Real-Time Measurement of Spontaneous Antigen-Antibody Dissociation

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ABSTRACT We report observations in real time of thermally driven adhesion and dissociation between a monoclonal IgE antibody and its specific antigen *N*-ϵ-2,4-dinitrophenyl-L-lysine. Both molecules were attached to the surfaces of different polystyrene microspheres trapped by optical tweezers. Monitoring spontaneous successive attachment and detachment events allowed a direct determination of the reaction-limited detachment rate k_{off} for a single bond and also for multiple bonds. We observed both positive and negative cooperativity between multiple bonds depending on whether the antigen was linked to the microsphere with or without a tether, respectively.

INTRODUCTION

A great many biochemical processes are mediated by specific receptor-ligand interactions, and often both molecules are attached to juxtaposed surfaces of different cells. Given the importance to accurately characterize the binding kinetics that governs such interactions, they have been extensively studied with a variety of methods such as shear flow (Pierres et al., 1995, 1998; Goldsmith et al., 2000; Swift et al., 1998), atomic force microscopy (Schwesinger et al., 2000; Florin et al., 1994; Lee et al., 1994), fluorescence quenching (Swift et al., 1998), surface plasmon resonances (Myszka et al., 1997; Yu et al., 1998), photobleaching recovery in combination with total internal reflection measurements (Lagerholm et al., 2000), and the quartz crystal microbalance (Ebato et al., 1994). In all these experiments the association and/or dissociation of bonds, typically multiple bonds, between the receptor and the ligand molecules is induced by an externally applied force. In nature, however, many of these binding and unbinding processes occur spontaneously, driven by thermal motion. In addition, adhesion and dissociation often begins and ends, respectively, with a single bond. Formation of subsequent bonds is likely to depend on the presence of already formed ones. Thus, on the one hand, accessing the single-bond regime may provide more insight into the initial adhesion mechanisms (see, for example, the March 12, 1999 special issue of Science on single molecule chemistry). On the other hand, the overall binding kinetics and the nature of the cooperative behavior between multiple bonds may be affected by existing bonds. Hence, it is important to study thermally driven formation and dissociation of single and multiple bonds under conditions that closely mimic these processes in nature.

In this article we report the observation in real time of thermally driven adhesion and dissociation between antibody (the receptor) and antigen (the ligand) molecules immobilized on the surface of microspheres. By measuring the time interval between attachment and subsequent detachment events, we determined a characteristic dissociation rate k_{off}. We varied the surface density of antigens such that we could study the detachment of either single or multiple bonds. For single bonds we thus directly inferred the spontaneous detachment rate. In the multiple bond regime, when the antigen molecule was directly (and rigidly) attached to the surface, we observed a negative cooperativity between bonds. Dissociation occurred at higher rates compared with the single-bond rupture rate. We attributed this effect to bond strain induced by the presence of competing bonds that may prevent sufficient penetration of the antigen into the receptor-binding pocket. When attaching the antigen molecule to the surface via a short, but flexible tether, we observed the opposite, more intuitive effect of positive cooperativity among bonds. In this case, increasing the number of bonds increased the tenacity of the adhesion, and accordingly the detachment rate decreased.

The experiment used two optical tweezers (Svoboda and Block, 1994) in which two microspheres, one coated with the antibody, the other one coated with the antigen, were trapped. The bond-forming probability between antibody and antigen molecules is given by a stochastic collision whose rate is determined by the Brownian motion of the microspheres in the optical traps. Once the bonds are formed, they are subject to a very small external force that is unable to induce their breakage. Detachment thus occurs spontaneously, i.e., driven by thermal fluctuations. Although spontaneous statistical breakage of single bonds of protein A-IgG has been observed by dynamic force spectroscopy with micropipettes (Simson et al., 1999), bond breakage is generally induced by application of a constant force (Swift et al., 1998; Pierres et al., 1995; Tempelman and Hammer, 1994; Chesla et al., 1998; Stout, 2001) or a

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Note that certain commercial equipment, instruments, or materials are identified in this article to specify the experimental procedure adequately. Such identification is not intended to imply recommendation or endorsement by the National Institute of Standards and Technology, nor is it intended to imply that the materials or equipment identified are necessarily the best available for this purpose.

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variable force (Schwesinger et al., 2000; Seifert, 2000). In these experiments, the lowest forces exerted on the bonds were on the order of the force required to rapidly break the bond (Pierres et al., 1995; Goldsmith et al., 2000). Forces much lower (on order of 1 pN, similar to the ones present in our experiment) were used to determine the interaction range and the association rate of individual surface-attached molecules (Pierres et al.; 1998) but not the dissociation rate.

In our experiments we studied spontaneous breakage of single and multiple bonds, and for all conditions we recorded the entire distribution of durations of adhesion events. The strong covalent attachment of the biomolecules to the microspheres precluded lateral diffusion along the surface as is often observed in cells. Our technique could, however, be applied to living cells for the purpose of studying their adhesion to various biomolecules or their mutual adhesion.

The influence of tether length in the binding kinetics between receptor and ligand has been studied previously (Jeppesen et al., 2001; Wong et al., 1997), and it is known that it affects or purposely alters the adhesion process. The effect of tethering versus nontethering, however, has not been investigated as extensively, and we were able to study this regime with sufficient sensitivity to observe changes in the detachment rates.

The biological system we use consists of monoclonal antibody IgE and its specific antigen *N*-ε-2,4-dinitrophenyl-L-lysine (DNP). Previously, studies of detachment kinetics of IgE and various derivatives of the DNP hapten, where one of the molecules is immobilized on a surface, were carried out using fluorescence methods (Erickson et al., 1991), combined with hydrodynamic flow (Swift et al., 1998; Tempelman and Hammer, 1994) and total internal reflection microscopy combined with fluorescence photobleaching recovery (Lagerholm et al., 2000).

MATERIALS AND METHODS

Coating of the microspheres

The antigen DNP and the monoclonal IgE antibody anti-DNP clone SPE-7 (both from Sigma Chemical Co., St. Louis, MO) were covalently attached onto carboxylated spheres (Bangs Laboratories, Fishers, IN) with diameters of 5.5 μm and 4.5 μm, respectively. The covalent coupling was carried out with 10 mg of either size microspheres. The spheres were washed with 0.1 M carbonate buffer (pH 9.6) and subsequently centrifuged. This procedure was repeated three times, and the microsphere pellet was then suspended in 2 ml of 0.02 M sodium phosphate buffer (pH 4.5). A freshly prepared 0.5-ml (2%) solution of water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide was reacted with the sphere suspension and then incubated and mixed for 4 h at room temperature on a rotator. Unreacted carbodiimide was removed by washing the microspheres three times with a 0.2 M borate buffer (pH 8.5). The antigens or antibodies were then added. and the suspension was incubated overnight at 4°C on a rotator and centrifuged afterwards. The resulting pellet was suspended in 0.25 M ethanolamine and incubated for 1 h at 4°C on a rotator to block unreacted sites on the microspheres. Excess ethanolamine was removed by centrifugation. The pellet was resuspended in bovine serum albumin (BSA) solution (10 mg/ml borate buffer) and incubated for 1 h at 4°C on the rotator to suppress nonspecific binding. Finally, excess BSA was removed, and the coupled microspheres were stored at 4°C in phosphate-buffered saline (pH 7.4) on a rotator. The final volume of the sphere suspension prepared as described above was 2 ml. Spheres for use in control experiments were similarly prepared but without adding antibodies or antigens.

Attachment of the DNP molecule to the microsphere via a flexible tether was performed in two steps. First, the DNP molecule was covalently attached to the tether, and second, a biotin-neutravidin link was used to attach the tether to the microspheres. The tether we used, EZ-link-sulfo-NHS-LC-biotin (Pierce, Rockford, IL), is 22.4 Å long. To covalently attach the DNP molecule to the tether, 20 mM DNP solution was reacted overnight at pH 7.2 with 14.3 mM tether solution to form DNP-LC-biotin. The neutravidin (Pierce) was covalently attached to the microspheres following the same carbodiimide chemistry described above for the attachment of DNP to the spheres in the absence of the tether. For this, 100 μl of 4 μM neutravidin solution was used. Unreacted sites on the microspheres were blocked with ethanolamine. The neutravidin-coated spheres were then reacted with the DNP-LC-biotin at the desired concentration. Again, unreacted sites on the microspheres were quenched with ethanolamine, and nonspecific binding sites were treated with BSA solution.

The concentration of DNP and DNP-LC-biotin in solution before attachment varied from 70 pM to 700 μM in the different experiments. The concentration of antibody in solution before attachment was 0.13 μM and was kept constant in all experiments. We found that higher concentration in solution before attachment did not change the surface coverage of antibodies.

A number of control experiments were carried out to assure that the adhesion we observed was indeed due to antibody-antigen binding. First, the adhesion experiment was carried out using both types of microspheres processed through the carbodiimide chemistry without the antibody and the antigen. No attachment could be observed. In a second series of experiments we looked for adhesion between spheres coated with the antibody (or the antigen) and spheres without antibodies or antigens, as used in the previous control experiments. Again, no attachment was observed. Finally, to block all specific binding sites, we incubated microspheres coated with antibody or antigen for 1 h at room temperature with free antigens or antibodies in solution, respectively. Subsequently, these microspheres were used in adhesion experiments with the corresponding antigen or antibody-coated spheres, prepared as described above for the experiments reported in this article, and no attachment was found. Adhesion was observed only between antigen and antibody-coated spheres.

Flow cytometric assays

Flow cytometry was used to estimate the surface density of antibodies that can bind to microspheres that are coated with antigens at different concentrations in solution. For these measurements a fluorescent marker (fluorescein isothiocyanate) was attached to the antibody molecules (using the fluorescein-Ex labeling kit, Molecular Probes, Eugene, OR). Subsequently, the antibodies were placed in solution (again at a concentration of 0.13 μM) with antigen-covered spheres. Unbound antibody molecules were removed through washing. The fluorescence per sphere was measured and calibrated against Flowcal 525 spectral standard microspheres by FAST Systems (Gaithersburg, MD). The number of fluorophores was determined from a calibration of the Flowcal 525 microspheres (Lenkei et al., 1998).

For the flow cytometric measurements, six samples with different surface densities of antigens, corresponding to concentrations of antigen in solution before attachment to the microspheres varying from 70 pM to 700 μM, were prepared. For each sample, $\sim 10^5$ spheres were analyzed, and the mean fluorescence intensity was determined. Because the mean fluorescence intensity at the two lowest concentrations was at the noise level of the instrument, only the fluorescence variation from the 70 nM to 700 μM samples was used to estimate the number of fluorophores per microsphere.

A plot of the mean fluorescence intensity versus the logarithm of the concentration of the antigen solution used to coat the spheres follows a sigmoid curve. This behavior is in agreement with cell surface receptor binding models, in which the ligand is free in solution (Lauffenburger and Linderman, 1993).

From the calibration of the fluorescence, we found that the surface density of antibodies that bind to the antigen-coated spheres varies from 50 μm^{-2} to 3.1×10^4 μm^{-2} for the 70 nM to 700 μM samples, respectively. For this determination we have assumed that two markers are attached to each antibody molecule. In practice, the number of fluorophores that may attach to each antibody can vary between 2 and 10 (Haughland, 1996), and fluorescence quenching of neighboring markers may take place (Haughland, 1996). Both factors would lead to lower surface densities than the ones indicated above. By extrapolation of the sigmoidal curve to concentrations of 700 pM and 70 pM we obtained surface densities of $\sim 5 \times 10^{-1}$ μm^{-2} and 5×10^{-2} μm^{-2}, respectively.

The maximum surface density of antibodies bound to the antigen-coated spheres obtained from the flow cytometry measurements was consistent with the maximum number of antibodies that could be attached to the microspheres directly. An estimate of the number of IgE molecules that can attach to the microsphere directly can be obtained by dividing the surface area of a sphere by the parking area of each molecule. The size of the antibody is ~15 nm in its longest direction and ~5 nm along the orthogonal direction (Swift et al., 1998). The parking area of an antibody molecule on the sphere, which we computed simply as the square of the linear dimension, can thus be estimated to vary between 2.5×10^{-5} μm^2 and 2.25×10^{-4} μm^2, or equivalently, the estimated antibody surface density ranges from 4×10^4 μm^{-2} to 4.4×10^3 μm^{-2}. The surface density of 3.1×10^4 μm^{-2} from the flow cytometric measurements was within this range, and therefore, we are confident of the estimates of the antigen surface density at lower concentrations. As mentioned earlier, during all experiments the surface density of antibodies covalently attached to the microspheres was unchanged. The quantity of antibodies offered during coating was sufficient to fully cover the spheres. Hence, estimates of the maximum density of antibodies on the surface of the microspheres were also estimates for the maximum density of antibodies available for adhesion during the experiment.

Estimating the number of bonds formed during adhesion

The average number of bonds, N, that could be formed during our experiments at any one encounter between the two microspheres depends on the surface density of antigens, the surface density of the antibodies, and the size of the contact area. To estimate the contact area, we needed to estimate to what distance the surfaces of the microspheres needed to approach for binding to be possible. This distance depends on the orientation of the IgE molecule with respect to the surface of the sphere. Attachment of the IgE molecule to the sphere can occur randomly to any part of the antibody molecule, as the amine groups are nearly uniformly distributed throughout the surface of the protein (Hermanson, 1996). Given the size of the antibody molecule, we could define the contact area to be a spherical cap of height equal to half the characteristic antibody dimension, 5 nm or 15 nm, respectively. We thus found that the contact area may range between 4×10^{-2} μm^2 and 12×10^{-2} μm^2, assuming an average microsphere radius of 5 μm.

Using the surface densities determined by flow cytometric assays and the larger contact area, we inferred the average number of bonds that can form (Table 1). We note that for 700 pM and below it appears that at most one bond could be formed. Assuming that under these conditions the antigen molecules follow a Poisson distribution on the surface of the microsphere, we estimated that the probability to form two bonds in the contact area was ~100 times smaller than the probability to form a single bond. The exact determination of the number of bonds formed at any encounter is very difficult. All estimates given above are conservative and most likely overestimate the number of bonds formed. The experimental results, which we present below, however, are consistent with these estimates.

TABLE 1 Estimated average number of bonds, N, in the contact area and the measured detachment rates k_{off} with and without a tether versus the DNP-antigen concentration in solution before coating of the microspheres

DNP concentration (μM)	N	k_{off} (s^{-1}), no tether	k_{off} (s^{-1}), with tether
700	3691	3.44 ± 0.13	≤0.04
0.7	2345	2.42 ± 0.21	1.05 ± 0.06
0.07	6	2.31 ± 0.05	1.03 ± 0.05
0.0007	0.06	1.58 ± 0.35	1.22 ± 0.07

For no tether, the uncertainties indicate the SD of the mean value from measurements performed on various days on the same sample. When the antigen is attached via tether, the uncertainties represent the SDs of the one-parameter fit (see Eq. 2) to the data.

Experimental setup

The experimental setup consisted of two optical tweezers (Mammen et al., 1996), one fixed and one mobile, which were used to independently trap microspheres and bring them into proximity. Both traps were obtained by focusing laser light through a ×100 oil immersion objective with a numerical aperture NA = 1.3 in an inverted microscope (Axiovert 100, Zeiss, Thornwood, NY). The sample was held between two glass coverslips that are attached to a perforated microscope slide. We observed the sample on a monitor using a CCD camera.

A diode laser supplied light at 830 nm for the fixed trap. The strength of the trap could be adjusted by changing the intensity of the light beam, and for the experiments presented here ~30 mW entered the objective lens. Excursions of the trapped microsphere from the center of the trap were monitored by optical trapping interferometry (Denk and Webb, 1990). In this method, two orthogonally polarized light beams, which function together as a single optical trap, experienced different retardations when the trapped microsphere was located asymmetrically in the illuminated region of the object plane. In this way displacement information was encoded in the ellipticity of the light polarization and measured as a normalized difference signal from two photodiodes.

In an isolated trap, at a given temperature (20 ± 1°C in our experiments), the trapped sphere experiences Brownian motion in the presence of a restoring force. The voltage signal measured fluctuated around the equilibrium position, which corresponds to 0 V. We calibrated the absolute distances over which the trapped spheres moved by taking two spheres that had associated and pulling on them with the mobile optical trap. We measure this displacement using video microscopy, knowing the magnification of our optics and the size of the pixels on our camera, and we correlated it with the voltage signal from the optical trapping interferometry. We did this for several values of the displacement and fit the dependence of voltage on displacement to a power series. We used the linear term in the power series as the conversion factor from voltage to displacement, after having verified that the voltage signal is linear in the displacement for displacements characteristic of the amplitude of the Brownian motion. The conversion factor that we determined in this way was 70 nm/V. The amplitude and the frequency of the fluctuations depend on the strength of the trap. Here, we used a relatively weak fixed trap, such that the amplitude of the motion of a trapped sphere was typically 100 nm. The spring constant of the trap, determined from the power density spectrum of the damped motion of the trapped microsphere in water (Svoboda and Block, 1994), is ~13 pN/μm. A typical signal from an unperturbed trapped microsphere is shown in the first part (for $t < 53$ s) of Fig. 1.

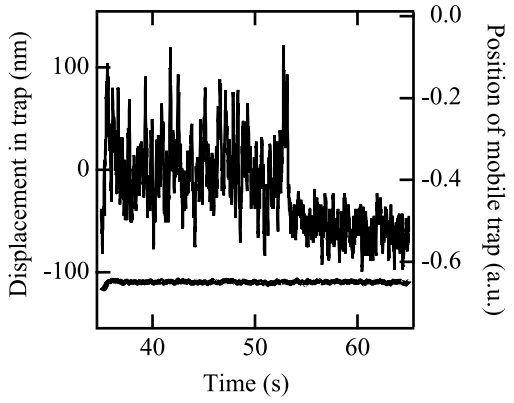

FIGURE 1 Brownian motion of microsphere trapped in the fixed optical trap as monitored by optical trapping interferometry. The trace shows the motion before ($t \leq 53$s) and after ($t \geq 53$s) adhesion. The lower trace represents the position of the mobile trap as recorded on a position sensitive photodiode. It is a measure of the relative position of the two optical traps. Adhesion occurred spontaneously, although the position of the mobile trap was kept unchanged.

The mobile trap was formed with light at 1064 nm from a Nd:YAG laser. This trap was stronger than the fixed trap and used ~200 mW of laser power. We could change the position of the optical trap in the object plane by steering the laser beam with a motorized mirror (Svoboda and Block, 1994).

In the experiments, we trapped a microsphere coated with antibodies in the fixed trap and one coated with antigens in the mobile trap. The two traps were then brought together to a distance comparable to the Brownian-motion-driven excursions of the sphere in the fixed trap. On the monitor the two spheres appeared to be in contact. The position of the sphere in the fixed trap was continuously recorded. Eventually, while keeping the distance between the two spheres unchanged, the two objects would associate. The recorded signal changed (see Fig. 1 for $t \geq 53$ s): its amplitude decreased, and its average value shifted. Because the two spheres were then attached to each other, the amplitude of the motion was characteristic of the motion in the mobile trap, which was much stronger and confined the trapped sphere more tightly. Typically the amplitude of the Brownian motion was reduced by a factor of 2 when the two spheres were attached to each other. The average value of the signal changed because the antibody-coated sphere would now oscillate around a new equilibrium position given by a balance between the two trapping forces. By measuring the voltage difference between the equilibrium values of the signal before and after attachment, we determined the displacement of the microsphere from the center of the weak trap. The force exerted on the bonds due to this displacement was then readily obtained from the trap spring constant and found to be on the order of 1 pN, which was much weaker than the typical bond strength of 60 pN measured over a 10-ms time scale (Ros et al., 1998). Although this force was measured by the displacement in the weak trap, an equal-in-magnitude but opposite-in-direction force was being applied by the strong trap. (Otherwise there would be a net force applied to the two microspheres, which would result in a net center of mass motion.) This force was also distributed among however many bonds were present at any given time, and thus the force per individual bond may have been even smaller. Therefore, due to the exponential dependence on the ratio between force and temperature (Bell, 1978) the effect of displacement from the equilibrium position in the weak trap resulted in a negligible reduction of the spontaneous dissociation rate of the molecules.

We reemphasize that in our experiments, the rupture of bonds was induced by the thermally driven motion of the microspheres. Although the presence of the optical traps (whose role was primarily to keep the two spheres in proximity to each other and to monitor bond formation and dissociation) resulted in a small, but constant, externally applied force, the loading rates on the bonds between the two microspheres were because of thermal fluctuations and thus existed naturally. In this respect, we were observing the spontaneous dissociation of bonds, under conditions similar to those that occur in biological systems. This is in contrast to some atomic force microscopy and micropipette experiments where a time-dependent force or loading rate is externally applied to induce bond breakage.

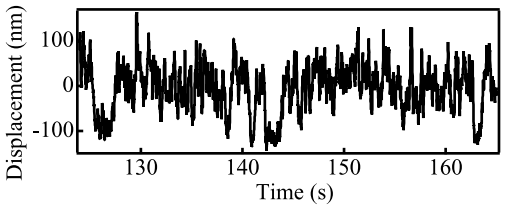

FIGURE 2 Typical time evolution of the signal showing successive association and dissociation events. The length of the association intervals varies stochastically.

RESULTS AND DISCUSSION

Distribution of attachment time intervals

Fig. 2 shows an interval of ~50 s from a typical time trace in which we observed subsequent association and dissociation events as they occurred on one pair of antibody-antigen-coated spheres. The measurements were performed at four different concentrations, 700 pM, 70 nM, 700 nM, and 700 μM, for both tethered and untethered attachment of DNP to the microsphere. At each concentration, the experiment was repeated on many different (at least 120) pairs of spheres. We measured the durations of the attachment events, or bound times, and found that the lengths of these intervals varied stochastically. Fig. 3, *a* and *b*, shows the distributions of the bound-time intervals for spheres coated with a 700 pM and 700 μM concentration of antigens without tether, respectively. Figure 3 *c* shows the distribution of the attachment time intervals at 700 nM for DNP attached through the tether. Similar curves were obtained for all other conditions. Note that when the antigen was attached using a tether, many more longer events were observed than when it was attached directly to the microsphere. When spheres were coated at a lower concentration, 70 pM with or without tether, only occasionally could attachment events be recorded, providing insufficient statistics for analysis.

To avoid falsely counting noise, we discarded time intervals shorter than 0.7 s. For this criterion the probability to count false events was smaller than 3%. (To determine the duration of an event, we set a threshold for the attachment events and determined the durations of all intervals during which the signal stayed below threshold. To discriminate real events from false ones, we used the same threshold, but with opposite sign, and determined the time intervals spent above threshold by the microsphere in the course of its random motion in the trap. From the above-threshold time

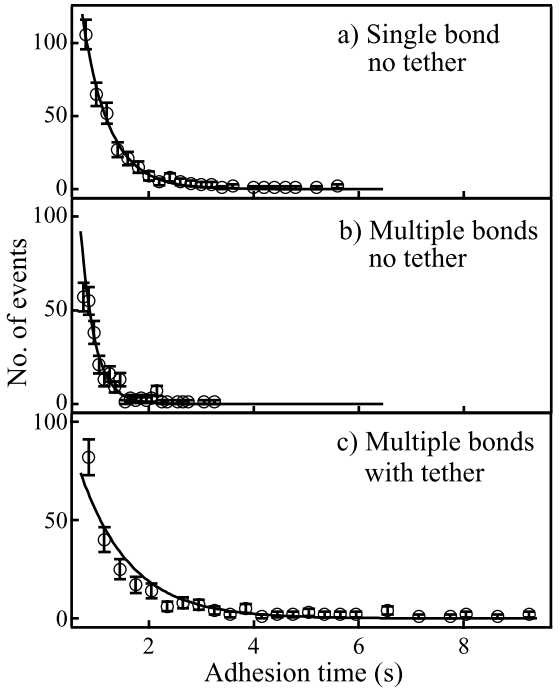

FIGURE 3 Histogram of the time intervals during which the antigen and antibody molecules were attached. (*a*) Case when only a single, nontethered bond could be formed during an adhesion event. The characteristic decay rate was 1.6 s^{-1}. The concentration of DNP in solution before coating was 700 pM. The data shown was obtained, during 1 day, from 88 time traces, each 50 s long. (*b*) Case of multiple bonds and no tether. The decay time was 3.4 s^{-1}. The concentration of DNP in solution was 700 μM. The line is also a one-parameter exponential fit to the data. The data were acquired during several days from ~120 pairs of microspheres. (*c*) Case of multiple bonds with tether. The decay time was 1.05 s^{-1}. The concentration of DNP in solution was 700 nM. For all cases, the error bars represent the statistical uncertainty computed as the square root of the number of events per bin, and all solid curves represent single-parameter exponential fits to the normalized data, which was multiplied by the integral of the data.

intervals, during which no binding could occur, we computed the probability that a time interval of a given length $\Delta\tau$ is a false event. For $\Delta\tau = 0.7$ s this probability is ≤ 0.03, and we arbitrarily decided that this is an acceptable level for discriminating between true and false events.) The time resolution of the measured intervals was, however, unaffected by this cutoff and is given by the characteristic time to detect motion in the weak trap, usually 5 ms. Also, we expected the dissociation to be a first-order process and thus to decay as a single exponential. Only in the unlikely case of a different underlying detachment mechanism occurring on a time scale shorter than 0.7 s would we not be able to identify its time evolution with the current experimental setup.

The different association-dissociation events that occur consecutively are expected to be independent of each other and obey Poisson statistics. We verified that under all experimental conditions, the average number of adhesion events that occur in a time interval of fixed length approximately equals the variance of the distribution of the number of events in this time interval. (Within 50 s on average three, and at the lowest surface density four binding events take place.) Therefore it appears natural to seek a stochastic analysis and interpretation of the data. The bond formation and rupture mechanisms are subject to fluctuations and their description may critically depend on how many bonds are involved in each association or dissociation event.

Single bonds

As discussed previously, for spheres coated at an antigen concentration in solution of 700 pM, at most a single bond could form on any encounter between the coated microspheres. The system can, hence, be described as occupying one of two possible states: a bound or an unbound state. Because of the specifics of our experimental setup, as soon as the bond was broken, the trapping force of the (fixed) optical tweezer, which was exerting a small force on the bond, pulled the microsphere into its natural equilibrium position in the center of that trap where it oscillated until the next adhesion event. Thus it seems unlikely that two subsequent adhesion events, because of bond reforming, would appear as a single adhesion event of duration greater than 0.7 s, because this time is longer than the characteristic time of motion in the trap. Therefore the statistical event we were considering is the first detachment event after attachment had occurred. We denoted $P_b(t)$ the probability that the system was in the bound state and no event had occurred during the time t. At $t = 0$ the system had just entered the bound state and the initial condition was given by $P_b(0) = 1$. The rate equation, which governs the time evolution of $P_b(t)$, then takes a very simple form:

$$\dot{P}_b(t) = -k_{off}P_b(t), \tag{1}$$

where k_{off} is the rate at which the detachment occurs. The solution of this equation, i.e., the probability that the system is still in the bound state at time t, is given by $P_b(t) = \exp(-k_{off}t)$. Accordingly, $F(t) = 1 - \exp(-k_{off}t)$ is the probability that the two biomolecules have dissociated during the time t. The derivative of this distribution, $R_{off} = dF(t)/dt$ is the probability density for the durations t during which the molecules are bound to each other. This distribution of bound times is given by:

$$R_{off}(t) = k_{off}\exp(-k_{off}t) \tag{2}$$

A very similar situation is encountered in the analysis of a random telegraph signal (Rice, 1954) or in the analysis of waiting times as they appear in queuing problems (van Kampen, 1992).

The distribution of measured attachment time intervals for single bonds is shown in Fig. 3 *a*. When normalized, the data shown are fit well by the expression given in Eq. 2. In

the figure, however, we chose to present the unnormalized distribution, to show the number of events that we typically recorded in one day. From the fit to the normalized data, we found the thermally driven dissociation rate of a single bond $k_{off} = 1.58\ s^{-1}$.

When the antigen molecule was attached via the short tether to the microsphere the distribution of bound times in this single-bond regime looked very similar to the one in the absence of the tether (Fig. 3 *a*) but with a slightly smaller decay constant $k_{off} = 1.22\ s^{-1}$. Tethered bonds appeared to be somewhat more robust because the tether may have allowed for additional flexibility. This observation was reflected by the fact that the fraction of long-time interval events that occurred in the tethered case was much higher than in the untethered case. For example, if we define a characteristic time for each decay as $\exp(-k_{off}t_c) < 1/100$, we find that in the untethered case less than 4% of the data occurred at $t > t_c$, whereas in the tethered case ~20% of the data appeared at $t > t_c$.

We note that from the analysis of a random telegraph signal pertinent information may be extracted from both times: for instance, the open times and the closed times in ion channel studies (Sakmann et al., 1980) or the bound times and the unbound times in single-bond kinetics. This was not possible with our data even when only a single bond was responsible for an attachment event. Although the bound time undoubtedly reflects the fact that the system was spending time in the bound state and could be used to extract k_{off}, the unbound time was determined by the Brownian motion of the microsphere in the optical trap. This time does not represent the time the two biomolecules, the antibody and the antigen, spent unbound in proximity of each other and can therefore not be used to infer k_{on}, the bond formation rate.

Forming multiple bonds

The interpretation of Eq. 2 outlined above is applicable not only to the single-bond case but also when multiple bonds are formed. However, only in the single-bond case does k_{off} represent the bond rupture rate, characteristic of the specific pair of molecules that bind to each other. If multiple bonds are formed during the attachment, the rate of detachment may be affected by several factors, which we address below. The overall detachment rate will no longer depend only on the bond breakage rate but also on the formation of additional bonds during an encounter between two microspheres. In particular, once the first bond is formed, other antibody-antigen pairs may be close enough to each other and form more bonds.

Intuitively, one might expect that the larger the number of bonds that can be formed, the longer the average time before the two microspheres come apart. As illustrated in Table 1 this was not the case in the experiments in which the antigen was attached directly to the microsphere. In the absence of the tether, we observed a small but systematic increase of the rate k_{off} with increasing antigen surface density. This suggests that the more molecules competing to form bonds, the smaller the apparent binding affinity, and we thus observed a negative cooperativity between bonds in this IgE-DNP binding assay.

On the other hand, when the antigen molecules were attached to the surface of the microsphere via the short tether, the binding became stronger as the surface density of exposed antigens increased, or equivalently the number of bonds that could form increased. This led to longer attachment time intervals and is evident when comparing Fig. 3, *b* and *c*. For tethered antigens, Table 1 also shows that, although we observed a decrease in the dissociation rate with increasing number of bonds per contact area, we could not distinguish between different small numbers of bonds (3 and 10 for the concentrations used in this experiment). At the highest surface density, when very many bonds may be formed, ~85% of the times in which attachment occurred, no dissociation was observed within 25 s. Therefore we were unable to determine a dissociation rate by fitting a distribution of attachment time intervals. Our observations, however, suggest that $k_{off} \leq 1/25\ s = 0.04\ s^{-1}$ is a very conservative upper estimate and in fact k_{off} is more likely to approach zero as the two spheres never came apart.

When the maximum number of bonds, N, that can be formed at any encounter between the microspheres is a small integer (≤ 5), modeling the bond formation and rupture processes using the master equation approach (van Kampen, 1992) seems appropriate. This consists of solving a set of N coupled rate equations with the appropriate initial and boundary conditions. For our experiment, we assumed that bond formation is not permitted once all bonds are broken and the formation and rupture of bonds was treated as a one-dimensional random walk in bond space. In the case of $N = 1$, the distribution of bound times predicted by this approach was the same as described above (see Eq. 2). In the case of $N \geq 2$, the model predicts a more slowly decaying distribution at times longer than 2 s, which is in contradiction to the experimental observations in the nontethered case for which we recorded all adhesion events of duration $t \leq 10$s. In addition, when multiple bonds may be formed, the model predicts a decay with multiple time constants. Unfortunately, although we recorded all events with $t \leq 20$s, the statistics of the data did not allow us to resolve multiple exponential decays. Therefore, the single exponential that we measured was most likely the weighted average of the different exponential decays that composed the solution of the rate equation for multiple bonds. Such an averaged distribution is expected to decay more slowly than the distribution of detachment times for a single bond, which is consistent with our observation for the tethered case (see Fig. 3, *a* and *c*). A more realistic description of the binding dynamics of only a few bonds might involve a bond formation rate that depends on the number of already ex-

isting bonds. To test different functional dependences of the bond formation and rupture rates on N, one would need more data at times shorter than the ones we could access with our setup.

At sufficiently high surface densities of antigens a quasicontinuous theoretical description in the form of a Fokker-Planck equation could be envisioned (van Kampen 1992; Plant et al., 1993). Although at our highest antigen densities such an approach seems appealing, it cannot describe our experiments. Complete detachment of the two microspheres relies on breakage of every bond, and in particular of the last bond, a regime in which the problem becomes discrete and the quasicontinuous theory breaks down. Also, because the k_{off} values measured for the multiple bond case were comparable to the single-bond value, it appears that breakage of the last few bonds is critical in determining k_{off}.

Experimentally we also measured the unbound time that elapsed between the end of an attachment event and the beginning of the next such event. We found that this unbound time was much shorter when multiple bonds could be formed than in the case when at most one bond could be formed. This observation is consistent with the greatly increased probability to form a bond when many more antigen molecules are available on the surface of the microsphere.

Cooperativity: role of the tether

In the absence of the tether the counterintuitive dependence of the rate k_{off} on the maximum number of bonds that could be formed may be due to steric constraints. Even if numerous antigen molecules were available on the surface, binding of adjacent molecules may be prohibited due to the immobilization of the molecules on the microsphere and due to surface density limitations. The largest antibody surface density is determined by the size of the antibody molecules or, equivalently, by the parking area required for each molecule. We suspect that, because of simple geometric constraints, once one bond is formed it is highly unlikely that a second antigen-antibody pair will be optimally aligned for binding. To form the second bond, the relative orientation of the spheres must change, and this could exert stress on the existing bond. Antigen-antibody pairs that are close enough to form a bond might only be able to do so once the existing bond has ruptured. This competition between already formed bonds and those wanting to form may induce and thus accelerate the rupture process: Formation of a bond after rupture is suppressed because the spheres move apart as soon as the last bond breaks because the two microspheres are pulled back into their individual traps. The faster dynamics observed when more than one bond is involved could also be a result of multiple weak bonds (as illustrated in the left side of Fig. 4), which are much more likely to rupture as they are weaker. Adjacent antigens may also hinder the complete penetration of any of them into the binding pocket of the antibody, thus resulting in a weaker bond that may rupture sooner.

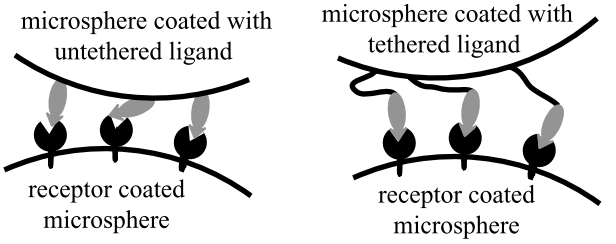

FIGURE 4 Schematic representation of the adhesion between two microspheres, mediated by receptor-ligand bonds. In one case (*left*) the ligand was attached directly to the microsphere, which restricted the orientation of the ligands and resulted in strained bonds when multiple bonds were formed. In the other case (*right*) the ligand was attached to the microsphere by a tether, which allowed greater freedom of orientation of the ligands to form multiple unstrained bonds.

The results we obtained when attaching the antigen molecules to the microspheres via an ~20-Å-long tether support this hypothesis. The tether allowed the antigen to penetrate completely into the binding pocket of the antibody (see right side of Fig. 4). Thus the bond appeared not only stronger but also more flexible. This is consistent with our measurement of a somewhat smaller detachment rate of a single bond in the tethered case as compared with the untethered case (see Table 1). When the DNP molecules are attached to the tether, adjacent antibodies and antigens may form independent bonds that do not strain the already existing one(s) and therefore delay the detachment event. This results in an increase of bond strength and leads to the observed positive cooperativity. The presentation of the ligand, here the DNP molecule, is known to significantly influence the receptor-ligand binding affinity (Ebato et al., 1994; Jeppesen et al., 2001; Wong et al., 1997). Figure 4 shows an illustration of the modified binding due to the tether. The occurrence of both positive and negative cooperativity depending on the epitope density in an enzyme-linked immunoabsorbent assay has been reported previously (Werthén and Nygren, 1993). The experiments presented here reveal the crucial role of the tether or, alternatively, the importance of flexibility in the cooperative behavior between multiple bonds in proximity to each other.

The values of the dissociation rates obtained for large numbers of bonds when the antigen was tethered to the surface of the microsphere was only slightly (less than a factor of 2) larger than values reported from fluorescence quenching experiments in which the antibodies were immobilized on cell surfaces (Swift et al., 1998; Erickson et al., 1991). Part of this discrepancy may be because in our attachment protocol of the antibody molecules to the microspheres the orientation of the antibody molecule to the surface was random, and on average, only one binding pocket was presented to the ligand. In the fluorescence quenching experiment the antibodies were attached to the

surface using the Fc portion, thus presenting both binding sites to the ligand. Our experimental situation was, in fact, equivalent to studies of binding and unbinding kinetics between the antigen and the Fab fragment of the antibody. To our knowledge, no studies exist to which a direct comparison can be made.

Summary and conclusions

We have developed a new technique to observe real-time adhesion between antibodies and antigens that are covalently attached to the surface of polystyrene microspheres. We attached the antigens to functionalized microspheres either directly or via a short, but flexible, tether. The two spheres were trapped in different optical tweezers, and both adhesion and detachment occurred stochastically and under essentially stress-free conditions. Unlike in other experiments (Chesla et al., 1998; Reichle et al., 2001; Schwesinger et al., 2000) the bond rupture was not induced by an external force or loading rate. Even though in this study we did not investigate the strength of the antibody-antigen bonds, the experimental setup is ideally suited for such studies as we can apply carefully calibrated forces along the direction of the bonds to induce their rupture.

We directly measured the rate k_{off} at which a single bond was broken as determined only by thermal fluctuations and under negligible external forces. We found the detachment rate for one bond to be $k_{off} \approx 1.22\ s^{-1}$ in the tethered case. When both molecules were directly attached to the microspheres, we observed an increase in k_{off}. We also observed that for no tether the distribution of detachment events depended counterintuitively on the maximum number of antibody-antigen bonds available in the contact area. The binding kinetics exhibited a negative cooperativity. When more flexibility was allowed by attaching the antigen molecule via a tether to the microsphere, the binding kinetics showed positive cooperativity and the dissociation rates decreased as the number of bonds formed increased.

The distribution of times after which the system returned to the unbound state always had the form of an exponential decay. Our experimental setup had the advantage of eliminating rebinding, which took place on time scales longer than 0.7 s, a process that complicates the analysis of the results of other techniques (Lagerholm et al., 2000). The observed adhesion dynamics is consistent with a stochastic description as developed for a bivalued random telegraph signal and directly predicts the distribution of attachment time intervals for the single-bond case.

The rate at which the two trapped microspheres collide depends critically on the distance from which the objects are brought together. It is difficult to accurately control this distance because all microspheres differ slightly in diameter. Furthermore, the orientation of the antibody on the sphere may also determine at what distance binding is most favorable. We plan to improve the experiment by better controlling this parameter using fluorescence resonance energy transfer. In this technique (Stryer and Haugland, 1967), the efficiency of the energy transfer between two molecules is a direct measure of the distance between them. In our experiment, we could attach one of the molecules on the antibody in proximity of the antigen-binding site, whereas the other molecule would be attached to the antigen on the sphere. A fluorescence resonance energy transfer signal at the single-molecule level (Schütz et al., 1997; Zhuang et al., 2000) may also allow us to extract information about k_{on}, the bond formation rate, in addition to k_{off}, the bond rupture rate.

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