Specific Antigen/Antibody Interactions Measured by Force Microscopy

U. Dammer,* M. Hegner,* D. Anselmetti,‡ P. Wagner,§ M. Dreier,* W. Huber,¶ and H.-J. Güntherodt*
*Institute of Physics, University of Basel, CH-4056 Basel; ‡Ciba-Geigy, Research Services, Physics, CH-4002 Basel; §ETH Zürich, Department of Biochemistry II, CH-8092 Zürich; and ¶F. Hoffmann-La Roche, CH-4002 Basel, Switzerland

ABSTRACT Molecular recognition between biotinylated bovine serum albumin and polyclonal, biotin-directed IgG antibodies has been measured directly under various buffer conditions using an atomic force microscope (AFM). It was found that even highly structured molecules such as IgG antibodies preserve their specific affinity to their antigens when probed with an AFM in the force mode. We could measure the rupture force between individual antibody-antigen complexes. The potential and limitations of this new approach for the measurement of individual antigen/antibody interactions and some possible applications are discussed.

INTRODUCTION

A fundamental prerequisite for the amazing complexity of life is specific recognition on the molecular level. The interplay of multiple noncovalent bonds (e.g., electrostatic, electrodynamic (van der Waals), or hydrogen) or hydrophobic interactions is the basis for highly specific interactions. Various assays and biosensors have been successfully developed over the last few decades (Chaiken et al., 1990). Recently, a new approach has been explored, i.e., the direct measurement of the intermolecular interaction by suitable "force measuring devices." It turns out that the atomic force microscope (AFM) (Binnig et al., 1986; reviews by Hansma and Hoh, 1994, and by Morris, 1994) fulfills important requirements for such a device, because measurements in the piconewton range are readily achievable under physiological conditions. Promising results were obtained from the biotin/streptavidin model system (Lee et al., 1994a; Moy et al., 1994; Florin et al., 1994; Dammer et al., 1995a), from complementary DNA strands (Lee et al. 1994b), and from adhesion proteoglycans (Dammer et al., 1995b). In addition, adhesion forces and the elasticity of proteins have also been mapped (Radmacher et al., 1994a,b). However, the biggest scientific challenges in this field, and potentially the most rewarding applications for the new technique, are related to the interaction between antibodies and antigens.

Although the antibody/antigen interaction is of great practical and theoretical relevance, its direct study by force measurement is rendered more difficult by several factors. Both antibodies and antigens usually have complicated tertiary structures and need firm attachment to the force-measuring device. Their interactions (Davis and Padlan, 1990; van Oss, 1994; Webster et al., 1994) depend on geometric factors, conformational state, and environment, and steric hindrance must be avoided. The expected forces are in the piconewton range, and the active sites of the antibodies are small compared with the antibody itself, so that nonspecific interactions must be considered.

In this study, immunopurified polyclonal goat IgG antibodies directed against biotin were used. In our case, the biotin is coupled via long (2.24 nm) spacer arms to bovine serum albumin (BSA), and together with the BSA forms an artificial antigen (biotinylated BSA or BBSA). The advantage of this is that the antigenic effect of the BBSA molecule containing 8–12 biotin moieties should be less sensitive to conformational changes, steric hindrance, and orientation. In our experiment, we have covalently immobilized the two components on the AFM sensor tip and on a flat gold surface via a newly synthesized ω-functionalized (NHS) dialkyldisulfide cross-linker to guarantee stable and reproducible conditions. The chemisorption of the long-chain cross-linker in a self-assembly process provides a highly reactive monolayer surface for coupling primary amines under mild conditions. Furthermore, we have used ultrasoft cantilevers for high force sensitivity and blocking molecules (BSA) to reduce nonspecific background. Several control experiments were carried out to check the validity of the force measurements.

EXPERIMENTAL

All proteins and immunocompounds, including the biotinylized BSA, were purchased from Pierce (Pierce Europe, Oud Beijerland, The Netherlands) and were of the highest available quality. The manufacturer modifies the BSA with NHS-LC-biotin (BSA has 59 lysines). About 30–35 are available for coupling with the NHS-LC-biotin. It is specified that 8–12 mol of NHS-LC-biotin was coupled per mole of BSA. Of the 30–35 lysines of the BSA there were still more than 20 free for coupling to the aminoreactive DSU SAM. Dithio-bis(succinimidylundecanoate) (DSU) cross-linker was synthesized according to the method of Wagner et al. (1994, 1995, and manuscript submitted for publication). Solvents, of the highest purity, were from Fluka (Buchs, Switzerland), and gold (99.99%) was from Balzers (Zürich, Switzerland). Flat gold surfaces were produced either conventionally by thermal evaporation on silicon

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Address reprint requests to Dr. M. Hegner, Institute of Physics, University of Basel, Klingelbergstrasse 82, CH-4056 Basel, Switzerland. Tel.: 41-61-267-3763; Fax: 41-61-267-3784; E-mail: hegner@ubaclu.unibas.ch.

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wafers at room temperature (Dammer et al., 1995b) or as ultraflat template-stripped gold as previously described (Hegner et al., 1993; Wagner et al., 1995; Hegner and Wagner, 1996). The two methods showed no effect on the results. The two gold surfaces, i.e., the tip and the substrate, were treated basically in parallel. Amino-reactive self-assembled monolayers of DSU on gold surfaces were prepared by immersion of the fresh gold in a 0.5 mM solution of DSU in 1,4-dioxane over night at room temperature. After careful rinsing with 1,4-dioxane and air drying, the surfaces were immediately subjected to the immobilization of antibodies and derivatized albumin (incubation 90–180 min, four times washing with 3 ml buffer). The washing steps guaranteed that no multilayers of protein absorbed, as they show no tendency to aggregate in solution. On the other hand, there might be some unspecifically bound molecules that are washed away, leaving some uncoated surface. AFM imaging always shows a coverage close to one monolayer (data not shown), proving that this effect plays only a minor role. As standard buffer, degassed phosphate buffer (165 mM NaCl, 2.7 mM KCl, 10.1 mM $Na_2HPO_4 \cdot 7H_20$, 1.8 mM KH_2PO_4, pH 7.3) was used. All measurements were performed in this standard buffer in a commercial fluid cell (Digital Instruments, Santa Barbara, CA). BBSA was used at 0.6 mg/ml buffer, BSA at 1 mg/ml, immunopurified polyclonal goat IgG anti-biotin antibodies (ABIO) and rabbit anti-mouse IgG control antibodies (AMOUSE) at 10 μg/ml. Most samples were immediately used, although some others were used after 1 day's storage at 4°C. A series of experiments with many different tips and substrates and with many hundreds of approach-retract cycles was carried out, taking into account the statistical nature of a single interaction event. The functionalized sensor tips and substrates were always kept under buffer. Blocking was done with a solution of streptavidin (1 mg/ml) or biotin (1 mg/ml; Fluka). All measurements were taken on a multimode NanoScope III (Digital Instruments). Approach/retract cycles were performed as gently as possible (at 0.5 Hz). Here, the cantilevers were calibrated by SEM and resonance frequency measurements (k = 0.014 N/m, 5% relative error, ~40% absolute error).

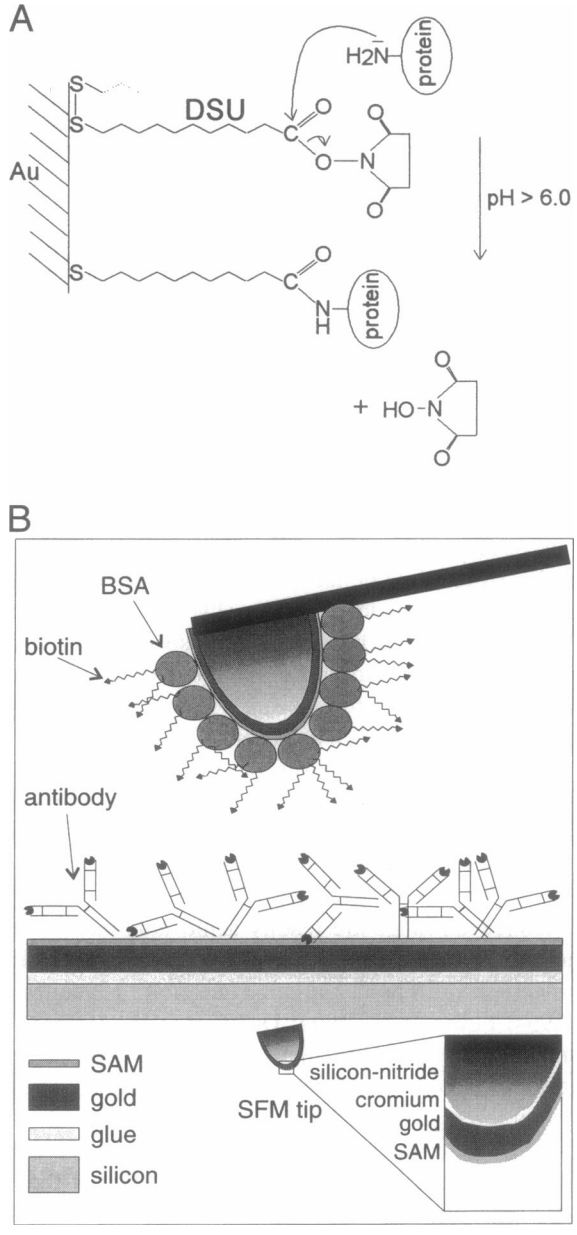

FIGURE 1 (*A*) Immobilization procedure. Both the antibodies and the antigen contain free amine groups, which can be coupled to gold surfaces via DSU cross-linker (see text; drawing not to scale; the thickness of the cross-linker monolayer is 1.7–1.9 nm). (*B*) Sketch of the experimental set-up. Biotinylated bovine serum albumin (BBSA) was covalently immobilized on a sensor tip. The anti-biotin antibodies (ABIO) are coupled to a flat gold substrate. In control experiments the BBSA is replaced by normal BSA or the ABIO by nonspecific antibodies. The interaction can be inhibited by adding biotin or streptavidin or suppressed by low- or high-pH buffer. The set-up can be reversed, i.e., the antibodies can be located on the sensor tip.

RESULTS

The coupling reaction of the proteins to the activated gold surface is illustrated in Fig. 1 *A*; a model of the set-up configuration is shown in Fig. 1 *B*. The bioreactive succinimide headgroup of the self-assembled monolayer is capable of binding primary amines, and the coupling reactions can be performed under physiological conditions. The reverse configuration, with the antibodies on the tip and the BBSA on the substrate, was also studied. Typical approach/retract cycles are shown in Fig. 2. Fig. 2, *A* and *B*, shows typical curves taken between a BBSA-covered sensor tip and an ABIO-coated substrate; Fig. 2 *C* shows a control where BBSA is replaced by BSA. To show that ABIO/BBSA curves originate from specific antibody/antigen interactions, a variety of control experiments were performed. In Fig. 3 *A* the whole series of experiments is summarized, and the percentage reduction in the mean adhesion force of the last jump during the experiments is shown. As a measure of the interaction strength, the size of the final jump from

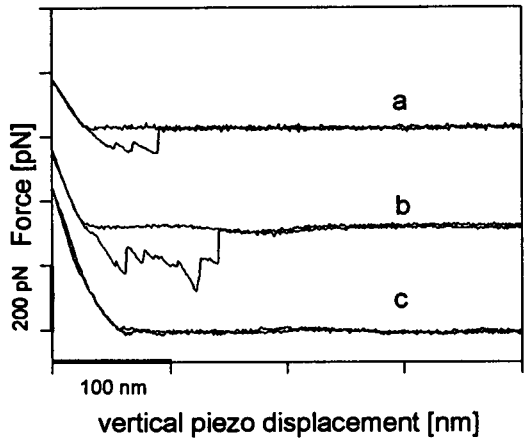

FIGURE 2 Typical approach/retract cycles taken between a BBSA-coated sensor and a ABIO-coated surface. The adhesion peaks are in the piconewton range and are often multiple (*curves a* and *b*) (discussion in text), whereas the BSA control (*curve c*) shows hardly any adhesion.

the surface peak in each curve was automatically taken and averaged over a large number (total >3000) of different curves ("mean adhesion force") (Fig. 3 *B*; total >1500). The mean adhesion force of the specific ABIO-BBSA is 111.5 pN. All measurements were included in the averaging process in to avoid misinterpretation due to arbitrary choice of "good" or "bad" curves. It turned out that there is a clear difference between the (specific) ABIO-BBSA interaction and the (nonspecific) ABIO-BSA interaction (Fig. 3 *A*, nos. 1 and 2). Blocking by either biotin or streptavidin is possible (Fig. 3 *A*, nos. 3 and 4). If we use an antibody that is not specific for biotin (rabbit anti-mouse), we obtain mean adhesion forces similar to the nonspecific background, again indicating the specificity of the BBSA-ABIO reaction (Fig. 3 *A*, no. 7). The results for the reverse set-up with the antibodies on the sensor tip were similar to those from the set-up sketched in Fig. 1 *B* (data not shown). However, the reverse set-up often shows lower specific binding, indicating that the antibodies at the tip are more vulnerable to misorientation. The specific interaction can be suppressed by low pH (10 mM citrate, pH 1.8; Fig. 3 *A*, no. 5) or high pH (100 mM trimethylamine·HCl, pH 11.5; Fig. 3 *A*, no. 6) (Goldblatt et al., 1993; Harlow and Lane, 1988). In all cases, the suppression is reversible, i.e., the full adhesion force is reconstituted when standard buffer is used again. Table 1 summarizes Fig. 3 *A*.

Fig. 3 *B* shows a histogram of the analysis of the final jump from the surface during the specific BBSA-ABIO interaction. We found maximum peaks of the measured adhesion forces composed of integer multiples of an elementary force of 60 ± 10 pN. Although we were dealing with polyclonal antibodies that have been affinity purified, a clear quantization could be observed.

DISCUSSION

The control experiments and the quantized force of the last jump out of contact have confirmed that the force measure-

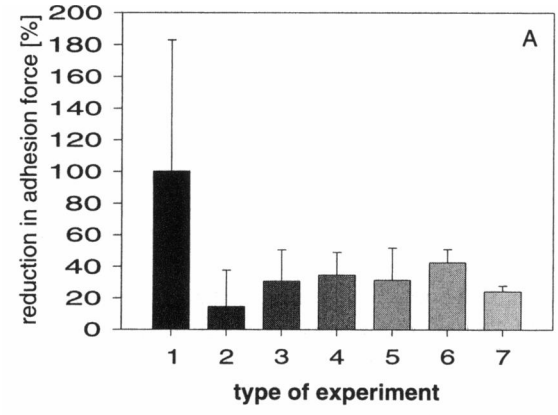

Table 1: Summary of the control experiments of the antibody-antigen interaction of Figure 3A (see text).

	surface/tip	surface/tip	medium	force [pN]	force [%]
1	ABIO	BBSA		111.5±98.6	100.0±88.4
2	ABIO	BSA		17.5±26.5	15.7±23.8
3	ABIO	BBSA	biotin	33.8±22.5	30.3±20.2
4	ABIO	BBSA	streptavidin	39.6±15.1	35.5±13.5
5	ABIO	BBSA	high pH	34.7±23.8	31.1±21.3
6	ABIO	BBSA	low pH	48.5±7.9	43.5±7.1
7	AMOUSE	BBSA		26.8±4.7	24.0±4.2

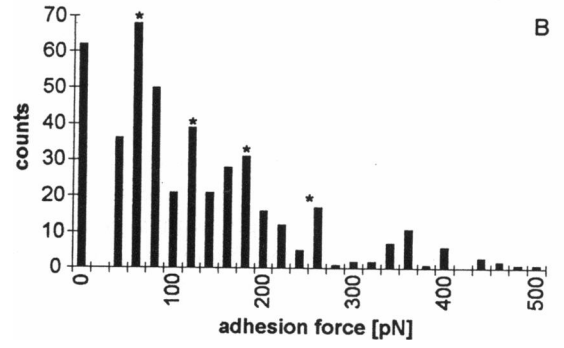

FIGURE 3 (*A*) Summary of the control experiments (>3000 curves). Bar no. 1 shows 100% force of the mean value of 111.5 pN ± 98.6 pN for the specific ABIO-BBSA interaction (last jump off the surface). The other bars show the percentage reduction of the interaction force corresponding to the 100% force of the specific interaction. In no. 2 BBSA was replaced by BSA; nos. 3 and 4 show blocking by biotin and streptavidin, respectively. Nos. 5 and 6 represent the (reversible) change of the buffer from PBS to low pH and high pH, respectively, whereas in no. 7 ABIO was replaced by a nonspecific antibody AMOUSE; errors are standard deviations. (*B*) Histogram of the adhesion force between biotinylized bovine serum albumin and antibiotin IgG. Analysis of the peak of the final jump off the surface during approach/retract cycles.

ments represent specific interactions between biotin and anti-biotin antibodies. Because of the covalent anchoring of the antibodies and the (artificial) antigens it was possible to use the same tip for 100 measurements and more. The forces that are exerted on the sample during the repulsive regime are in the range of 100 to 400 pN. The radii of the silicon-nitride tips used are specified by the manufacturer to be around 50 nm. This repulsive force is then active on an area

of a least 100 nm^2. Because the biomolecules show a quantized interaction force during the jump out of contact, no irreversible damage of the biomolecules occurred. Although we have no evidence of possible pressure effects, a feedback, limiting the repulsive force on the biomolecules, will be implemented in the approach cycles of future experiments. Some major points have still to be discussed: Can an estimate for the binding force of an individual biotin and antibiotin pair be given? What are the possible applications of this approach in view of the experimental results, and how should future force experiments be designed?

The size of the peak of the final jump from the surface in the approach/retract cycles that we used as a measure for the interaction force is certainly a direct determination of the strength of the bond formed between one or multiple individual biotin and antibiotin pairs. Such a value will also depend on external parameters such as temperature, pH, or buffer conditions, and on intrinsically statistical parameters such as the exact relative orientation and state of the complex, and is within the scope of future experiments. Thus, meaningful statements can only be made about force distributions. Whether statements about individual bonds can be made will depend on the sharpness of the force maxima in this distribution and on the force sensitivity of the instrument used. One solution is to look at the detailed shape of the approach/retract curves and focus on discrete steps. This will work well if the width of the force distribution is relatively small, for then multiples of a force quantum can be identified as such (Moy et al., 1994). In our experiments we analyzed the size of the final jumps (Fig. 3 *B*), and a force quantization of 60 $\pm$ 10 pN was observed. About 6% of the curves show no adhesion, and the others form a broad distribution ranging up to 500 pN with a concentration between 40 and 260 pN. In the future we plan to study systems with monoclonal antibodies well oriented on the surface, allowing a more pronounced observation of force quantization, and thus a correlation with data obtained from thermodynamic experiments.

When discussing the force of separation of individual antibody/antigen pairs, it is important to compare numbers obtained from the energetics of the reaction. If the free enthalpy ΔH is known and the effective range of the potential d can be estimated, then the unbinding force is given by $F = \Delta H/d$. Typical antibody/antigen complexes with K_{ass} from 10^2 to 10^{10} M^{-1} have free enthalpies of about 30 to $100 \cdot 10^{-21}$ J/pair (van Oss, 1994). With a binding pocket of 0.6 nm from Davis and Padlan (1990; obtained for vitamin K_1 and antibody) or 0.93 nm for biotin/streptavidin (Moy et al., 1994), this gives a force of about 35 to 165 pN (20 to 100 pN, respectively). In round numbers, this corresponds with steps ($n \cdot 60 \pm 10$ pN) that we commonly see in the final jump from the surface in our approach/retract curves (see Figs. 2 and 3 *B*). It is not entirely clear whether ΔH or rather the free energy ΔG describes the antibody/antigen complex formation better (Kelly and O'Connell, 1993; Webster et al., 1994; Moy et al., 1994). In this study, however, we are dealing with polyclonal antibodies and cannot distinguish between the two. However, from the above estimate and from geometrical considerations (the radius of our tips with the gold coating is about 60 nm, the diameter of an antibody is about 15 nm, BBSA is slightly smaller), it is clear that only very few (say less than ten but often only one) antibiotin/biotin complexes are formed and subsequently broken in an approach experiment.

There is one important point to be taken into account when looking at the detailed shape of approach/retract curves. We interpret the fine structure (multiple jumps) as the breaking of different contact points that are likely to be antibody/antigen pairs but nonlinear convolution of multiple unbinding processes and might also include nonspecific background as given by van der Waals or electrostatic interactions. Furthermore, the effect of lateral movement of the sensor tip during the process of approach or withdrawal must be considered. For geometrical reasons, there will be a force on the tip that is parallel to the surface, reflecting the finite angle between the cantilever and the surface (typically 10–20°). To a first approximation (no bending) the lateral movement Δx is proportional to the vertical movement Δz, with $\Delta x = \tan \theta \, \Delta z$, where θ is the mounting angle of the cantilever. In future experiments, this effect should be software-corrected by moving the sample laterally while taking approach/retract cycles. This effect is rather small (a 10-nm vertical movement in contact might cause a 2-nm lateral drift) but makes it more difficult to interpret the detailed shape of the fine structure. Moreover, we observe a vertical lever movement of about 30–50% of the total piezo excursion in the attractive regime before the last jump. Because the biomolecules are covalently attached to both surfaces via a heterobifunctional cross-linker, a lateral displacement and therefore a piling up of some molecules is unlikely. The resulting tip/sample distance can be explained by a "spring" that is loaded during retraction and bridges between tip and sample. The extra spring could be represented by a partial unfolding of the participating biomolecules. This observation will be the topic of future investigations.

Another important question to be addressed in light of these experiments concerns the future applications of the force method. Direct measurements with a force-measuring device such as the AFM are complementary to the existing immunotechnology based on biosensors or labeled antibodies and can give additional information about the spatial distribution of the receptors or the antigens. New immunosensors of high throughput rate, combined with minute sample quantities, could be used in the screening of novel active substances. Such applications might be within reach if some requirements can be fulfilled. The main design principle for future experiments will be that the reaction between the bioactive molecules takes place under well-controlled conditions as close as possible to the physiological. Therefore, the force-measuring device must be extremely gentle, to avoid conformational changes or even destruction of the molecules. Furthermore, lateral drift must be corrected. Taking the rapid progress of electronics into account, these conditions might be realized soon. In addi-

tion, the preparation of the sensor tips is essential, and should include well-oriented antibodies (Brada and Roth, 1984; Ill et al., 1995) or F_{ab} fragments, dense packing without steric hindrance, and the suppression of unspecific background.

In conclusion, the specific interaction between polyclonal biotin-directed antibodies and biotinylated bovine serum albumin has been measured directly by force microscopy. Specificity has been demonstrated by a number of control experiments. It has been estimated that only a very few antigen/antibody complexes contribute to the measured binding force. Provided that certain limitations can be overcome, the correlation to data from thermodynamic experiments of individual antigen/antibody interactions should be possible. In future research, this technique has obvious applications in many different fields.

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