

# Adhesion Forces Between Individual Ligand-Receptor Pairs

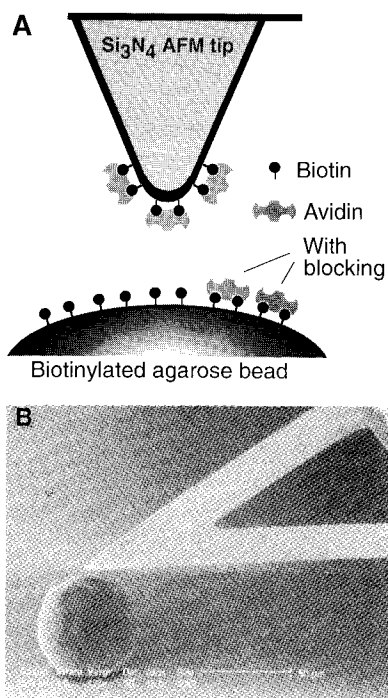
Ernst-Ludwig Florin, Vincent T. Moy, Hermann E. Gaub\*

The adhesion force between the tip of an atomic force microscope cantilever derivatized with avidin and agarose beads functionalized with biotin, desthiobiotin, or iminobiotin was measured. Under conditions that allowed only a limited number of molecular pairs to interact, the force required to separate tip and bead was found to be quantized in integer multiples of  $160 \pm 20$  piconewtons for biotin and  $85 \pm 15$  piconewtons for iminobiotin. The measured force quanta are interpreted as the unbinding forces of individual molecular pairs.

Specific molecular interaction is a distinguishing aspect of the life sciences. Such interactions, derived from multiple weak bonds between geometrically complementary surfaces of recognition sites, are short-range, noncovalent, and can be very strong. Examples are molecular recognition between receptor and ligand, antibody and antigen, and complementary strands of DNA. Attempts to investigate the forces that characterize these interactions have been limited by the lack of suitable techniques to measure the forces between individual molecules. Although the optical trapping technique is very sensitive, its use has been limited to certain samples and to measurements of forces less than tens of piconewtons and it is not suitable for studies where greater applied forces are needed (1). Magnetic force experiments (2), pipette suction experiments (3), and experiments with the surface force apparatus (4) are sensitive but lack spatial resolution. With the development of the scanning probe microscopes, particularly the atomic force microscope (AFM), new instruments and techniques have become available that have the precision and sensitivity to probe surfaces in physiological environments with molecular resolution and at forces down to the piconewton range (5).

Here we used an AFM to measure the

specific interaction between biotin and avidin, a 67-kD protein with four identical subunits (6). We chose this ligand-receptor pair as a model system because of its high affinity,



**Fig. 1. (A)** Schematics of the avidin-functionalized AFM tip and the biotinylated agarose bead, shown here partially blocked with avidin. **(B)** Scanning electron micrograph of an AFM cantilever on an agarose bead.

Physikdepartment der Technischen Universität München, 85748 Garching, Germany.

\*To whom correspondence should be addressed.

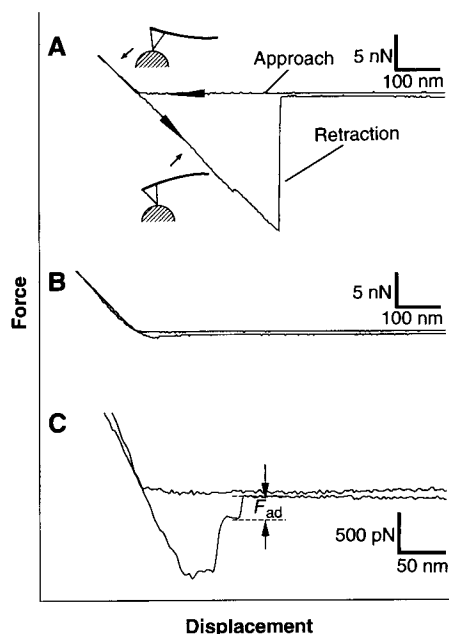
the availability of thermodynamic and structural data on it (7), and its broad range of applications. In view of further applications, we used commercially available AFM tips without modification and functionalized them with avidin. For samples, we chose biotinylated agarose beads. The minuteness of the

tip-sample interaction area in combination with the polymeric nature of the sample greatly helped to reduce nonspecific binding (8) and allowed us to resolve the forces between individual molecular pairs.

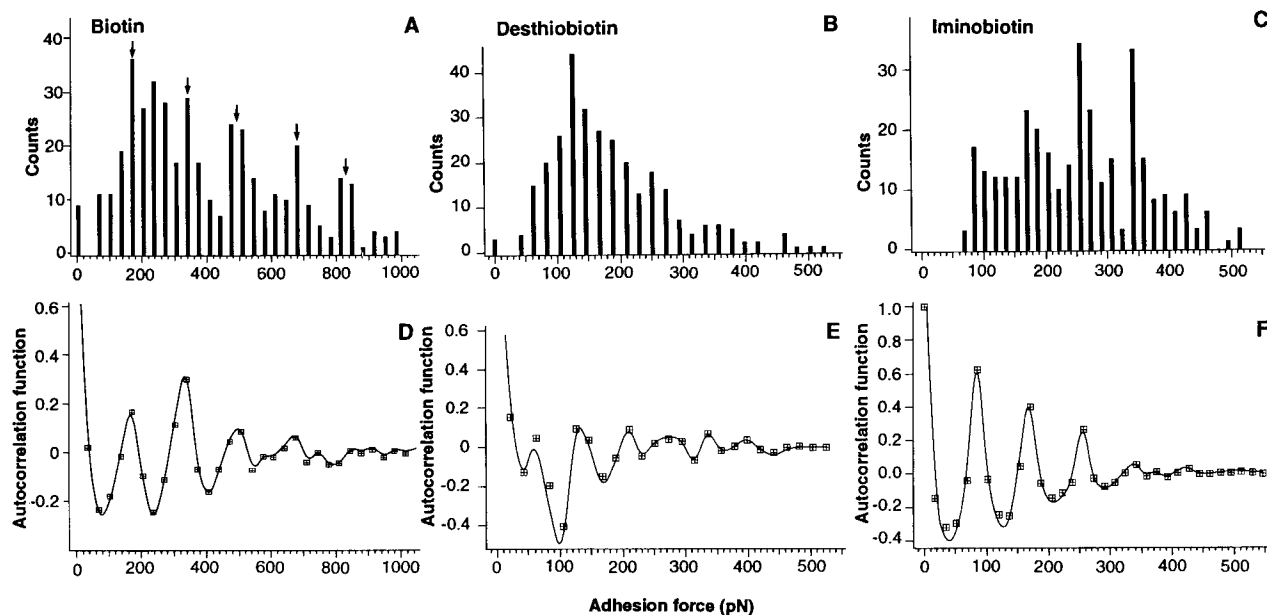
Various strategies for the functionalization of the AFM tip have been investigated in previous studies (9). We have shown that covalent immobilization of one of the binding partners on the tip [such as through cross-linkers (10, 11)] is not necessary and that a coupling through bovine serum albumin (BSA), which nonspecifically adsorbs to the silicon nitride surface of the tip, results in a sufficiently strong bond (Fig. 1) (12). For the force measurements, the tips were mounted in a scanned stylus AFM (13) and positioned on top of the bead, guided by an epifluorescence microscope. In so-called force scans (14), the deflection of the cantilever was recorded on approach to the bead and on retraction of the cantilever from the bead (15) and was directly converted into force by means of the measured spring constant of the cantilever (16). A typical force scan taken with an avidin-functionalized tip on a biotin bead is shown in Fig. 2A. The adhesive force between tip and bead results in a deflection of the cantilever toward the bead on retraction of the cantilever. With increasing bend of the cantilever, the tension on the adhesive bonds between tip and bead increases until the bonds yield. In contrast to force scans on most rigid surfaces, where the tip abruptly dissociates, the interaction between the AFM tip and the bead involves as many as 100 biotin-avidin pairs that typically break in multiple steps. [The time dependence of this forced dissociation could supply much additional information (3) but was not investigated here.] When

the tip again approaches the bead, the bonds reform, which allows the force scans to be repeated several hundred times before the yield force gradually diminishes to half its original value. The interaction shown in Fig. 2A is more than 95% blocked by an excess of free avidin (Fig. 2B) or biotin but not by free BSA (17), which confirms that the measured adhesive bond is specific, dependent on the presence of both biotin and avidin.

In order to detect single binding events, we reduced the total number of interacting molecules by blocking most of the biotin on the agarose bead with avidin. A representative force scan obtained under these conditions is shown in Fig. 2C. Most force scans displayed multiple stepwise breaking processes. One feature of the scans is the quantization of the adhesive force. A histogram derived from more than 300 force measurements is plotted in Fig. 3A. In our analysis, we considered only the last step of the unbinding, because it is conceivable that the other steps are a nonlinear convolution of multiple unbinding processes. The histogram shows five maxima highlighted with arrows. The autocorrelation analysis of the histogram is plotted in Fig. 3D. The pronounced periodicity of the autocorrelation function shows that the measured adhesion forces are composed of integer multiples of an elementary force quantum of  $160 \pm 20$  pN (18). We attribute this force quantum to the interaction of a single biotin-avidin pair. As a confirmation of the specificity of these measurements, the tip was additionally blocked by the addition of an excess of free biotin (19). This resulted in a shift of the relative population of the force quanta to lower values but did not



**Fig. 2.** Cantilever deflection curves on approach and retraction of an avidin tip on a biotinylated agarose bead (A) before and (B) after blockage with an excess of free avidin (200  $\mu$ g/ml). (C) Magnification of a force scan on a biotinylated agarose bead approximately 95% blocked with free avidin.  $F_{ad}$  is the measured unbinding force plotted in Fig. 3, A through C.



**Fig. 3.** Histograms of the adhesion forces between an avidin tip and agarose beads with (A) covalently bound biotin, (B) desthiobiotin, and (C) iminobiotin after 95% blockage with free avidin. (D through F) Autocorrelation analyses of the histograms A through C, respectively (25).

change the value of the adhesive force quanta. We found that the frequency of the nonbinding contacts was increased by a factor of 2, from 30 to 60%, after the addition of free biotin.

In comparable experiments carried out on hard samples, such as silicon oxide, instead of the agarose beads, we (9) and others (11, 20) did not find quantized adhesion. A likely explanation is that in a stiff geometry between parallel binding sites, the yielding force is also a function of their relative positions. The soft agarose bead, however, allows the force on parallel binding sites to be built up through the tension of soft springs (agarose), so that the sites' relative positions are of negligible importance. An additional advantage of the agarose bead is that, by rolling, it may release lateral stress that was built up by a lateral displacement of the tip during the force scan.

Experiments with the surface force apparatus (21) and with pipette suction techniques (3) have revealed that lipids can be pulled out of a membrane by means of receptor-ligand bonds. These studies provide a lower limit for the biotin-avidin bond of 10 pN. As a crude estimate, the adhesion force may be calculated from the gradient of the binding enthalpy. With a binding enthalpy of 35 kT, our measured adhesion force translates into a characteristic decay length of the bond of 9 Å. This value represents much more closely the overall depth of the binding pocket (7) than does the typical decay length of the short-range interactions (such as hydrogen bonds) that constitute the adhesion. This finding supports the idea that molecular recognition is a delocalized process in which, driven by thermal fluctuations, the ligand-receptor complex samples its conformation space (22). In this scenario, the external force imposes a gradient on the potential that favors steps toward dissociation. A quantitative evaluation of the mechanism, however, will require a detailed analysis of the time and force dependence of the unbinding process.

To further demonstrate the specificity of the measured force quanta, we carried out measurements with desthiobiotin and iminobiotin. Both of these biotin analogs have lower binding constants than does biotin (6). We did the desthiobiotin experiment by simply repositioning the cantilever used in the experiments in Fig. 3A over a desthiobiotin bead that was placed on the same support used for the biotin bead. The histogram from a series of more than 200 force scans (Fig. 3B) is clearly shifted to lower forces. The maximum peaks at  $125 \pm 20$  pN (23). The peaks in the autocorrelation function (Fig. 3E), however, are not meaningful enough to allow us to conclude that this is the value of the force quantum. Force measurements carried out on an iminobiotin bead that was approximately 95% blocked with avidin are summarized in the histogram in Fig. 3C. Here we find a force

quantization of  $85 \pm 15$  pN, which is also pronounced in the autocorrelation function (Fig. 3F). The overall maximum of the histogram is shifted to higher quantum numbers, which indicates a larger number of interacting pairs as compared to the previous experiment. These lower values of the adhesion force for the biotin analogs are consistent with the significantly lower value of the binding enthalpy as compared to that of biotin.

Both control experiments demonstrate the specificity of the measured interaction. As we have reported previously, this experimental approach can be applied to the investigation of other ligand-receptor systems (9). In particular, surface epitopes on soft samples such as living cells promise to be ideal systems for this approach. The theoretical limitation in sensitivity of this kind of experiment is imposed only by thermal fluctuations of the cantilever and is well below typical adhesion forces between biomolecules (24). The high force resolution of softer cantilevers will therefore allow not only the detection of weaker bonds but also the investigation of unbinding kinetics as a function of imposed force. A more immediate limitation of these measurements is the nonspecific interaction between specimens and functionalized tips. This effect can be minimized by decreasing the tip size. Smaller tips will also help to fully exploit the potential of this kind of experiment for in situ or in vivo imaging of cellular epitopes by means of specific molecular recognition mechanisms.

## REFERENCES AND NOTES

1. A. Ashkin, J. M. Dziedzic, T. Yamane, *Nature* **330**, 769 (1987); S. C. Kuo and M. P. Sheetz, *Science* **260**, 232 (1993); K. Svoboda, C. F. Schmidt, B. J. Schnapp, S. M. Block, *Nature* **365**, 721 (1993).
2. N. Wang, J. P. Butler, D. E. Ingber, *Science* **260**, 1124 (1993); S. B. Smith, L. Finzi, C. Bustamante, *ibid.* **258**, 1122 (1992).
3. E. Evans, D. Berk, A. Leung, *Biophys. J.* **59**, 838 (1991).
4. J. N. Israelachvili, *Intermolecular and Surface Forces* (Academic Press, London, 1992).
5. G. Binnig, C. F. Quate, C. Gerber, *Phys. Rev. Lett.* **56**, 930 (1986); B. Drake *et al.*, *Science* **243**, 1586 (1989); M. Radmacher, R. W. Tillmann, M. Fritz, H. E. Gaub, *ibid.* **257**, 1900 (1992).
6. N. M. Green, *Adv. Protein Chem.* **29**, 85 (1975).
7. O. Livnah, E. A. Bayer, M. Wilcheck, J. L. Sussman, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5076 (1993); S. A. Darst *et al.*, *Biophys. J.* **59**, 387 (1991).
8. Trial experiments carried out on silicon wafers and glass resulted in more nonspecific interactions than occurred on the beads. Because the agarose beads were originally engineered for affinity chromatography, we expected a minimal number of nonspecific interactions. It is also known that high pressure exerted on molecules affects their function. The soft elastic bead augments the contact area and hence buffers the avidin from pressure-induced denaturation during measurements.
9. V. T. Moy, E.-L. Florin, H. E. Gaub, unpublished results.
10. S. Karrasch, M. Dolder, J. Ramsden, F. Schabert, A. Engel, *Biophys. J.* **65**, 2437 (1993); M. Egger, S. P. Heyn, H. E. Gaub, *Biochim. Biophys. Acta* **1104**, 45 (1992).
11. M. Pierce, J. Stuart, A. Pungor, P. Dryden, V. Hlady, unpublished results.
12. The modification of the AFM cantilever was carried out as follows: 50  $\mu$ l of biotinylated BSA (Sigma, Deisenhofen, Germany) at 1 mg/ml was adsorbed onto the tip of an  $\text{Si}_3\text{N}_4$  cantilever (Digital Instruments, Santa Barbara, CA) overnight at 37°C. After rinsing with phosphate-buffered saline (10 mM  $\text{PO}_4^{3-}$  and 140 mM NaCl at pH 7.2), the cantilever was incubated with avidin (50  $\mu$ l at 1 mg/ml) for 5 min and washed with phosphate-buffered saline. Functionalized tips were used immediately. We use the term "avidin" here, although the protein used in these experiments was an avidin derivative, NeutrAvidin (Pierce, Oud-Beijerland, Netherlands). NeutrAvidin is derived from avidin after deglycosylation and has a lower isoelectric point and exhibits lower nonspecific binding.
13. E.-L. Florin, M. Radmacher, B. Fleck, H. E. Gaub, *Rev. Sci. Instrum.* **65**, 639 (1994).
14. J. B. Pethica and W. C. Oliver, *Phys. Scr.* **T19** 61, 61 (1987).
15. Force measurements were made with a home-built scanned stylus AFM based on digital electronics (13). All experiments were confirmed with exclusively analog electronics. The scan rate was typically 1 Hz. The beads were localized under an inverted optical microscope (Zeiss Axiomat, Oberkochen, Germany), and the AFM was positioned with the cantilever directly over the bead. The resolution of the instrument, which was supported on an isolated optical table, was better than 10 pN.
16. The cantilevers were calibrated as follows: A small reference lever (30  $\mu$ m by 2 mm by 30 mm) was constructed and calibrated with known weights. Calibration force scans were carried out on a rigid substrate (glass) and on the reference lever. To dampen the vibration of the lever, measurements were done in water. The spring constants of the cantilever  $k_c$  and the reference lever  $k_r$  are related by  $k_c = k_r (s_g/s_r - 1)$ , where  $s_g$  and  $s_r$  are the measured slopes of the force scans on glass and the reference lever, respectively. The spring constant of the cantilevers used was measured to be  $k = 61 \pm 10$  mN/m.
17. Addition of BSA did not interfere with the binding. The force scan was indistinguishable from the one in Fig. 2A. After addition of an excess of free biotin, the force scan was comparable to the one in Fig. 2B.
18. The histograms in Fig. 3, A and C, were repeated five times with different batches of modified cantilevers and on different beads. The value of the force quantization did not vary within the given error range. The number of force quanta varied between two and five from cantilever to cantilever and from bead to bead.
19. The histograms in Fig. 3, A and B, were recorded with the same cantilever on beads with different ligands. The control experiment, where the cantilever was blocked, was done in a separate experiment with a different cantilever.
20. G. U. Lee, D. A. Kidwell, R. J. Colton, *Langmuir* **10**/2, 354 (1994).
21. C. Helm, W. Knoll, J. Israelachvili, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8169 (1991).
22. H. Frauenfelder, F. Parak, R. D. Young, *Annu. Rev. Biophys. Chem.* **17**, 451 (1988).
23. The lower amplitudes of the higher order peaks may be due to a greater nonspecific interaction between the tip and the desthiobiotin or may reflect a reduced number of binding partners caused by the degradation of the tip.
24. E. Evans, R. Merkel, K. Ritchie, S. Tha, A. Zilker, in *Studying Cell Adhesion*, P. C. P. Bongrand and A. Curtis, Eds. (Springer-Verlag, Berlin, in press).
25. The autocorrelation function was calculated from the histogram after subtraction of its envelope function. The latter was calculated by the overlay of a sixth-order binomial filter [P. Marchand and L. Marmet, *Rev. Sci. Instrum.* **54**/8, 1034 (1983)].
26. This work was supported by the Deutsche Forschungsgemeinschaft. Technical support from Digital Instruments is gratefully acknowledged. We thank M. Benoit for providing Fig. 1B.

17 November 1993; accepted 15 February 1994