Intermolecular Forces and Energies Between Ligands and Receptors

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The recognition mechanisms and dissociation pathways of the avidin-biotin complex and of actin monomers in actin filaments were investigated. The unbinding forces of discrete complexes of avidin or streptavidin with biotin analogs are proportional to the enthalpy change of the complex formation but independent of changes in the free energy. This result indicates that the unbinding process is adiabatic and that entropic changes occur after unbinding. On the basis of the measured forces and binding energies, an effective rupture length of 9.5 ± 1 angstroms was calculated for all biotin-avidin pairs and approximately 1 to 3 angstroms for the actin monomer-monomer interaction. A model for the correlation among binding forces, intermolecular potential, and molecular function is proposed.

The elaborate organization of life requires specific molecular recognition. Although nonspecific interactions may contribute to adhesion in molecular and cellular assemblies, the selectivity of ligand and receptor interactions provides the specificity required to regulate multicomponent systems. Over the past decade, significant progress has been made to elucidate the nature of these interactions, particularly in the isolation, cloning, and structural analysis of numerous ligand-receptor complexes. Along with structural analysis, the investigation of ligand-receptor dynamics is essential for the understanding of the system's functions (*1*). Approaches that were developed to directly examine interaction forces have provided valuable insights into the mechanics of various biological systems (*2*). Recently, we modified the atomic force microscope (AFM) (*3*) to measure the unbinding force of individual ligand-receptor pairs (*4*). As a model system, we focused on the biotin-avidin complex (*5*).

The forces between ligand and receptor were measured with the AFM in the force scan mode (*4*). The receptor molecules, either avidin or streptavidin, were attached to the AFM tips, and the ligands (biotin, iminobiotin, or desthiobiotin) were immobilized on a polymer bead (*6*). The tip was brought into contact with the bead, and the adhesion was measured directly from the bend of the cantilever upon retraction. The strength of the adhesion was regulated by inhibitors to functional groups on either surface. In experiments where the interaction was limited to a few molecules, the force required to rupture contact between ligand and receptor-functionalized surfaces occurred in integer multiples; the force required to rupture a single ligand-receptor pair will be referred to as the unbinding force of the particular molecular pair.

The unbinding force of five different avidin-biotin pairs varied from 85 ± 10 pN for avidin-iminobiotin to 257 ± 25 pN for streptavidin-biotin (Fig. 1A). An examination of the corresponding thermodynamic energies revealed no obvious correlation between unbinding forces and changes in free energy ΔG (Fig. 1B). However, a linear relation between unbinding forces and enthalpy changes ΔH is evident (Fig. 1C). The proportionality factor is given by the effective width of the binding potential $r_{eff} = \Delta H/F_u$, where F_u is the unbinding force (*7*). The observation that r_{eff} is 9.5 ± 1 Å for all avidin-biotin pairs studied reflects the structural similarity of the binding pockets and is a good internal criterion for the accuracy of our force measurements.

A comparison between the unbinding force of individual molecular pairs and thermodynamic potentials is appropriate if the single molecular pair can sample its conformation space. Because our experiments are slower, by orders of magnitude, than the molecular dynamics, the ergodic assumption is justified. Under the influences of an external force, it is possible that transitions between conformational intermediates become anisotropic. However, if we assume that the forced unbinding of biotin and avidin proceeds along a trajectory in phase space that resembles the thermodynamically favored path, then the measured proportionality between the enthalpy change and the unbinding force implies that the ligand-receptor unbinding has negligible entropic contributions. Furthermore, if we also assume that the deformation of the ligand-receptor complex before rupture is reversible, then the process must be adiabatic.

The complete ligand-receptor dissociation process can thus be summarized in the model illustrated in Fig. 2. Up to the point where the external force exceeds the unbinding force, which in a mechanical model is defined as the gradient of the potential at its deflection point (designated as U^r in Fig. 2), the restoring force of the system is conservative, and free energy and enthalpy are indistinguishable (*8*). Dissipative and entropy driven processes occur beyond the rupture point and are thus irrelevant to the unbinding force, although they may dominate the free energy of the system.

A

Ligand-receptor	F_u (pN)	ΔH (kcal/mol)	ΔG (kcal/mol)	r_{eff} (Å)
Avidin-biotin	160 ± 20	−21.5	−20.4	9.3
Avidin-iminobiotin	85 ± 10	−11.6	−14.3	9.5
Streptavidin-biotin	257 ± 25	−32.0	−18.3	9.3
Avidin-desthiobiotin	94 ± 10	−13.5	−16.5	10
Streptavidin-iminobiotin	135 ± 15	NA	−12.2	

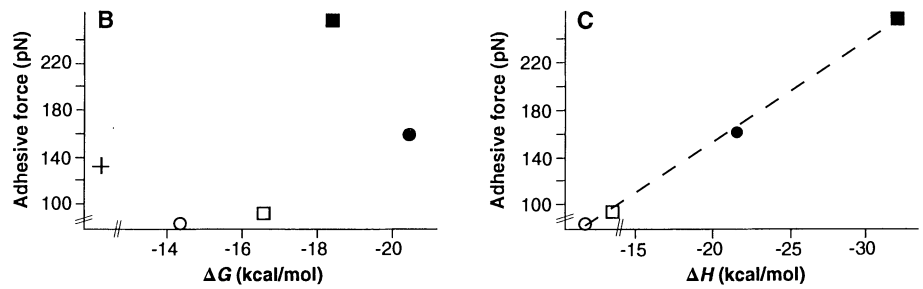

Fig. 1. (**A**) Tabulation of ligand-receptor unbinding forces and the corresponding thermodynamic values. Force measurements were carried out with a scanned-stylus–type AFM (*14*). Thermodynamic values were taken from Green (*5*), except those for streptavidin-biotin, taken from Weber *et al.* (*15*). Calorimetric measurements for avidin-desthiobiotin were performed at 25°C in a MicroCal Omega titration calorimeter. Forty 2-μl injections of ligand solution were titrated at 4-min intervals into 60 μM solutions of receptor. (**B**) Plot of unbinding force versus free energy for avidin-biotin (●), avidin-iminobiotin (○), streptavidin-biotin (■), avidin-desthiobiotin (□), and streptavidin-iminobiotin (+). (**C**) Plot of unbinding force versus enthalpy.

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To support this generalized interpretation of the correlation among unbinding force, free energy, and enthalpy of the ligand-receptor complex, we investigated a reaction in which the contributions to the free energy from processes occurring outside the binding pocket can be regulated and determined. It is known that the formation of the iminobiotin-avidin complex occurs in two steps (9):

$$ImH^+ + B^- \rightleftharpoons Im + BH$$

$$Av + Im \rightleftharpoons AvIm$$

Here, Av, Im, and B denote avidin, iminobiotin, and base, respectively. The initial reaction, corresponding to the deprotonation of the guanidine group of iminobiotin (pK_a, where K_a is the acid constant, is 11.95), is pH-dependent. Although this reaction contributes to the observed energy change, it should not effect the energetics of the free base reaction (that is, Av + Im = AvIm). In this system, our model predicts that the equilibrium constant of the complex, but not its unbinding force, will depend on the conditions of the buffer.

The decrease in the formation of avidin-iminobiotin complex observed in equilibrium binding experiments at lower pH values was also detected in our force measurements carried out on iminobiotin beads with avidin-functionalized tips (Fig. 3). To determine whether the decrease in the adhesion stems from the decrease in the number of complexes formed alone or if a change in the binding force of the individual molecular pairs also contributes, we blocked the majority of the iminobiotin binding sites on the beads with free avidin and measured the bond rupture forces. The distributions of rupture forces measured at different pH reveal that the unbinding forces are independent of the pH and buffer conditions (Fig. 4).

These experiments demonstrate a two-step process in the dissociation of the avidin-iminobiotin complex that results in the decoupling of the unbinding force of individual complexes and the free energy change of the overall reaction. The force measurements indicate that the initial unbinding of the complex (AvIm $\rightleftharpoons$ Av + Im) is insensitive to the environment (within pH 4 to 10). This is followed by the protonation of iminobiotin, which contributes to the total energy but is not directly relevant to the interaction between iminobiotin and avidin.

As a comparison to the avidin-biotin system, we examined the interaction between monomers in an actin filament, a system that is designed to support mechanical loads (*10*). An effective rupture length of 3 Å was obtained from the rupture force of a single actin filament and an estimate of the enthalpy of activation (*11*). Alternatively, a rupture length of 0.5 Å can be calculated from the elastic property of the F-actin polymer (*12*). Both estimates reveal that the actin monomer-monomer bond is stiffer and acts over shorter distances than the avidin-biotin bond. These differences in the characteristics of the pair potentials are reflected in the nature of the molecular recognition and the functions of the two systems. A scavenger system such as the biotin-avidin system favors a large negative free energy change and an interaction potential that extends from the entrance of the binding pocket and guides the ligand into the binding pocket. In contrast, the recognition between actin monomers is along flat surfaces of the monomers (*13*) and is effective over the range of van der Waals contact. This shorter interaction distance allows the system to support a large load with low binding energy.

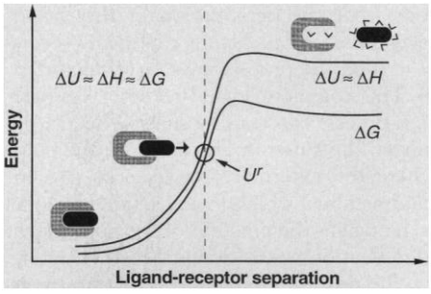

Fig. 2. Schematics of the intermolecular potential of ligand-receptor dissociation. The quantities ΔU, ΔH, and ΔG are the changes in the internal energy, enthalpy, and free energy, respectively, of the process; U^r corresponds the position of bond rupture. Receptor and ligand are illustrated in gray and black, respectively. The contribution from the solvent is depicted in the dissociated molecules.

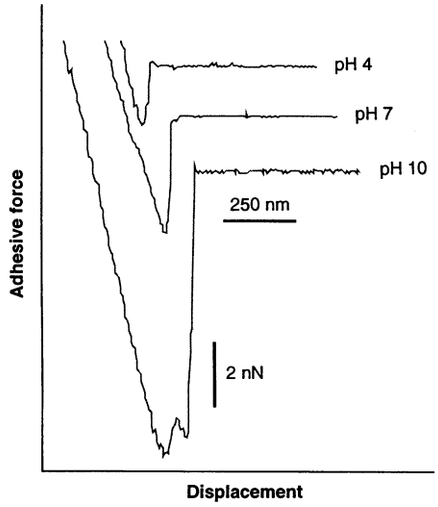

Fig. 3. Dependence on pH of the adhesive force between an avidin-functionalized tip and an iminobiotin-labeled agarose bead. The retraction traces of AFM force scans carried out at pH 4, 7, and 10 with adhesive forces of 2, 4, and 10 nN, respectively, are shown. Buffers: 100 mM sodium phosphate at pH 7 and 10, 100 mM glycine at pH 4.

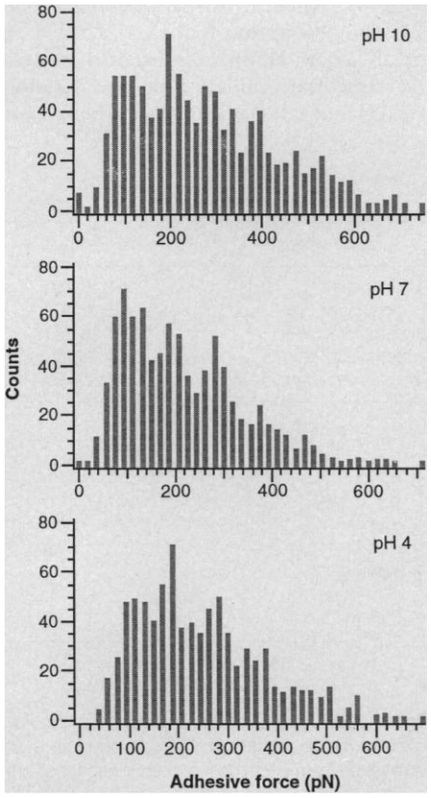

Fig. 4. Histograms of rupture force between avidin-functionalized AFM tips and iminobiotin-labeled beads measured at pH 4, 7, and 10. Buffers were the same as in Fig. 3.

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6. Silicon nitride tips (Digital Instruments) were incubated in 50-μl drops of biotin-labeled bovine serum albumin (BSA) [1 mg per milliliter of phosphate buffered saline (PBS)] overnight at 37°C. After being rinsed five times with PBS, avidin or streptavidin solution (50 μl of 1 mg of protein per milliliter of PBS) was added to the biotin-adsorbed tips. The binding of avidin (or streptavidin) to the tips was complete after 5 min. Tips were used for measurements after rinsing with PBS. Agarose beads derivatized with biotin, iminobiotin, or desthiobiotin were washed three times and equilibrated with buffered solutions before measurement. Deglycosylated avidin (Neutra-

vidin, Pierce) was used in place of conventional avidin in all measurements. Streptavidin from *Streptomyces avidinii*, derivatized agarose beads, and biotin-BSA were purchased from Sigma.
7. This equation is not valid for all ligand-receptor systems. Because of the complexity of the pair potential, there is no simple relation between unbinding force and binding energy for the general case. A more appropriate expression is $F_u = \Delta H^*/r_{eff}$, where ΔH^* is the enthalpy of activation.
8. This approximation is based on the assumption that the effective spring constant of the system is significantly smaller than the spring constant of the avidn-biotin complex. Strictly speaking, separation occurs when the gradient of the external force exceeds the second derivative of the pair potential.
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11. The force required to rupture a single actin filament was measured with microneedles to be approximately 110 pN [A. Kishino and T. Yanagida, *Nature* **334**, 74 (1988)]. A derivation of r_{eff} requires knowledge of the enthalpy of activation. The enthalpy of the actin monomer-monomer bond is endothermic with a value of about 15 kcal/mol. Assuming an upper limit of 20 kcal/mol for the enthalpy of activation, the enthalpy of activation for the backward reaction (monomer-monomer dissociation) is estimated to be 5 kcal/mol.
12. On the basis of an elastic model, the microscopic elastic constant of the monomer-monomer bond was calculated from the bending elastic modules [F. Oosawa, *Biorheology* **14**, 11 (1977)]. Assuming an elastic constant of 2 N/m, Hooke's Law yields a r_{eff} value of 0.5 Å.
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14. There is as much as 20% variability among the different methods used in the calibration of cantilevers [V. T. Moy, E.-L. Florin, H. E. Gaub, unpublished results; J. P. Cleveland, S. Manne, D. Bocek, P. K. Hansma, *Rev. Sci. Instrum.* **64**, 403 (1993); J. L. Hutter and J. Bechhoefer, *ibid.*, p. 1868; T. J. Senden and W. A. Ducker, *Langmuir* **10**, 1003 (1994)]. The values reported here are based on cantilevers calibrated with a macroscopic reference lever in a method that does not depend on the high-frequency response of the cantilever.
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16. This work was supported by the Deutsche Forschungsgemeinschaft. We are grateful to S. Kilger and G. Cevc for their assistance in the titration microcalorimetry measurements; to E. Sackmann, H. M. McConnell, and J. Brauman for inspiring discussions; and to Digital Instruments for technical support.

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Partial Control of an Ion-Molecule Reaction by Selection of the Internal Motion of the Polyatomic Reagent Ion

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The ion-molecule reaction $NH_3^+ + ND_3$ has been studied at various collision energies (1 to 5 electron volts in the center of mass) with preparation of the NH_3^+ reagent in two nearly isoenergetic vibrational states. One state corresponds to pure out-of-plane bending of the planar NH_3^+ ion (0.60 electron volts), whereas the other state is a combination of in-plane and out-of-plane motion (0.63 electron volts). The product branching ratios differ markedly for these two vibrational-state preparations. The differences in reactivity suggest that the in-plane totally symmetric stretching mode is essentially inactive in controlling the branching ratio of this reaction.

Most chemical reactions are statistical in nature, and their course cannot be controlled by the excitation of different vibrational motions of the reactants, but outstanding exceptions are known to occur for small reaction systems (*1*, *2*). For example, H (*3*–*6*) or Cl (*7*) can selectively abstract the H or D atom from HOD depending on the vibrational excitation of the HOD reagent. The results for the H + HOD and Cl + HOD reactions conform to intuition in that the bond being stretched reacts; that is, motion along the reaction coordinate promotes reaction (*8*).

In the case of more complex reactants, almost no experimental information exists on the role of reagent internal motion in directing the outcome of a reactive encounter (*9*, *10*), although some theoretical calculations (*11*, *12*) suggest that such effects may be important. We present here a study of the ion-molecule reaction

$$NH_3^+(\nu_1,\nu_2) + ND_3 \rightarrow NH_3D^+ + ND_2 \quad \text{(deuterium abstraction)} \quad (1a)$$

$$\rightarrow NH_3 + ND_3^+ \quad \text{(charge transfer)} \quad (1b)$$

$$\rightarrow NH_2 + ND_3H^+ \quad \text{(proton transfer)} \quad (1c)$$

in which the NH_3^+ reagent ion is prepared in nearly isoenergetic internal states having different vibrational motions. The NH_4^+ isotopomer channels (Eq. 1, a and c) have an exothermicity of ~0.9 eV (*13*), whereas the charge transfer product channel (Eq. 1b) is nearly thermoneutral. The branching ratio among these three product channels can be partially controlled by the choice of reagent ion internal motion.

The NH_3^+ ion is an ideal reactant for state-selected studies because multiphoton ionization can be used to prepare the ion with a wide range of controlled vibrational excitation (*14*, *15*). A resonant two-photon excitation from the pyramidal ground state to the planar $\tilde{B}$ or $\tilde{C}'$ Rydberg state of the ammonia molecule selects an intermediate vibrational state. The large change in geometry that results from these transitions allows excitation to be placed into two vibrational modes of the molecule. The pyramidal-to-planar change in geometry allows access to an extended progression of out-of-plane totally symmetric bending vibrations (*16*), referred to here as the umbrella bending mode (ν_2) (Fig. 1A). In addition, a slight lengthening of the N–H bond can excite the in-plane totally symmetric stretch vibration (*15*, *17*), referred to here as the breathing mode (ν_1) (Fig.

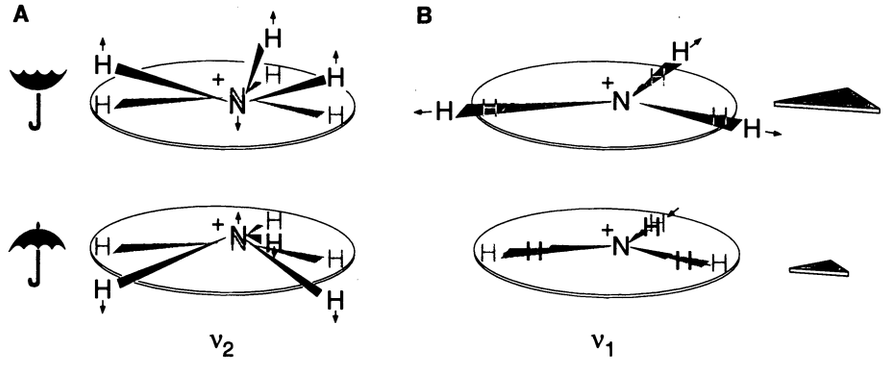

Fig. 1. Two of the six vibrational modes of the planar NH_3^+ ion: (**A**) the umbrella bending mode (ν_2) (out-of-plane totally symmetric bending mode) and (**B**) the breathing mode (ν_1) (in-plane totally symmetric stretching mode). The extremum of the vibrational motion, in black, is compared to the equilibrium geometry, in gray.

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