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ORIGINAL PAPER

Optical tweezers to study *single* Protein A/Immunoglobulin G interactions at varying conditions

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Abstract Optical tweezers (OT) are ideally suited to study the interaction of single receptor-ligand bonds. Here we introduce a newly developed assay using OT to investigate the interactions between Protein A from *Staphylococcus aureus* and Immunoglobulin G from rabbit serum (RIgG). We demonstrate that the rupture forces depend on the loading rate and on the sodium chloride concentration. The measured loading rate effect is well known in the literature and the data we obtained were found to be in good agreement with an already published theoretical model. The dependence of the rupture forces on the salt concentration demonstrates the influence of hydrophobic interactions on the bond strength. Our experimental setup can probe the interaction between a single receptor and its specific ligand under changing conditions and hence offers manifold applications in single molecule biotechnology.

Introduction

In the recent years single molecule analysis turned out to be a revolutionary method in biotechnology and biophysics. The use of atomic force microscopy, microneedles and magnetic or optical tweezers (OT) opened up spectacular insights into cellular, molecular and even atomic structures and processes. OT for example are applied for studying the binding and unbinding of histones and histone-like proteins to DNA (Bennink et al. 2001a, b; Brower-Toland et al. 2002; Gemmen et al. 2005; Salomo et al. 2006a, 2007; Cui and Bustamante 2000) or the function of RNA polymerases (Wang et al. 1998; Yin et al. 1995). Furthermore they are used to investigate the elastic properties of single DNA-molecules (Marko and Siggia 1995; Wenner et al. 2002; Williams et al. 2001; Bouchiat et al. 1999; Smith and Cui 1996; Wang et al. 1997; Salomo et al. 2006b). By mechanically unfolding single proteins or RNA aptamers new insights can be gained about the energetics of molecular folding processes (Cecconi et al. 2005; Liphardt et al. 2001; Onoa et al. 2003). In biophysical applications the flow resistance of DNA grafted colloids (Gutsche et al. 2006) respectively their interaction with each other (Kegler et al. 2007) was characterized. Protein/protein interactions represent another challenging topic in single molecule analysis. In this field the interaction between several antigens and antibodies (Chen et al. 2007; Kulin et al. 2002; Helmerson et al. 2001) as well as between cellular receptors and their specific ligands was studied.

In the work presented here we focused on the interaction between Immunoglobulin G (IgG) and Protein A, a 42-kDa protein that originates from the cell wall of *Staphylococcus aureus*. The expression of Protein A, which is coded by the *spa*-gene, is controlled by cellular osmolarity, DNA-topology and a system consisting of two components and which is called ArIS-ArIR (Fournier and Klier 2004). Protein A is able to bind IgG's within the heavy chain of the Fc-region. It exhibits the highest affinity to human IgG1 and IgG2 and also to mouse IgG2a and IgG2b. Furthermore

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it binds human IgA, IgE and IgM as well as mouse IgG1 with reduced affinity and shows no interaction with human IgD or IgG3 or mouse IgA, IgE, IgG3 and IgM.

Staphylococcus aureus as a pathogen uses Protein A along with several surface proteins to ensure its survival in host organisms (Cheung et al. 1997; Hartleib et al. 2000). Protein A binds host antibodies in the opposite direction to their original orientation to the cell wall of *S. aureus*. Hence the pathogen can not be recognized by the immune system and is protected against phagocytosis by macrophages. Furthermore Protein A is able to kill B-Lymphocytes efficiently which play a crucial roll in immune defense against bacterial infections (Goodyear and Silverman 2003). It is also involved in different protective biological functions such as antitoxic (Ray et al. 2000; Ray 1999) and antitumor activities (Shukla et al. 1996; Kumar et al. 1992; Bansal et al. 1992) and has additional antiparasitic (Ghose et al. 1999) and antifungal properties (Srivastava et al. 1997). Recombinant Protein A overexpressed in *Escherichia coli* is used in chromatographic applications for antibody purification (Hober et al. 2007). Labelled with fluorescence (Biberfield et al. 1975) or radioactive (Cleveland et al. 1979) markers it is utilized in antibody detection assays.

In single molecule experiments Protein A embodies a model system to study protein interactions (Stout 2001; Strigl et al. 1999; Touhami et al. 2007; Simson et al. 1999, 2004). We utilized it to investigate its interaction with Immunoglobulin G from rabbit serum (RIgG) under varying conditions by the use of OT. Besides the already described effect of the loading rate (Strigl et al. 1999; Simson et al. 1999) the aim of this work was to demonstrate for the first time on a *single* molecule level the influence of sodium chloride on the interaction strength between these two binding partners.

Materials and methods

Instrument design, calibration and force measurement

The OT set-up is based on an inverted microscope (Axiovert S 100 TV, Carl Zeiss, Jena, Germany). For the optical trap a diode pumped Nd: YAG laser (1,064 nm, 1 W, LCS-DTL 322, Laser 2000, Wessling, Germany) was coupled in to the bottom TV-port of the microscope. Our sample cell consists of a stainless steel corpus with a volume of $\sim$300 µl which is enclosed by two cover slips on bottom and top (not shown). For media exchange additional in- and outlets are incorporated. In order to enable the immobilization of a micrometre-sized bead a micropipette is inserted into the sample cell (inner diameter: $\sim$1 µm, Fig. 1a). The sample cell is mounted onto an optical stage which can be moved in three dimensions with nanometre resolution by using piezo-actuators (P-5173CD, PI, Karlsruhe, Germany). More details on the setup can be found here (Salomo et al. 2006b).

Force detection is carried out by video imaging. An online procedure was developed to convert the microscope picture into an intensity profile allowing to determine both the position of the beads and their separation to each other with an accuracy of $\pm$1 nm and a temporal resolution of 16.67 ms. A typical image is shown in Fig. 1a. Out of these data the force acting on the particle held in the optical trap is calculated with a resolution of $\pm$0.1 pN.

The optical trap was calibrated according to Stokes law $F = 6\pi\eta r v$ where η is the viscosity of the medium, r the radius of the particle an v is the velocity relative to the surrounding medium. By varying the latter between 50 and 1,000 µm/s the optical forces are calibrated with an absolute accuracy of $\pm$10%. The relative uncertainty of the force measurement is typically $\pm$0.5 pN. The whole experimental set-up is placed in a temperature controlled room (temperature stability $\pm$1 K).

Coating of polystyrene beads with Protein A and Immunoglobulin G

The coating of microparticles with Rabbit Immunoglobulin G (RIgG) (Sigma Aldrich, Taufkirchen, Germany) and Protein A from *S. aureus* (Sigma Aldrich, Taufkirchen, Germany) is mainly deduced from already published protocols and adapted to our needs (Nustad et al. 1982; Quash et al. 1978; Siiman et al. 2001). For the preparation carboxylated particles with a diameter of 2.0 µm (Kisker, Steinfurth, Germany) are used. The beads from a 50-µl suspension (50.0 mg/ml) are washed twice with 200 µl of carbonate buffer [100 mM sodium carbonate, 100 mM sodium bicarbonate (pH 9.6)] and three times with 200 µl buffer A [20 mM phosphate buffer (pH 4.5)]. In between beads are collected by centrifugation (2 min, 8,000$\times g$). After resuspension of the final bead pellet in 50 µl of buffer A, the surface carboxyl groups are activated by a 4 h incubation at room temperature with 50 µl of a 2% EDC solution [ethyl-3-(3-dimethyllaminopropyl)-carbodiimide] (Applichem, Darmstadt, Germany) in the same buffer. Particles are then washed three times with buffer A. After resuspension in 80 µl buffer B [200 mM borate buffer (pH 8.5)], 10 µl of a RIgG-solution (0.75 mg/ml) respectively 10 µl of a Protein A-solution (1.0 mg/ml) are added followed by overnight shaking at room temperature. To avert unspecific binding at remaining free reactive groups, the pellet is washed twice with 200 µl of BSA solution (10 mg bovine serum albumin/ml; buffer B) and incubated under shaking for 30 min at room temperature. This blocking step is then repeated one more time. Finally, the particles were

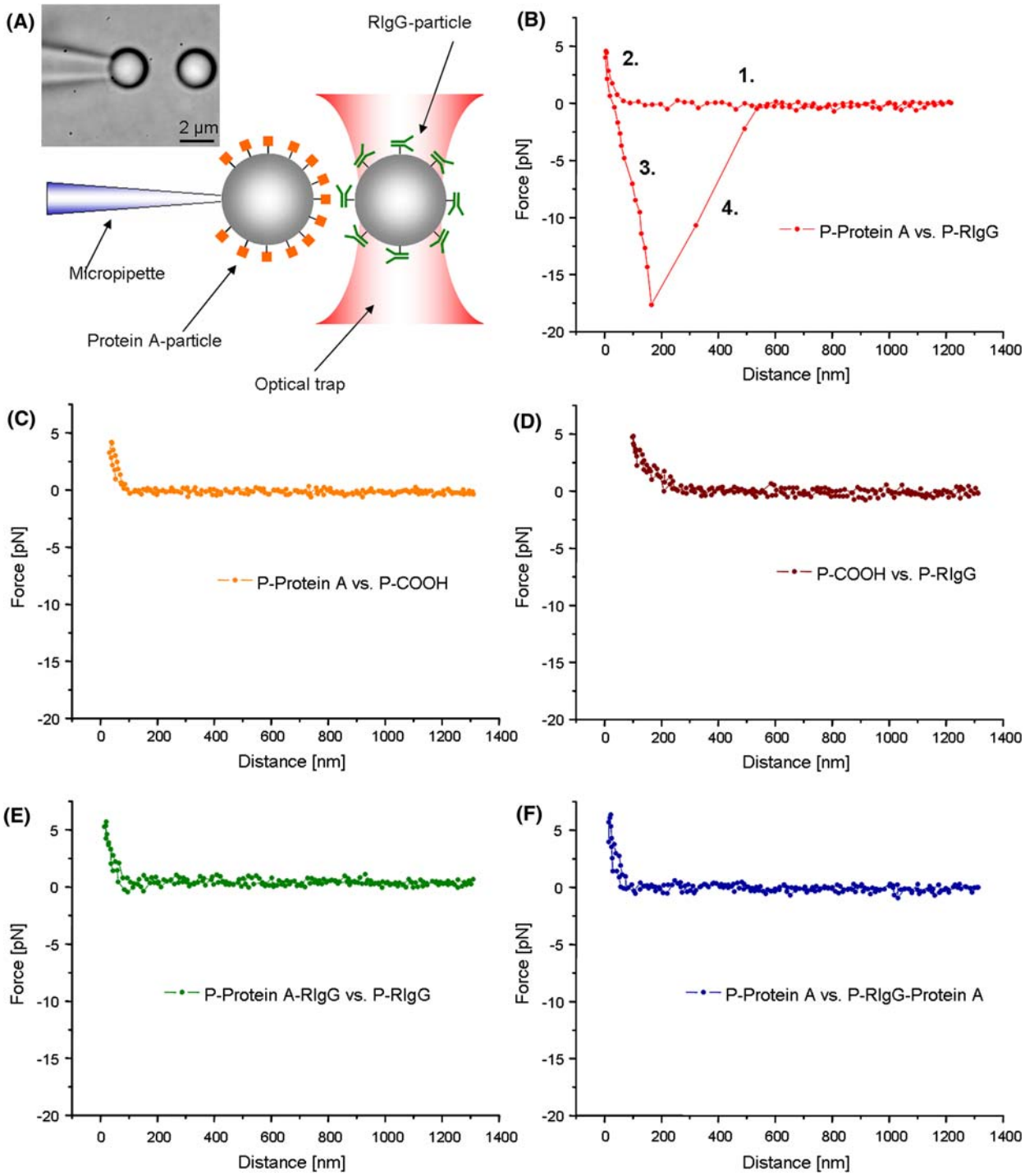

Fig. 1 Experimental setup and control experiments. **a** Experimental setup. One particle coated with Protein A is fixed at the tip of a micropipette. The other particle carrying Immunoglobulin G on its surface is held in the focus of a force measuring optical trap. Inset: Microscope picture of the experimental setup. **b** Original data from a measurement with a Protein A coated particle (*P-Protein A*) and a RIgG modified bead (*P-RIgG*). The particles were approached (*1.*) and brought in contact until a force level of 5 ± 2 pN was reached (*2.*). Afterwards the colloids were pulled apart (*3.*) until the bond broke (*4.*). **c, d** Control experiments with unmodified beads. The developed experimental procedure was applied to Protein A- (*P-Protein A*) and RIgG-beads (*P-RIgG*) that were measured against unmodified carboxylated particles (*P-COOH*). No interaction could be detected. **e, f** Control experiments with saturated beads. A similar experiment was carried out with RIgG-saturated Protein A-beads (*P-Protein A-RIgG*) that were measured against RIgG-particles (*P-RIgG*) and with Protein A-beads (*P-Protein A*) that were brought in contact with RIgG-olloids saturated with Protein A (*P-RIgG-Protein A*). Again no specific bonding events were observable

Table 1 Mean rupture forces at different loading rates

Loading rate (pN/s)	Mean rupture force (pN)	Confidence interval		Standard deviation (pN)	Number of single rupture events
		Lower limit ($P = 95\%$) (pN)	Upper limit ($P = 95\%$) (pN)		
0.3	6.5	5.2	7.9	5.6	102
3.0	12.8	11.3	14.3	7.8	112
6.0	15.9	14.4	17.4	9.4	152
12.0	19.8	17.8	21.7	11.3	130
24.0	21.3	19.5	23.2	11.1	138
50.0	24.0	22.4	25.6	10.1	155
140.0	27.5	25.8	29.2	10.3	141
215.0	29.8	28.0	31.7	12.3	172
300.0	30.0	27.8	32.2	12.5	132
415.0	31.2	29.1	33.3	11.8	125
500.0	32.1	29.9	34.2	13.5	149

resuspended in 20 µl of PBS (pH 7.4) (136 mM NaCl, 2.7 mM KCl, 15.6 mM Na_2HPO_4, 17.6 mM KH_2PO_4) and stored at 4°C.

By determining the residual protein in the supernatant via the Bradford protein assay (Bradford 1976) after the overnight incubation the number of immobilized molecules per particle is calculated to range between 3,000 and 4,000 in case of RIgG and between 8,000 and 9,000 for Protein A.

Measuring Protein A/Immunoglobulin interactions

In order to measure the interaction strength between Protein A and RIgG the following experimental procedure turned out to be effective and reliable: A bead modified with Protein A is captured and brought in contact with the tip of the micropipette were it is fixed by capillary forces. Subsequently a second particle with RIgG on its surface is captured and brought in the immediate neighbourhood of the already immobilized bead at the micropipette. A schematic of the experimental setup is depicted in Fig. 1a.

In a computer controlled measurement procedure the particles are brought in close proximity until a force level of 5 ± 2 pN is reached. This state is maintained 1 s enabling the proteins on the particle surfaces to interact with each other. Then the beads are pulled apart until the bond breaks (Fig. 1b). During the whole process which was looped and repeated several times the position of both beads and their distance to each other was recorded constantly. All measurements were done in 10 mM phosphate buffer. Depending on the experimental requirements the moving speed of the optical stage or the sodium chloride concentration (20–4,000 mM) of the buffer was altered. The obtained data were used to calculate the rupture forces that were analysed afterwards.

Statistical data analysis

From the obtained data sets (at least 102 disruption events) the mean values of the rupture forces were calculated. In order to show significant differences between the distributions of the data sets a thorough data analysis was necessary. Therefore the confidence interval with a probability of 95% was calculated for all data sets. Using this value one can directly decide whether two distributions are significantly different from each other, if their confidence intervals do not overlap or have to be considered as similar, if they coincide. As a precondition for the applicability of the confidence interval the data have to show a normal distribution. To proof normal distribution the Kolmogorov–Smirnov-test was applied.

Results

Specific interactions and control experiments

A typical force distance plot used to determine the rupture forces is depicted in Fig. 1b. After approaching the two particles coated with Protein A respectively RIgG a strong interaction can be detected by pulling the two particles apart and that breaks if a distinct force level is exceeded. To test weather the observed interactions originate from specific Protein A/Immunoglobulin G interactions or are of unspecific nature RIgG- or Protein A beads were brought in contact with an unmodified carboxylated bead (Fig. 1c, d). No interaction could be detected. Additional similar measurements carried out using RIgG saturated Protein A-particles and RIgG-beads (Fig. 1e) did not exhibit any interactions too. The same holds for Protein A-beads that were brought in contact with RIgG-colloids saturated with Protein A (Fig. 1f).

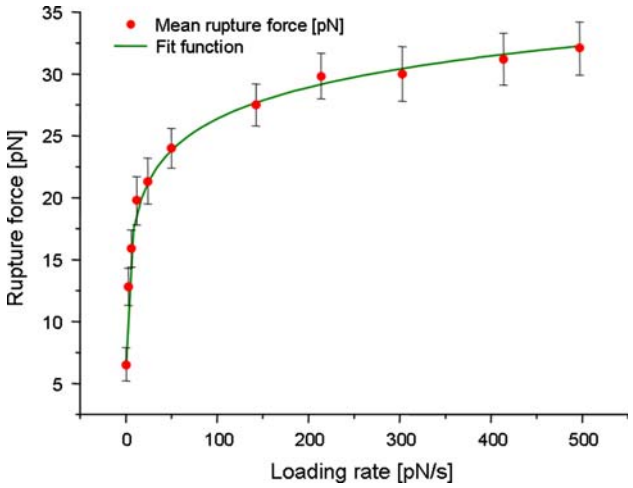

Fig. 2 Loading rate dependence of the median rupture forces. The mean rupture forces were plotted against the applied loading rate (*red full circles*, 95%-confidence intervals are added in *black*). A steep increase of the rupture forces at rising loading rates is followed by a pronounced plateau if loading rates exceeding 215 pN/s. The data were fitted by applying Eq. 2 (*green line*)

Loading rate dependence

To study the dependence of the Protein A/Immunoglobulin G interaction on the applied loading rate (rate of force appliance in pN/s) the velocity used for pulling the interacting particles apart was altered. Measurements were done in 10 mM Phosphate buffer pH 7 with 20 mM NaCl. The different resulting loading rates ranged between 0.3 and 500.0 pN/s (Table 1). For each velocity more than 100 disruption events were recorded. To determine the mean rupture force for each loading rate the observed forces directly before bond breakage were determined from the force distance plots and averaged. The Kolmogorov–Smirnov-test was applied to each data set and they were found to be normal distributed. Subsequently the confidence interval with a probability of 95% was determined. Together with the mean rupture forces its lower and upper limits are plotted as error bars in a orce/loading rate-diagram (Fig. 2). Within this plot a pronounced increase of force can be observed which levels off at $\sim$30 pN if the loading rate exceeds 215 pN/s.

Sodium chloride dependence

Besides the loading rate also the effect of salt on the interaction strength between Protein A and RIgG was studied. Therefore the rupture forces were measured at a constant loading rate (50.0 pN/s) but under varying sodium chloride concentrations (10 mM Phosphate buffer pH 7.0 with 20–4,000 mM NaCl).

For data analysis the same procedure as for the data obtained from the loading rate dependency experiments was applied. Only the data for the 500 mM sodium chloride concentration barely miss the criteria for normal distribution. Nevertheless also for this value the upper and lower limit of the confidence interval was determined. Subsequent to the statistical analysis (Table 2) the mean rupture forces were plotted against the salt concentration (Fig. 3). With rising molarity of sodium chloride (20–500 mM) an initial decrease of the rupture forces can be observed. But exceeding this value a pronounced increase of the interaction strength can be observed leveling off above 3,000 mM salt.

Discussion

Loading rate

In our measurements we found a marked dependence of the mean rupture forces, from the applied loading rate (0.3–500.0 pN/s). They range between 6.5 and 31.9 pN (Fig. 2 and Table 1). The loading rate effect has also been demonstrated by Strigl et al. (1999). For their experiments they used a micropipette-based picoforce transducer consisting of a red blood cell fixed by capillary suction. A microparticle carrying one type of adhesion proteins was biochemically linked to the immobilized blood cell. The second type of particle coated with the respective ligand

Table 2 Mean rupture forces at different sodium chloride concentrations

NaCl (mM)	Mean rupture force (pN)	Confidence interval		Standard deviation (pN)	Number of single rupture events
		Lower limit ($P = 95\%$) (pN)	Upper limit (pN)		
20	21.3	19.5	23.8	11.1	138
150	19.8	18.2	21.5	10.4	156
500	17.4	15.7	19.2	10.9	151
1000	18.1	16.7	19.5	9.3	177
2000	24.4	22.3	26.5	12.9	145
3000	26.9	24.9	29.0	12.3	151
4000	26.6	24.2	28.9	13.1	125

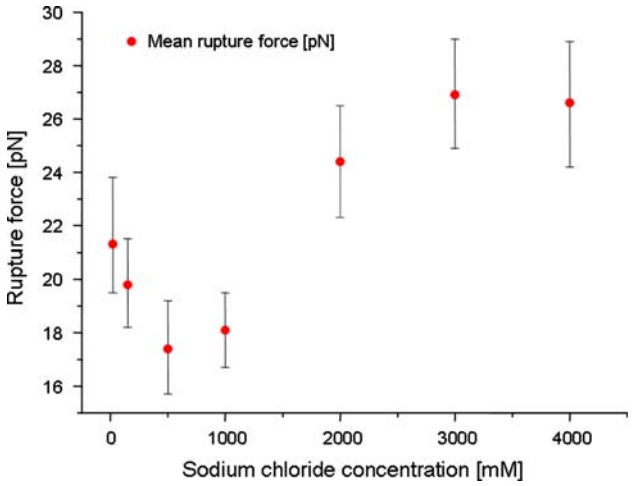

Fig. 3 Salt dependence of the median rupture forces. The mean rupture forces are plotted against the sodium chloride concentration (20–4,000 mM) (*red squares*, 95%-confidence intervals are added in *black*). With rising salt concentration the rupture forces initially decrease and reach their minimum at 500 mM NaCl and a force of ~17 pN. Further increase of the sodium chloride concentration stabilizes the Protein A/Immunoglobulin G interaction and leads to a steep increment of the forces necessary to destabilize the bond which levels of between 3,000 and 4,000 mM and rupture forces of ~27 pN

was held in a second microtip. As interaction partners also Protein A and Immunoglobulin G (rabbit anti mouse IgG) were used.

The dissociation of a specific bond as those between Protein A and Immunoglobulin G has to be considered as a statistical process for spontaneous as well as for force induced dissociation. By applying external forces the enthalpic barrier of bond dissociation is lowered. Other effects that are comparably small are negligible in this regime (Strigl et al. 1999). Based on this assumption the rate of bond dissociation (k_{off}) can be described by

$$k_{off}(f) = k_0 \exp(f/f_0) \quad (1)$$

In this equation, proposed by Bell et al. (1978), f stands for the force that acts on the bond and k_0 for the rate of spontaneous dissociation. The value f_0 is defined as the characteristic force and describes the intrinsic properties of the bond. Based on Bell's model a fit function was developed by Strigl et al. (1999) that describes the measured loading rate dependence of the rupture forces:

$$F_{1/2} = f_0 \ln\left[\exp(F_1/f_0) + \frac{f \ln 2}{k_0 f_0}\right] \quad (2)$$

In this expression $F_{1/2}$ represents the mean rupture force and F_1 is the detection limit for bond dissociation. By fitting the mean rupture forces with Eq. 2 the value for f_0 and k_0 were determined to be 3.67 (±0.12) pN and 0.0143 (±0.0029) s. The obtained curve is depicted in Fig. 2.

The rupture forces exhibit a relatively wide distribution as documented by the high standard deviation (see Table 1). As already pointed out by Strigl et al. (1999) this effect has different types of origin: Protein A owns four IgG binding sites that may show different affinities to IgG. This effect can be amplified by influences due to the immobilization of the proteins onto microparticles. The coating procedure can lead to sterical hindrance of the interaction and results in varying yield forces. Furthermore Protein A exhibits very variable affinities to different subclasses and isoforms of IgG.

Salt concentration

In our measurements it turned out that with rising salt concentrations the yield forces initially decrease from ~21 pN at 20 mM NaCl to ~17 pN at 500 mM NaCl. After this minimum the rupture forces rise and reach a maximum at ~27 pN (at a salt concentration of 3,000 mM). Sodium chloride concentrations above this value exhibit no further increase in rupture force (Fig. 3).

In the literature a stabilization of the interaction strength by sodium chloride at concentrations between 500 and 3,000 mM was already reported (Salinas and Ott 1987; Rehm 2006; Arouri et al. 2007), which is in agreement with our data (Fig. 3). The course of the salt dependence can be explained as follows: At low ionic strength the destabilizing effect of salt on to the protein/protein interaction outbalances. Exceeding the critical value the stabilizing effects overcome the destabilizing one and the rupture force increases. As destabilizing factor at rising salt concentration the lowering of electrostatic forces between the interaction pair must be considered. If the interaction would be of pure electrostatic nature one would expect a continuing decrease of bond strength with increasing sodium chloride concentration (Arouri et al. 2007). Therefore as a second, stabilizing factor hydrophobic interactions have to be taken into account. The increase of salt concentration leads to a loss of protein hydration. Hence hydrophobic side chains are exposed and can interact with other hydrophobic partners via Van der Waals forces (Arouri et al. 2007).

Conclusions

In our measurements we studied the interaction of Protein A with Immunoglobulin G on a single molecule level by the use of force measuring OT. By applying different loading rates a pronounced dependence on this value was found. The resulting data could be fitted with an ansatz developed in (Strigl et al. 1999; Simson et al. 1999). The changes in interaction strength with increasing sodium

chloride concentration could be connected to decreasing electrostatic and increasing hydrophobic forces and was found to be consistent with literature data.

In general it was shown that OT with their high resolution in space, force and time are in particular suitable for topics in the field of single molecule biotechnology like the receptor ligand interactions studied here. It was proven that the experimental setup developed in our group can be applied to manifold questions in this field. Perspectively it is planed to adopt this technique to high throughput analysis of receptor/ligand interactions in combination with micro arrays.

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