Title: Investigating the Binding Behaviour of two Avidin-based Testosterone binders using Molecular Recognition Force Spectroscopy

Short title: Investigation of testosterone binding proteins by force spectroscopy

Keywords: Atomic Force Microscopy, Molecular Recognition Force Spectroscopy, testosterone, Avidin, single molecule, protein recognition

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Abstract

Molecular Recognition Force Spectroscopy (MRFS), a biosensing Atomic Force Microscopy technique allows to explore the dissociation characteristics of ligand-receptor complexes at the molecular level. Here we used MRFS to study the binding capability of recently developed testosterone-binders. The two avidin-based proteins called sbAvd-1 and sbAvd-2 are expected to bind both testosterone and biotin, but differ in their binding behaviour towards these ligands. To explore the ligand binding and dissociation energy landscape of these proteins we tethered biotin or testosterone to the AFM probe while the testosterone-binder was immobilized on the surface. Continuously forming and rupturing a ligand-receptor complex at different pulling velocities allowed determining the loading rate dependence of the complex-rupturing force, thereby yielding in the molecular dissociation

rate (k_{off}) and energy landscape distances (x_β) of the four possible complexes: sbAvd-1-biotin, sbAvd-1-testosterone, sbAvd-2-biotin and sbAvd-2-testosterone. We found that the kinetic off-rates for both proteins towards both ligands are similar. In contrast, the x_β values as well as the probability of complex formations varied considerably. In addition, competitive binding experiments with biotin and testosterone in solution differ significantly for the testosterone-binding proteins implying a decreased cross-reactivity of sbAvd-2. Unrevealing the binding behaviour of the investigated testosterone-binding proteins is expected to improve their usability for possible sensing applications.

Introduction

Avidin is known to bind biotin highly specific and very tight with femto-molar affinity [1]. Hence it is wildly used in biomedical and biotechnical applications like protein purification and labelling, drug pre-targeting, and nanostructure-building [2]. Beneath these important applications the avidin scaffold is a promising candidate for protein engineering. Alterations of the amino-acids in the (biotin) binding pocket allow generating new proteins, combining the excellent properties of the wild type avidin with the ability to bind other small ligands like hormones. This makes it to an attractive alternative to specific antibodies [3] overcoming their general limitations like low stability, large size and laborious and expensive fabrication.

Within this study we investigated two recently developed mutations of avidin optimized for steroid hormone binding named sbAvd-1 and sbAvd-2 [4]. The mutant sbAvd-1 has been selected by phage display method from a library of proteins with random mutagenesis focused at a loop area participating to the ligand-binding site, showing micro-molar affinity towards testosterone. To lower the affinity to biotin, further mutations of this protein by randomizing the loop area between the β strands 3 and 4 were performed and resulted in sbAvd-2 as described previously [4]. Both mutants preserved the high stability and

the tetrameric structure of avidin as proven with differential scanning calorimetry and analytical gel-filtration, and show micro-molar affinity towards testosterone. We explored the binding behaviour of these mutants towards testosterone as well as d-biotin at the molecular level by performing molecular recognition force spectroscopy experiments. In MRFS interaction forces of a single ligand-receptor bond are detected and measured. Furthermore, the energy landscape of the dissociation process of the binding, responsible for the kinetics of the interaction, can be explored. For this, increasing forces are applied to a previously formed ligand-receptor complex and the energy barrier gets thereby lowered. The dependence of rupture forces on the loading rate is then used to calculate energetic and kinetic data. The effect of a single point mutation of an avidin-family protein on the energy landscape was investigated previously [5]. There the lowering of affinity was demonstrated by comparison of W120F (trytophan 120 was replaced by phenylalanine) streptavidin mutant to the wild type protein. Within this study we explored the energy landscape of sbAvd-1 and sbAvd-2 towards both, testosterone and biotin and investigated the competitive binding towards both ligands.

Materials and Methods

Tip Chemistry: Testosterone was covalently coupled to the AFM tip via a heterobifunctional poly(ethylene glycol) (PEG) crosslinker in a four step procedure. First, the inert silicon-nitride AFM tips (MSCT cantilever, Bruker, Germany) were amino-functionalized by performing gas phase silanization with aminopropyl-triethoxy-silane (APTES, Sigma-Aldrich, Austria) [6]. In the second step, Fmoc-NH-PEG-CO-NHS linker (α-([fluoren-9-yl-methoxycarbonyl]aminopropyl)-ω-{2-[4-(N-succinimidyloxy-carbonyl)butanoyl amino]propyl}-poly(oxyethylen)-800) [7] was coupled to the amine-modified tip with its NHS ester end by forming an amide bond. Thirdly, the Fmoc-protecting group was removed from the amine end of the linker by immersion in a solution containing N,N-dimethylformamide/piperidine (4:1, v/v). Finally testosterone was bound to the terminal

amine of the crosslinker in close analogy to Wildling et al. [8]. d-Biotin (Sigma-Aldrich, Austria) was coupled to the AFM tip via an NHS-CO-PEG-biotin crosslinker according to [9]. For this, amine groups were generated on the tip surface using APTES and, in a second step the linker including biotin was directly bound to the tip.

Surface Chemistry: Genetically modified avidin proteins were covalently immobilized on mica surface using the protocol adapted from [10]. For this, amine groups were generated on freshly cleaved mica sheets using APTES silanisation method. The amine-functionalized mica sheets were then immersed for 2 hours in a chloroform solution containing 1 mg/ml ethyleneglycol-bis(succinimidylsuccinate) (EGS, Sigma-Aldrich, Austria) and 0.5% (v/v) triethylamine, subsequently washed in chloroform, and dried in a gentle nitrogen gas stream. In order to couple the avidin forms to the surface, 0.1 mg/ml of sbAvd-1 or sbAvd-2 respectively, dissolved in PBS buffer (150 mM NaCl, 5 mM NaH_2PO_4, pH 7.5) were placed on functionalized mica for 3 hours resulting in a covalent coupling of the proteins via their lysine residue. Finally the functionalized mica sheets were washed with PBS buffer and stored at 4°C until further use.

AFM Measurements: All MRFS experiments were performed on a Pico SPM I (Agilent, USA). The spring constant of the cantilevers used for force spectroscopy experiments were determined by using the thermal noise method [11]. Force distance cycles were performed at pulling velocities between 100 and 2400 nm/s. During one data set of 1000 force distance curves, the tip position was changed ~4 times (all 200 curves) to avoid position dependent artefacts. Empirical force distributions of the rupture forces of the last unbinding event (pdf) were calculated as described earlier [12]. The loading rates were calculated by multiplying the pulling velocity with the effective spring constant, i.e. the slope of the unbinding event. In the single-barrier model [13], the rupture force F* is given as function of the loading rate with $F^* = f_\beta \cdot \ln(r/ k_{off} \cdot f_\beta)$, where f_β is the ratio of the thermal energy k_BT and x_β with the latter marking the thermally averaged projection of the transition state along the direction of the force. The

parameters x_β and k_{off} were determined by fitting F* against ln(r). The accuracy of the parameters was calculated by using propagation of errors [14] assuming that the standard error of F* is 15% (10% for the spring constant determination and 5% to account for the uncertainty in determining the most probable rupture force). After performing force distance cycles a specificity proof of the binding events was performed. For this, free testosterone or biotin (5 µM) were added into the measuring solution and incubated for about 1 hour to block the binding sites of sbAvd-1 and sbAvd-2, respectively.

Results

For performing MRFS experiments, the AFM tip had to be upgraded into a molecular biosensor [15] by covalent immobilisation of testosterone or biotin, respectively. In contrast to the coupling of biotin, which is commonly used as stable and robust test system for force spectroscopy [9], testosterone was not tethered to an AFM tip previously. We developed a coupling strategy for testosterone which is strongly related to a protocol developed for binding the steroid based hormone aldosterone [8]. For both ligands, the first step in these anchoring protocols was the generation of reactive amine-groups on the inert silicon-nitrite tip surface [6]. Secondly, a heterobifunctional poly(ethyleneglycol) (PEG) linker molecule was bound to the amino group on the surface, resulting in a stable amide bond. For biotin as ligand a biotin-PEG-CO-NHS linker [9] was used, yielding in biotinylated tips directly after this coupling step [16]. In contrast, testosterone was bound via the heterobifunctional PEG linker Fmoc-NH-PEG-CO-NHS [8] in two additional steps: (i) the deprotection of the fluorenylmethoxycarbonyl (Fmoc) group and (ii) the coupling of testosterone to the free amine-reactive site. The testosterone binding proteins sbAvd-1 and sbAvd-2 itself were immobilized to previously amino-functionalized mica sheets using a commercial short amino-

reactive homobifunctional ethylene glycol bis[succinimidylsuccinate] linker as described previously [10].

The scheme of the four investigated configurations is depicted in Figure 1A. The ligand-receptor complex rupture forces between the small ligands (biotin or testosterone) with their corresponding receptor (sbAvd-1 and sbAvd-2) were measured in force-distance-cycles (Figure 1B) whereby the tip carrying the particular ligand was repeatedly approached and retracted from the sample surface and the cantilevers deflection is recorded as a function of the tip-samples distance. At the beginning of the approaching period (Figure 1B, red line, right side) the cantilever deflection remained zero until the tip touched the testosterone-binder functionalized surface. By further approaching the cantilever bent upwards until the force indentation limit reached. Subsequently, the cantilever was retracted (Figure 1B, blue line), resulting in a relaxation of the cantilever deflection until it reached its resting position again. A protein-ligand complex was potentially formed during the time the tip was in close contact to the protein-functionalized surface. Complex formation resulted in a physical ligand receptor connection via the PEG linker yielding a downward bending of the cantilever in the retraction period. By further retraction, the linker got increasingly stretched until the bond ruptured and the cantilever jumped back into its resting position. For statistical evaluation 1000 force-distance-cycles were recorded at a certain pulling velocity. The detected binding events between biotin (or testosterone) and sbAvd-1 and sbAvd-2, respectively were analysed with respect to their interaction force and probability density functions (pdf) were generated [12]. The maxima in the pdf reflect the most probable unbinding force of the complex. In Figure 2A force distributions of all four interactions are shown at a loading rate of about 3000 pN/s. The interaction forces of sbAvd-1 and sbAvd-2 with both ligands are in the same range of about 50pN which is comparable to rupture forces of biotin with wild type avidin at this loading rate [17].

To gain insights into the energy landscape of the investigated ligand receptor complexes, the force necessary to cause a rupture was determined at different force loading rates. A loading rate range from 1,000 to 30,000 was achieved by varying the pulling velocities from 100 to 2,400 nm/s. Interestingly all four ligand-receptor combinations resulted in a well comparable dependence of the rupture force on the loading rate. Figure 2B shows linear fits of the loading rate dependence taking every rupture event into account. From these fits the molecular dissociation rates (k_{off}) and x_β values, describing the distance of the energy minimum to the maximum in the energy potential of the dissociation process, were estimated. The results of the calculated kinetic rates are listed in table 1. Consistently with the interaction forces all off-rates of the steroid binders with testosterone as well as with biotin as ligand are in the same range. In detail, the dissociation rate (k_{off}) of the biotin sbAvd-1 complex is 7.2 ± 0.37 s^{-1} and 11.4 ± 0.41 s^{-1} for the complex with sbAvd-2. These dissociation rates are comparable to off-rates of the wild type avidin – biotin complex measured by De Paris et al [18] performing biomembrane force probe experiments. Additionally also the dissociation rates of the testosterone complex with sbAvd-1 (10.0 ± 0.57 s^{-1}) or with sbAvd-2 (8.3 ± 0.25 s^{-1}) are very similar, indicating that a complex – once formed – shows the same stability independent from the mutations. Although the x_β values of the sbAvd-1 and sbAvd-2 interaction with testosterone are quite similar, the x_β of biotin binding differs by a factor of two. While the wild type avidin - biotin interaction shows an x_β of about 3Å [17, 19], sbAvd-1 and sbAvd-2 have an x_β of 1.4 and 0.8 Å, respectively.

The specificity of the measured interactions between testosterone and biotin with sbAvd-1 and sbAvd-2 was proven by the addition of free ligands in solution. This resulted in a preoccupation of the binding sites and inhibited the complex formation between tip-tethered ligand and the testosterone-receptors, yielding in a significant reduction of the complex formation probability. Figure 3 shows the average binding probabilities of the biotin (Figure 3A) and testosterone (Figure 3B) interaction with sbAvd-1 and sbAvd-2 in absence and

presence of free ligands (either testosterone or biotin). The addition of free ligands significantly lowered the binding probability. However, the effectiveness of the "blocking experiment" varied dependent on both, the type of ligand and the tested steroid binder. Both ligands in solution, free biotin as well as testosterone, were equally able to block the interaction of tip-bound biotin with both avidin mutants (Figure 3A). In contrast, the number of binding events between sbAvd-2 and tip-bound testosterone was only reduced to about 50 % by the addition of biotin. However, by subsequent addition of testosterone in solution the binding probability was further decreased to about 2%. This demonstrates the higher efficiency of sbAvd-2 to form a complex with testosterone in the presence of biotin compared to sbAvd-1. In contrast to the mutants, wild type avidin, known to form a stable complex with biotin with a binding probability of typically ~20%, did not show any specific interaction with testosterone (0.5 ± 0.3 % binding probability). Beneath the differences in the competitive binding behaviour a general trend in showing higher binding probability of biotin with sbAvd-1 and of testosterone with sbAvd-2 was observed. The mean binding probability of sbAvd-1 was 17% for biotin but only 14% for testosterone, whereas sbAvd-2 yielded in 23% binding probability with testosterone, while the complex formation with biotin occurred only in 16% of the investigated force distance cycles.

Discussion

The decreased binding probability, the lower capability of biotin to saturate the binding pockets of sbAvd-2 as well as the determined x_β values indicate that sbAvd-2 shows lower binding affinity towards biotin compared to sbAvd-1 or wt avidin, which is in agreement to earlier SPR experiments [4]. However, experiments in solution revealed that sbAvd-1 and sbAvd-2 show significantly more pronounced reduction of biotin-binding affinity compared to wt avidin in terms of kinetic rates [4]. Therefore a question rises: why AFM reveals high similarity in between biotin dissociation process among these proteins?

One important feature of avidin-biotin interaction is the loop connecting beta strands 3 and 4 (L3-4). This loop is known to act as a lid closing the open end of the beta barrel, thus shielding the bound ligand from surrounding solvent [20]. Furthermore, this particular loop contributes to biotin recognition via hydrogen bonding. When a tip-tethered ligand is used, these interactions are severely disturbed. This allows to explain, that the dissociation kinetics of sbAvd-1 and sbAvd-2 differ only marginally from each other in this analysis because they are identical to each other except few mutations in L3-4 - including T35A, which has been shown to cause moderate decrease in biotin-binding affinity to avidin in solution [21].

Conclusion

In conclusion we could proof and quantify the specific binding of biotin and testosterone with sbAvd-1 and sbAvd-2 respectively at the single molecule level. The different specificity proofs demonstrate that biotin and testosterone use the identical binding pocket, the β-barrel, of the avidin mutants. Furthermore, the comparison of binding probabilities and the estimation of x_β values indicate that sbAvd-2 shows lower binding affinity towards biotin compared to sbAvd-1 or wt avidin. The characterization and the molecular understanding of this binding behaviour of the recently developed testosterone binding proteins is expected to be beneficial for sensor based applications.

Acknowledgements

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Tables

	k_{off}	x_{β}
sbAvd-1 – biotin interaction	7.2 ± 0.37	1.40 ± 0.03
sbAvd-2 – biotin interaction	11.4 ± 0.41	0.81 ± 0.01
wt avidin – biotin interaction [18]	~ 10	~ 3
sbAvd-1 – testosterone interaction	10.0 ± 0.57	1.55 ± 0.03
sbAvd-2 – testosterone interaction	8.3 ± 0.25	1.16 ± 0.02
wt avidin – testosterone interaction	no interactions detectable	

Figure Legends

Figure 1: A) Scheme of the experimental setups. Using Molecular Recognition Force Spectroscopy the interaction of biotin and testosterone with both genetically modified avidins sbAvd-1 and sbAvd-2 were studied and compared. **B)** Typical Force-Distance Cycle. For measuring interaction forces the cantilevers deflection is recorded in respect to the samples distance. At a fixed lateral position the cantilever is approached to the surface (red line). Its deflection remains zero until the tip touches the surface. Upon further approach the cantilever bends upward and a linear increasing force is applied to the surface. Tip retraction (blue line) results first in relaxation of the cantilevers bending until it comes into its resting position again. However, if a ligand-steroid binder complex was formed the AFM tip is connected to the surface resulting in a downward bending of the cantilever and a non-linear stretching of the PEG chain until the ligand-receptor bond breaks at a certain rupture force (*F*).

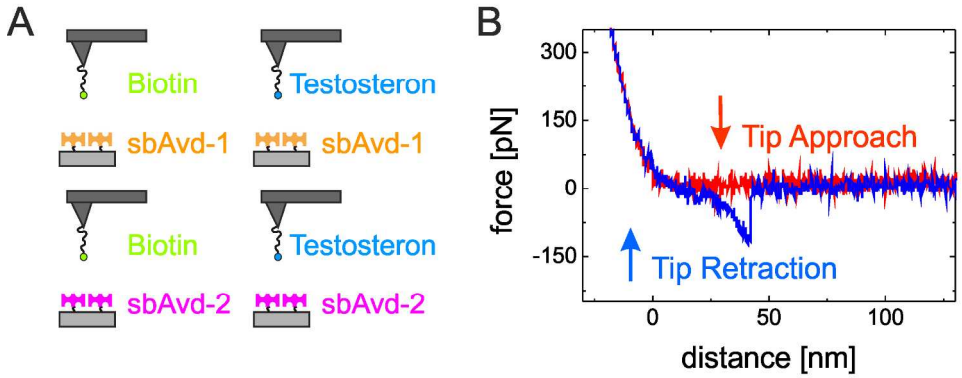
Figure 2: A) Probability density functions of the rupture force of the testosterone and biotin interaction with the sbAvd-1 and sbAvd-2 steroid binders. The rupture forces of all bonds are in the same range of ~50 pN at a loading rate of about 3000 pN/s. **B)** Loading Rate dependence of the unbinding forces. The linear fits were generated by taking every unbinding event in account. From the slope and the intersection with the y-axis from the fit the kinetic off rate k_{off} and x_{β} values can be estimated.

Figure 3: Comparison of binding probabilities of testosterone and biotin interaction with both testosterone binders with or without the presence of free ligands. **A)** Biotin Interaction. After the interaction of tip-bound biotin and sbAvd-1 and sbAvd-2 was investigated two different specificity proofs were performed by the addition of whether free biotin or free testosterone in

solution. The probability of a binding event was then reduced dramatically**. B)** Testosterone Interaction. To prove the specificity of the interaction between tip-tethered testosterone and the testosterone-binders free testosterone or free biotin molecules were added.

Table 1: Estimated kinetic off rates and x_β values from the linear fit of the loading rate dependences of the particular unbinding forces.

Figure 1



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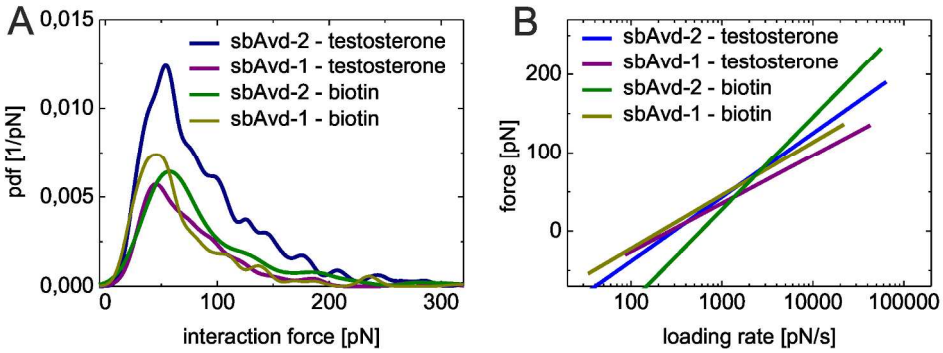
211x113mm (300 x 300 DPI)

Figure 2



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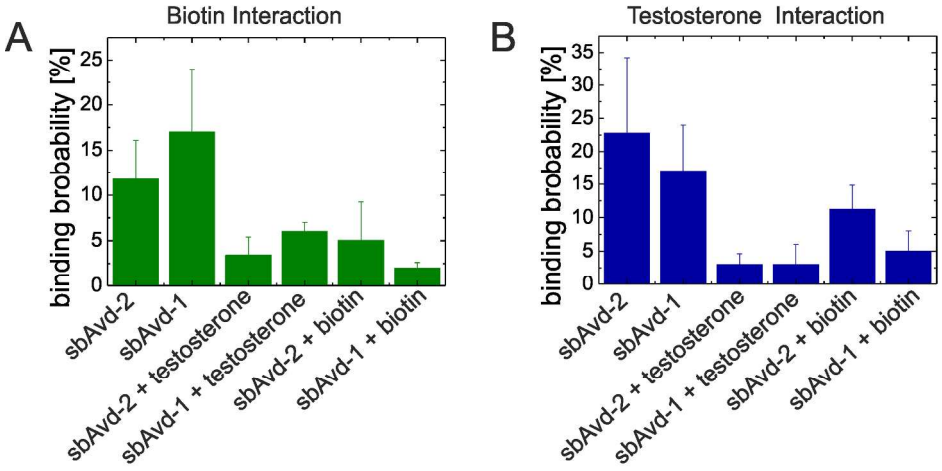
216x104mm (300 x 300 DPI)

Figure 3



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209x128mm (300 x 300 DPI)

Table 1

	k_{off} [s^{-1}]	x_β [Å]
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184x63mm (300 x 300 DPI)