading rate dependence of the rupture force for the streptavidin–biotin interactions (\square) and the avidin–biotin interactions (\circ).

Symbols represent experimental data. Lines are theoretical fits applying the Bell model for the two force regimes: a solid line for the streptavidin–biotin complex and a dotted line for the avidin–biotin complex.

in the absence of an external force. Later Evans and Ritchie (1997) using the Bell model and the Kramer theory on reaction kinetics (Kramers, 1940) derived a formula for the dependence of the unbinding force F_i on the force loading rate L_r :

$$F_i = \frac{k_B T}{r_0} \ln \left(\frac{L_r r_0}{k_0 k_B T} \right) \quad (2)$$

This exponential dependence of the inter- and intramolecular unbinding forces on the loading rate has already been demonstrated (Rief *et al.*, 1997; Fritz *et al.*, 1998; Oberhauser *et al.*, 1998). Plotting the rupture force F_i vs. $\ln(L_r)$ one can estimate the distance between the energy potential minimum and the energy barrier maximum, r_0 , for a given regime of applied external forces from the slope of the fitted line. The dissociation rate constant, k_0 , can be calculated from the intercept with the abscissa at zero force. According to the formula given in Eqn. 2, the parameters characterizing the binding states are expressed as follows:

$$r_0 = \frac{k_B T}{\text{slope}} \quad (3)$$

$$k_0 = \frac{L_r (F_i = 0) r_0}{k_B T} \quad (4)$$

The dissociation rates and the width parameters of the energy wells obtained from the linear fits to our experimental data of rupture forces for avidin/streptavidin–biotin (Fig. 4) are collected in Table 1. In the region of high loading rates, above 1700 pN/s, a potential barrier at a distance of about 0.024 nm from the center of the binding pocket dominates and it has a similar width for both avidin–biotin and streptavidin–biotin interactions. The dissociation rates are also equal within the error bars for both systems in the regime of loading rates above 1700 pN/s and are about 3 s^{-1} . However, for lower force loading rates, that is from 300 pN/s to about 1700 pN/s, the measured force slope of avidin–biotin differs from that of streptavidin–biotin. The width of the potential barrier is slightly lower for the avidin–biotin complex (0.07 nm) than for the streptavidin–biotin complex (0.08 nm) and this difference is statistically significant. The dissociation rate of the avidin–biotin pair (0.25 s^{-1}) is about two times lower than of the streptavidin–biotin pair (0.56 s^{-1}), but both dissociation rates for lower rupture forces are lower than k_0 obtained for the region of high unbinding forces. This means that the avidin/streptavidin–biotin complexes are more stable for force loading rates $< 1700 \text{ pN/s}$. The relative stability of the avidin–biotin and streptavidin–biotin pairs can be estimated from the ratio of the dissociation

rates (k_{0av}/k_{0str}) of these two systems (see Table 1). Our data shows that the avidin–biotin interaction is stronger than the streptavidin–biotin one due to the higher outer activation energy barrier of $0.86 k_B T$ (at room temperature it is about 2.1 kJ/mol). The inner activation energy barriers for these systems are similar to each other. The same conclusions we obtained from the estimated bond lifetimes of the complexes in the presence of a disrupting force, by dividing two standard deviations of the unbinding force by the force loading rate. The bond lifetimes for both systems are roughly constant and similar to each other at large force loading rates $> 4000 \text{ pN/s}$ and are about $5.0 \pm 1.7 \text{ ms}$. In the range of low force loading rates from 300 pN/s to 1700 pN/s the bond lifetimes vary: they decrease with the increasing force loading rate from about 80 ms in the case of the avidin–biotin complex and from about 40 ms in the case of the streptavidin–biotin complex for 300 pN/s to about 10 ms in both cases for 1700 pN/s.

The multiple transitions between avidin/streptavidin–biotin intermediate states formed during the unbinding process observed in our experiments are in agreement with the predictions of molecular dynamic simulations, which have shown several potential energy wells for avidin–biotin and streptavidin–biotin interactions (Grubmüller *et al.*, 1996; Izrailev *et al.*, 1997). Thus, the slope of each section in Fig. 4 represents the characteristic force loading rate scale for a specific energy barrier (an energy well) that dominates in the region of the unbinding pathway. We observed similar regimes of the force loading rate for the state transitions of avidin/streptavidin–biotin interactions as Yuan *et al.* (2000) did but different relations between the subsequent states of avidin and streptavidin. Our data shows the existence of equilibrium binding states similar for avidin–biotin and streptavidin–biotin for higher force loading rates but different for lower loading rates, in agreement with Merkel *et al.* (1999), albeit at different ranges of the force loading rates. Using the data of the biomembrane force probe (BFP) experiments (Merkel *et al.*, 1999) one can show that the inner activation energy barriers are similar to each other but the outer activation energy barrier for the avidin–biotin complex is higher by about $9.4 k_B T$ (at room temperature it is about 23 kJ/mol). Thus, only the outer activation energy is responsible for the stronger interaction between avidin and biotin, as was observed in our case. Yuan *et al.* (2000) applying AFM have also found that the avidin–biotin system is more stable than the streptavidin–biotin one but as a result of an increase of both activation energy barriers inner and outer ones. In their case the contribution of the inner energy barrier is about three times higher than the outer energy barrier, being equal to 10.95

Table 1. Bell model parameters obtained from the dependence of F_i on $\ln(L_r)$, where F_i – the unbinding force and L_r – the force loading rate

Range of L_r [pN/s]	Slope [pN]	r_0 [nm]	Intercept [pN]	k_0 [s ⁻¹]	References
Streptavidin–biotin					
300–1700	50.1±9.4	0.081±0.002	-167.7±65.9	0.56±0.46	
1700–9600	166.3±20.9	0.024±0.003	-1032.3±171.5	2.98±2.61	
100–1000		0.49		1.67×10^{-5}	Yuan <i>et al.</i> , 2000
1000–5000		0.05		2.09	Yuan <i>et al.</i> , 2000
0.05–10000		0.5		1.614×10^{-5}	Merkel <i>et al.</i> , 1999
10000–60000		0.12		12.9	Merkel <i>et al.</i> , 1999
Avidin–biotin					
300–1700	55.6±15.5	0.073±0.002	-146.1±103.2	0.25±0.18	
1700–9600	168.6±14.8	0.024±0.002	-1047.6±125.2	2.97±2.71	
100–1000		0.53		6.45×10^{-6}	Yuan <i>et al.</i> , 2000
100–5000		0.2		0.08	Yuan <i>et al.</i> , 2000
0.05–30	1.4	3.0			Merkel <i>et al.</i> , 1999
30–10000	13–14	0.3		10.74	Merkel <i>et al.</i> , 1999
10000–60000		0.12		12.9	Merkel <i>et al.</i> , 1999

¹The dissociation rates are estimated by us from extrapolation of the rupture force to zero for subsequent regimes (Fig. 3b, Merkel *et al.* 1999).

$k_B T$ (at room temperature it is ~24 kJ/mol) and 3.3 $k_B T$ (at room temperature it is about 8.2 kJ/mol), respectively.

The higher stability of the avidin–biotin than the streptavidin–biotin system is in accordance with the chemical and structural differences between the biotin binding pockets of avidin and of streptavidin. There are three extra hydrogen bonds and hydrophobic interactions in avidin (Weber *et al.*, 1989; Livnah *et al.*, 1993). In addition, in avidin the L3,4 loop is longer and can bind biotin more tightly (Weber *et al.*, 1989; Livnah *et al.*, 1993; Freitag *et al.*, 1997). It has been recently proved that hydrophobic interactions (Li *et al.*, 2003) and structural complementarity (Morttilo *et al.*, 2003) are the most important factors determining the strength of non-covalent bonds. In our experiments the avidin–biotin complex is more stable than the streptavidin–biotin one for the range of low loading rates. The similar stabilities of the avidin–biotin and streptavidin–biotin interactions at the regime of higher loading rates are due to the diminishing importance of structural factors in the interactions under such experimental conditions.

One may expect that the bond rupture forces should increase with the increasing strength of ligand–receptor interactions. Indeed, in our experiments, for the force loading rates of 300–1700 pN/s the unbinding forces of the avidin–biotin pair are

higher by about 20 pN than for the streptavidin–biotin pair. Surprisingly, weaker rupture forces are reported for the avidin–biotin complex than for the streptavidin–biotin one usually (Wong *et al.*, 1999; Merkel *et al.*, 1999; Yuan *et al.*, 2000). This effect may result from less rigid interface layers anchoring the ligand–receptor systems. In the case of AFM measurements (Wong *et al.*, 1999; Yuan *et al.*, 2000) (strept)avidin was coupled to the cantilever tip *via* biotinylated bovine serum albumin (BSA) and biotin was bound to agarose polymers. For the biomembrane force probe (BFP) (Merkel *et al.*, 1999) biotinylated polyethylene glycol (PEG)-polymers were used as the substrate for (strept)avidin molecules whereas PEG-biotin polymers were linked to a red blood cell.

From a comparison of the parameters describing the dynamic behavior of the unbinding forces collected in Table 1 and the measured bond-rupture forces (Merkel *et al.*, 1999; Yuan *et al.*, 2000; this work) it is obvious that interpretation of the measured rupture forces is a difficult and complex task. One has to take into account possible modifications of the compliance of the ligand–receptor system due to the experimental protocol because the primary source of errors in this analysis comes from the uncertainty of the spring constant value of the whole system including the cross-linkers. Therefore, in or-

der to understand the relation between the rupture forces measured by AFM and the real non-covalent bonds in the studied biocomplexes, more systematic investigations of ligand–receptor interactions on model systems are necessary.

Summarizing, the results presented in this paper show that the rupture forces measured for the avidin–biotin and streptavidin–biotin complexes depend on the rate of the applied external force. We observed transitions between intermediate states formed during the unbinding processes of both studied systems. Using the Bell model we were able to derive the width of the energetic barriers and dissociation constants for two different regimes of the dissociation process studied for the avidin– and streptavidin–biotin systems. The results are explained in the frame of the known structural and hydrophobic differences between the biotin binding pockets of avidin and streptavidin. The arrangement of the biotin binding sites is important in the regime of the low force loading rates but its influence on the detected rupture forces diminishes in the regime of the higher force loading rates. This indicates that the enhanced outer activation energy barrier for the avidin–biotin complex is responsible for its higher stabilization. The difference between the outer potential barriers is estimated to be about $0.86 k_B T$. The rupture forces stronger by about 20 pN for the avidin–biotin pair than for the streptavidin–biotin pair observed at force loading rates below 1700 pN/s are in agreement with this conclusion. This observation that the avidin–biotin complex is more stable than the streptavidin–biotin one is independently supported by the bond lifetimes evaluated for the studied systems at different ranges of applied force loading rates.

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