DOI 10.1515/revac-2013-0004 — Rev Anal Chem 2013; 32(3): 197–208

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Mechanical affinity as a new metrics to evaluate binding events

Abstract: Binding affinity is measured by dissociation constant, K_d, which uses concentration as units. The universal concentration units facilitate direct comparison of affinities for different binding events. However, K_d is a thermodynamic parameter, which lacks kinetic information of a binding event. In addition, K_d does not reveal the mechanical property of the binding, which emerges as a critical element for many physiologically significant processes such as DNA replication, RNA transcription, and protein translation. Here we propose a new parameter, mechanical affinity, to delineate kinetic and mechanical features of a binding event. The mechanical affinity is equivalent to the work required to dissemble the chemical binding between a ligand and a receptor. During this process, it must cover dissipated heat that originates from the relative movement between a ligand and a receptor. Because dissipated heat varies with unfolding direction or rate of mechanical perturbation, the mechanical affinity is a function of these two variables. Screening of chemicals using rupture force of a ligand-receptor complex or mechanical affinity is discussed at the end of this review. The interrogation on the mechanical interaction between a ligand and a receptor provides a new perspective not available in conventional thermodynamic evaluation of binding processes.

Keywords: chemical affinity; ligand-receptor interaction; mechanical affinity; mechanical stability; single-molecule methods.

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Introduction

Binding between two components represents a universal yet pivotal step for numerous processes. In the human body, binding between antibody and antigen initiates immune responses. For cells, binding between a receptor and a ligand is the first step to trigger signal transduction that involves a multitude of biochemical reactions, most of which occur after an enzyme is bound with a substrate. In materials synthesis, binding of reactants to catalysts produces organic and inorganic compounds. Self-assembly is a general strategy by which many materials are prepared. Association or binding between materials with similar properties is a driving force in the self-assembly. In biosensing, binding between an analyte and a receptor constitutes the recognition stage that generates signals to be amplified in subsequent steps.

Dissociation constant, K_d, measures chemical binding affinity. It depicts the concentration at which half of the binding sites are occupied by ligands. Binding with higher affinities suggests that fewer ligands (lower concentration) are required to occupy the binding sites, which yields a smaller K_d value. The clear-cut physical significance with universal concentration units in K_d renders the comparison between different binding events obvious and intuitive. In addition, measurement of K_d is straightforward. A series of ligand concentrations is all that is required to obtain a K_d value.

Binding is a dynamic process in which association and dissociation proceed simultaneously. Although K_d provides a measurement of binding strength, it does not reveal dynamic nature in a binding, which is better described by the association (k_{on}) and the dissociation (k_{off}) rate constants. Interpretation of these rate constants is not straightforward as different units may exist. It is even more problematic to compare k_{on} or k_{off} between the binding events that have different reaction orders. To collect dynamic information of binding events, it is desirable to adopt a universal variable, similar to the K_d, that can compare different binding processes.

In this review, we propose such a variable, mechanical affinity, to represent both energetic and dynamic aspects of binding events that are essential for many vital biological processes such as enzymatic actions of motor proteins and rolling and adhesion of leukocytes during inflammation. First, we will discuss dissociation constant, K_d, that reflects the thermodynamic nature of a binding process. Conventional methods to measure this variable will be introduced next. In a subsequent section, we will elaborate the concept of mechanical stability and mechanical

affinity. These two variables will be compared to the chemical affinity that has been commonly used to define the strength of binding. In the final section of this review, we propose screening methods that exploit mechanical property as a new metrics to evaluate binding processes.

K_d as a common variable to measure chemical affinity

For a simple two-component reaction, A+B→C, the reaction equilibrium constant for association (K_a) is expressed as $K_a=[C]_{eq}/([A]_{eq}[B]_{eq})$, where $[A]_{eq}$, $[B]_{eq}$, and $[C]_{eq}$ represent the concentrations of specific species at equilibrium. The dissociation constant, K_d, is a reciprocal of K_a. As a thermodynamic variable, K_d is related to free energy change of binding, $\Delta G_{binding}$ (or $\Delta G_{affinity}$), by the expression $\ln K_d=-\Delta G_{binding}/RT$, where T is absolute temperature and R is the molar gas constant.

Several methods exist for K_d determination. The most widely used method is based on the Langmuir isotherm approach (Langmuir 1918). In this method, as shown in Figure 1, the fraction of ligand-bound receptors increases with increasing ligand concentration until a plateau is reached, indicating that binding sites are saturated. The Langmuir model [Eq. (1)] (Langmuir 1918) is based on the assumption that all binding sites are equivalent and that the binding ability of molecules is not affected by the occupancy of nearby sites.

$$\Gamma=\Gamma_{max}[K_aC/(1+K_aC)] \quad (1)$$

where C is the concentration of adsorbate (or ligand), Γ is the amount of adsorbed ligand, and Γ_{max} is the maximum amount of adsorbate on a surface.

Different techniques have been employed to measure K_d of the interaction between a ligand and a receptor (Connors 1987). Among label-free approaches, UV-visible absorption spectroscopy is the simplest and commonly used technique (Xia et al. 2010). Isothermal titration calorimetry not only can measure K_d of the binding but also reveal thermodynamic parameters associated with the binding (Jelesarov and Bosshard 1999, Belozerova and Levicky 2012). Recently, surface plasmon resonance has become a popular optical technique to probe binding on a metal surface (Homola 2003, Karlsson 2004, Mayer and Hafner 2011). It provides quantitative information on the binding affinity as well as the binding kinetics. All these experiments require increasing concentrations of either a ligand or a receptor (Figure 1). The other challenge in these methods is their low sensitivity. Owing to the high sensitivity and broad dynamic range, fluorescence-based approaches have become useful techniques to monitor binding interactions. Among different fluorescence methods, fluorescence polarization anisotropy (Laurence 1952, Jameson and Sawyer 1995), fluorescence resonance energy transfer (FRET) (De Cian et al. 2007), and laser-induced fluorescence capillary electrophoresis (German et al. 1998, Yangyuoru et al. 2012) have been extensively used. However, labeling with a fluorophore may interfere with the binding. In addition, fluorescence signal from environment presents noise to the measurements. To address this issue, recently K_d has been determined by mechanical force that is required to dissemble a ligand-receptor complex (Koirala et al. 2011a, Nguyen et al. 2011, Paramanathan et al. 2012, Yangyuoru et al. 2012).

Mechanical properties of the binding

Mechanical force and its measurement

Mechanical force is a universal parameter that can be used to characterize a wide range of systems ranging from chemical bonds, intermolecular interactions, macromolecular structures, and many biological and biochemical processes. The basic interaction between individual atoms is described in covalent or noncovalent bonds, whose strength can be characterized with force. Change in the conformation of a macromolecule is often accompanied by a variation of the tension sustained by that molecule. Many vital biological processes can be monitored and characterized in terms of mechanical forces. For example, the biophysical aspects of the attachment and the rolling of leukocytes along the wall of blood capillaries during inflammation involve a complex balance of forces arising from blood-flow-induced hydrodynamic shearing effects and the adhesive forces between the leukocytes and the capillary wall (Konstantopoulos et al. 1998, Simon and Goldsmith 2002, Simon and Green 2005). Biochemical actions of many proteins, such as DNA (Wuite et al. 2000) or RNA polymerase (Yin et al. 1995, Davenport et al. 2000), exonuclease (Perkins et al. 2003), myosin (Finer et al. 1994), and kinesin (Visscher et al. 1999), generate forces up to tens of piconewtons. Moreover, activities of those proteins are often influenced by an externally applied force (Davenport et al. 2000). In ligand-receptor binding, the strength of the interaction between two binding partners can be measured in terms of the mechanical force that

is required to destroy the interaction from a specific direction (Ainavarapu et al. 2005, Koirala et al. 2011a, Nguyen et al. 2011, Paramanathan et al. 2012, Yangyuoru et al. 2012). In such a case, force is applied at a constant rate to overcome the energy barrier. The dynamics of the binding process can be retrieved by measuring the unbinding rate at a constant force or by measuring rupture force as a function of the force loading rate.

Despite its importance, there exists a lack of ensemble-average methods to interrogate force in solution. Hydrodynamic forces from a flowing fluid have been used to monitor the mechanical aspect of biologically relevant processes such as adhesion and rolling of white blood cells inside blood capillaries during inflammation (Dong and Lei 2000, Bianchi et al. 2013). However, such methods have difficulties to explore the roles of individual binding units at the molecular level. As a result, only little information has been collected to understand the mechanochemistry (Keller and Bustamante 2000), an emerging field to study the coupling between chemical energy and mechanical energy. Single-molecule techniques, however, start to address this problem. Compared to bulk assays where ensemble information is obtained, single-molecule experiments produce stochastic and discrete signals that can be statistically analyzed to reveal the property of subgroups in a population and the energetics of a particular reaction trajectory in a transition process (Koirala et al. 2013, Yu and Mao 2013). Among many tools to manipulate single molecules, force-based techniques, such as atomic force microscopy (AFM) (Binnig et al. 1986, Rief et al. 1997), optical tweezers (Ashkin et al. 1986, Smith et al. 1996, Visscher et al. 1999), microneedles (Kishino and Yanagida 1988), and magnetic tweezers (Smith et al. 1992, Strick et al. 1996), provide a unique capability to investigate the force generated by or applied to individual molecules. Details of each method are reviewed in recent publications (Bustamante et al. 2000, Neuman and Nagy 2008, De Vlaminck and Dekker 2012). Here, we present a brief introduction of these methods.

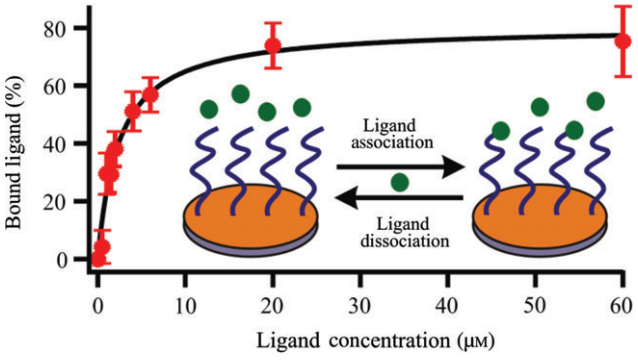

Figure 1 Determination of dissociation constant (K_d). Fraction of ligand-bound receptors is plotted with concentration of a ligand. The solid curve is a Langmuir isotherm fitting to obtain K_d. Inset shows a schematic of a binding process.

AFM is commercially available. It uses a microfabricated cantilever to attach a biomolecule through one of its ends (Figure 2A). The other end of the biomolecule is immobilized onto a substrate surface. The displacement between the cantilever and the surface is often controlled by a piezoelectric device. Force signal, which ranges from 10^{-11} to 10^{-7} *N*, in the biomolecule is measured by the deflection of the cantilever. This method has nanometer spatial resolution and piconewton force resolution. The stiffness of a cantilever ranges from 0.001 to 100 Nm^{-1}. It is noteworthy that the stiffer the cantilever, the lower the force sensitivity. As the dynamic range of the force measurement is broad, this method has been widely used to investigate the strength of intramolecular and intermolecular interactions in biomolecules.

Microneedles are usually 50–500 μm long and 0.1–1 μm in diameter. Because of the lower stiffness (10^{-6} to 1 Nm^{-1}), this method has an advantage over AFM to study delicate systems. Setup is similar to AFM in which a biomolecule is sandwiched between the tip of a microneedle and a substrate surface (Figure 2B). Force signal can be measured by observing the displacement of a bendable microneedle via imaging the microneedle itself or by using a chemically etched optical fiber that projects a reflected light from its tip onto a photodiode. This method can measure force from 10^{-12} to 10^{-10} N with a minimum distance of 10^{-9} m. The instrument is not commercially available.

Magnetic tweezers use permanent or electromagnets to trap magnetic microparticles (Figure 2C). Biomolecules can be tethered between magnetic particles and a nonmagnetic surface. This method can measure force from 10^{-14} to 10^{-11} N with a minimum distance of few nanometers. Because it can trap multiple particles, throughput can be higher compared to other force-based single-molecule approaches. It has a unique capability to rotate magnetic particles, thereby allowing torque measurements of torsionally constrained biomolecules. Recently, various types of magnetic tweezers have been developed (Yan et al. 2004, Lipfert et al. 2011), however, the major disadvantage associated with these methods lies in the fact that force is not measured directly (Bustamante et al. 2000, Neuman and Nagy 2008, De Vlaminck and Dekker 2012).

Optical tweezers can trap dielectric microparticles at a laser focus (Figure 2D). Trap stiffness of a laser trap (10^{-10} to 10^{-3} Nm^{-1}) is much smaller than that of AFM cantilevers, which allows a better force resolution in the range

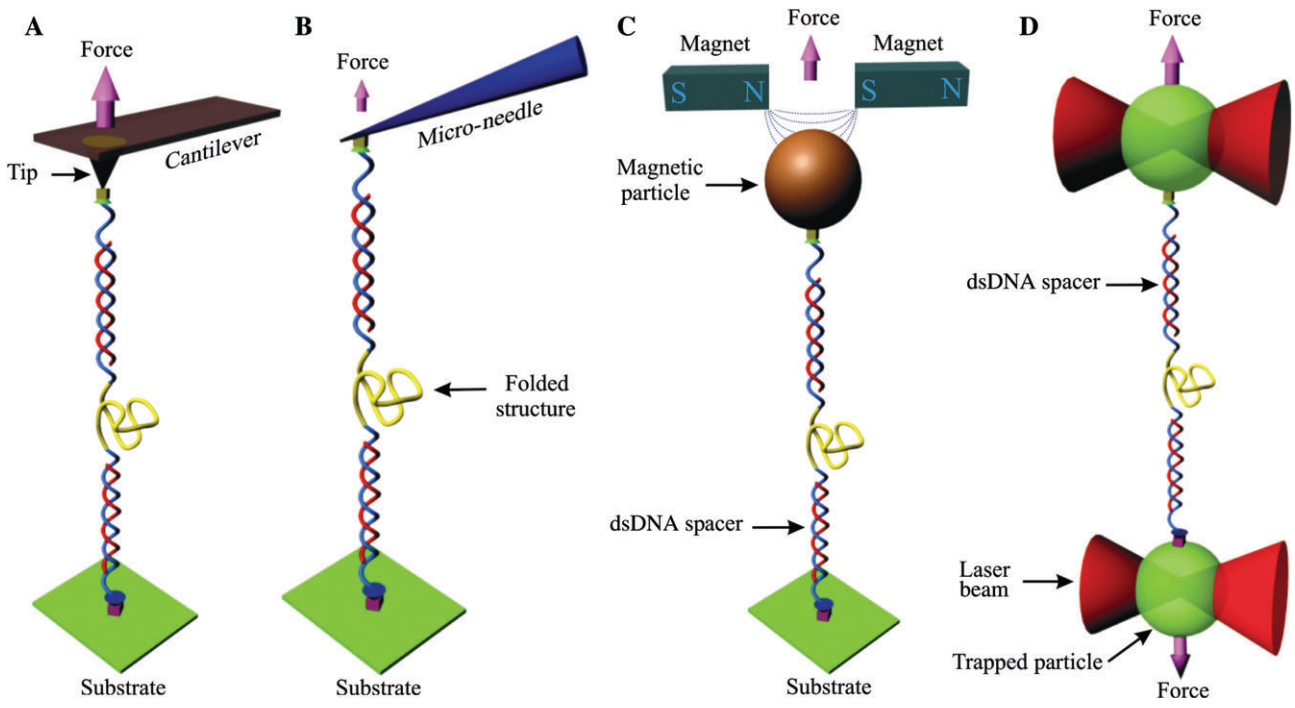

Figure 2 Schematic of commonly used single-molecule methods for mechanical unfolding and refolding of biomolecules. A biomolecule of interest (yellow) is sandwiched between two spacers, dsDNA for example, which can be tethered between two immobilized surfaces by affinity interactions or covalent linkages in AFM (A), microneedle (B), magnetic tweezers (C), or laser tweezers (D) instrument. When a tension is applied to stretch the tethered molecule, the biomolecule of interest is unfolded at a certain force. Reducing the tension allows refolding of the biomolecule. This unfolding-refolding process can be repeated many times until the tether is detached from the surfaces. To observe multiple unfolding and refolding events, the surface attachment should be stronger than the unfolding force ($F_{rupture}$) of the structure formed in the biomolecule.

of 10^{-13} to 10^{-10} N. It has a spatial resolution of 10^{-9}–10^{-10} m, which allows resolving single base pairs in DNA. The force exerted on a microparticle can be flexibly controlled by parameters such as laser power, particle size, and difference in the refractive index between the particle and the trapping medium. In single-beam optical tweezers, a biomolecule is usually tethered between a trapped particle at the laser focus and a surface of a substrate or a particle held at the tip of a micropipette by suction (Kellermayer et al. 1997, Wang et al. 1997, Cecconi et al. 2005, Moffitt et al. 2008). In dual-beam optical tweezers, the molecule is often tethered between two trapped particles at two separate laser foci (Moffitt et al. 2008, Woodside et al. 2008, Yu et al. 2009). The major disadvantage of this method is laser-induced damage of biomolecules.

Using these single-molecule tools, different approaches can be adopted to investigate the mechanical property of a biomolecule and its interaction with a ligand. In one setup, two binding partners can be immobilized to two surfaces separately. After binding between these two partners is accomplished by bringing two surfaces together, moving one surface away from the other increases the mechanical tension. The increased tension eventually destroys the binding interaction. However, the major challenges in this approach are the precise measurement of the distance change during the unfolding and the reversibility of the process. In another approach, an intramolecularly folded biomolecule is first tethered between two surfaces using affinity or covalent linkages (Figure 2). As one surface is moved away from another by a computer-controlled motor, the tension in the biomolecule increases until unfolding occurs, which is manifested by a sudden change in the tension or the end-to-end distance in a force-extension (*F-X*) curve recorded for this process. During the opposite process in which the end-to-end distance becomes shorter, the biomolecule refolds. The same processes can be performed in the presence of the ligand to evaluate ligand-receptor interactions.

Mechanical unfolding or refolding processes present a completely different mechanism compared to the temperature- or chemical denaturant-mediated unfolding or refolding. Whereas the force-induced processes have localized effects on a molecule, the temperature- or chemical-assisted events are global in nature. The

localized force effect suggests that the direction of unfolding is rather critical for the process. However, it is expected that unfolding/refolding should be an ensemble average of all unfolding directions that are presented in the temperature- or the chemical-assisted transition processes owing to their global nature.

Mechanical stability

From energy perspective, force (F) reduces the free energy of a system by a factor of $F \times x$, where x is the unfolding distance of a biomolecule. As shown in Figure 3, such a reduction brings down the energy barrier of a two-state folding/unfolding system by $F \times x^{\dagger}$, where $x^{\dagger}$ is the transition distance, or the distance between the folded structure and the transition state. With application of a specific force ($F_{rupture}$), the energy barrier is reduced to a level within reach of the thermal energy of the environment ($k_B T$, where k_B is the Boltzmann constant and T is absolute temperature). This leads to unfolding of the structure. Assuming $x^{\dagger}$ does not change during the application of force, the magnitude of $F_{rupture}$ is inversely dependent on the transition distance with a larger distance corresponding to a smaller $F_{rupture}$ value (Evans 2001). The relationship between $F_{rupture}$ and $x^{\dagger}$ can be understood by the elasticity of a molecule. A soft and flexible molecule requires a long distance to be unfolded (larger $x^{\dagger}$). The force ($F_{rupture}$) along this long distance is expected to be small. Moreover, a rigid but fragile molecule only needs a little stretch in its structure (smaller $x^{\dagger}$) to induce unfolding. However, the force leading to this little perturbation ($F_{rupture}$) is expected to be significantly higher than the previous case.

Because $F_{rupture}$ alone does not fully account for the mechanical perturbation defined by $F \times x^{\dagger}$, there is a caveat to use this parameter to compare the mechanical stability of folded molecules. Comparison of $x^{\dagger}$ among DNA secondary species, such as hairpins (Woodside et al. 2006), DNA G-quadruplexes (Yu et al. 2009, Yu et al. 2012a,b, Koirala et al. 2011a), DNA i-motifs (Dhakal et al. 2010), and their intermediates (Dhakal et al. 2012, Koirala et al. 2012), has suggested that $x^{\dagger}$ varies with different molecules. Within the same molecule, $x^{\dagger}$ is anisotropic among different unfolding trajectories (Yu et al. 2012a). Therefore, mechanical aspects of unfolding can be described accurately only when both $F_{rupture}$ and $x^{\dagger}$ are taken into account. The other caution to use $F_{rupture}$ for the comparison is that magnitude of $F_{rupture}$ is dependent on the force loading rate. The dependence is rather pronounced for processes with slow unfolding rates (Evans and Ritchie 1997, Strunz et al. 1999). In such a case, $F_{rupture}$ for reversible folding and unfolding processes occurs in a timescale often not reachable in an experiment. Although

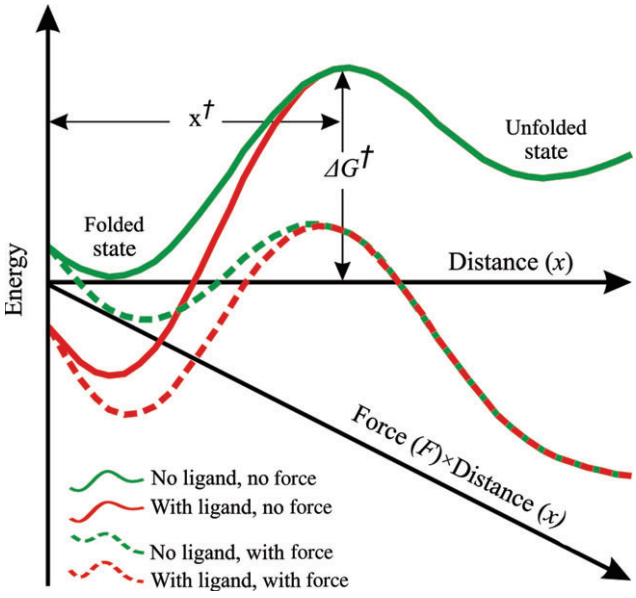

Figure 3 Effect of applied force on the energy landscape of unfolding and refolding of a biomolecule. Solid and dotted green curves represent energy profiles of a free biomolecule before and after application of force, respectively. Solid and dotted red curves depict those of the biomolecule-ligand complex before and after a force is applied, respectively.

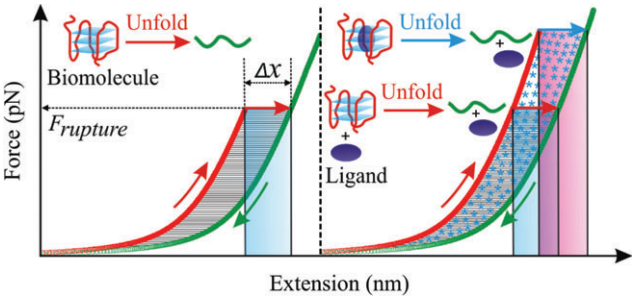

Figure 4 Typical force-extension (*F-X*) curves of unfolding (red) and refolding (green) of a biomolecule with (right) and without (left) a ligand. Change in extension (Δx) and the rupture force ($F_{rupture}$) for the unfolding of the biomolecule are measured directly from the *F-X* curves. The work to unfold the structure in the absence of ligand can be obtained from the hysteresis area (gray shaded region), which is equivalent to the area under the rupture event (colored rectangle) after energetic correction for a stretched DNA from 0 pN→$F_{rupture}$ using a worm-like chain model (Liphardt et al. 2001). In the presence of ligand, ligand-bound and ligand-unbound populations can be differentiated based on the unfolding force or the unfolding work that is estimated from the hysteresis area or the area under the unfolding event after similar energetic corrections shown above.

$F_{rupture}$ is straightforward to understand and has universal units in newtons, the above arguments have made $F_{rupture}$ a not-so-ideal parameter to compare different binding processes.

We propose that unfolding work, $W_{receptor\text{-}unfold}=F_{rupture}\times\Delta x$, where Δx is the difference in the end-to-end distance between a folded and an unfolded structure along a specific unfolding trajectory (see shaded region in the *F-X* curve in Figure 4, left panel), is a suitable parameter to account for mechanical properties of an unfolding event. With the single-molecule techniques described above (see Figure 2), $F_{rupture}$ and Δx can be directly measured from the *F-X* curves (see Figure 4, left panel, for example). $F_{rupture}$ and Δx provide the information on the mechanical stability and the size of the folded biomolecule, respectively.

$W_{receptor\text{-}unfold}$ has universal energy units, J/mol. Therefore, it can be used among different systems to facilitate direct comparison. This parameter not only considers the work to disrupt the folded structure per se, it also accounts for the work to extend the folded structure from 0 pN to $F_{rupture}$ and the work to stretch the unfolded structure from 0 pN to $F_{rupture}$ (Liphardt et al. 2001). These works represent an actual mechanical stability of a folded structure, which must resist all external perturbations. Because $F_{rupture}$ is a kinetic parameter that is dependent on the force loading rate (see above), $W_{receptor\text{-}unfold}$ is expected to have a similar feature. In fact, it has been shown that $F_{rupture}$ and $W_{receptor\text{-}unfold}$ are dependent on the force loading rate in single molecular force spectroscopy experiments (Evans and Ritchie 1997, Rief et al. 1997). $W_{receptor\text{-}unfold}$ is anisotropic because different unfolding trajectories often suggest different interactions between a receptor and surrounding solvent molecules. This property has been confirmed in the mechanical unfolding of a human telomeric G-quadruplex from different trajectories, which is made available by a click-chemistry modification of specific nucleotides in human telomeric DNA sequences (Yu et al. 2012b, Dhakal et al. 2013).

Mechanical affinity

With a ligand bound to a molecule, the folding and unfolding energetic landscape of the molecule changes. Due to the stabilization effect of the ligand, the free energy of the molecule bound with ligand decreases, whereas that of the unfolded state with a dissociated ligand does not vary significantly (Figure 3). Assuming the energy for a transition barrier does not vary significantly in the presence of a ligand, the transition barrier to unfold the ligand-bound receptor increases.

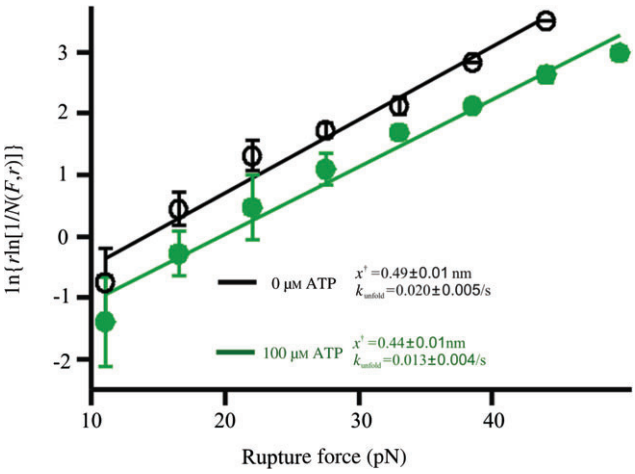

Figure 5 Determination of the distance from the folded to the transition state ($x^{\dagger}$) and the unfolding rate constant k_{unfold} for a free DNA aptamer (black) and an aptamer-ATP complex (green). The $x^{\dagger}$ was obtained from the slope of the linear fit (solid lines) to the data points shown in the graph using the Evans model (Evans and Ritchie 1997, Li et al. 2006) $\ln[r\ln[1/N(F,r)]]=\ln[k_{unfold}/(x^{\dagger}_{f\to u}/k_BT)]+(x^{\dagger}_{f\to u}/k_BT)F$, where r is the loading rate (5.5 pN/s), $N(F,r)$ is the fraction of folded molecules at force F and loading rate r. The k_{unfold} is obtained from the intercept of the linear fit to the data points in the graph.

On the other hand, the transition distance ($x^{\dagger}$) of a ligand-bound molecule is likely different from that of a free, folded molecule. Retreatment of our recent data on an ATP-bound DNA aptamer (Yangyuoru et al. 2012) shows

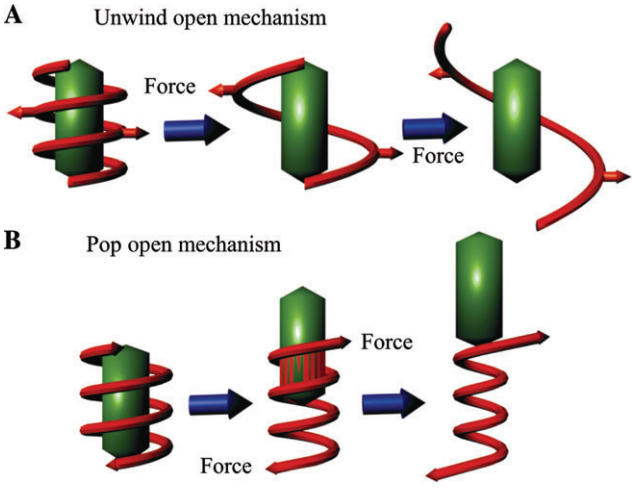

Figure 6 Disassembly of a receptor (strand) and a ligand (rod) complex by force from different directions. The ligand, such as histone (green), wrapped by a receptor, such as DNA (red), can be either unwound-open when the DNA is pulled from the middle (A), or popped-open when the two ends of the DNA are pulled (B). It requires a larger force to overcome the large friction in the latter process compared to the former event.

that the transition distance becomes shorter with respect to that of a free DNA aptamer (Figure 5). Because rupture force ($F_{rupture}$) is related to the magnitude of $x^{\dagger}$ by $F_{rupture} \times x^{\dagger}$ (see Figure 3), it is possible that $F_{rupture}$ shows a marginal increase or even a decrease when $x^{\dagger}$ increases significantly. Such a variation in $F_{rupture}$ may hold even though energy barrier increases as a result of ligand binding. Therefore, $F_{rupture}$ may not accurately represent the mechanical property of a ligand-bound receptor, which is similar to the case for a folded molecule without a bound ligand as discussed above.

Instead, we propose that the work to unfold a ligand-receptor complex, $W_{complex\text{-}unfold}$, can better reflect the mechanical property of the complex. This work can be dissected into two components,

$$W_{complex\text{-}unfold} = W_{receptor\text{-}unfold} + W_{affinity}, \quad (2)$$

where $W_{receptor\text{-}unfold}$ is the work to unfold a free, folded receptor as defined above, and $W_{affinity}$ corresponds to the work required to dissemble the ligand-receptor complex, which is equal to the mechanical affinity. Measurement of mechanical affinity is straightforward from Eq. (2): It is equivalent to the difference between the mechanical work to unfold a free receptor and that to dissemble a receptor-ligand complex, $W_{affinity} = W_{complex\text{-}unfold} - W_{receptor\text{-}unfold}$.

Similar to $W_{receptor\text{-}unfold}$, $W_{complex\text{-}unfold}$ is anisotropic and dependent on the force loading rate. This is because energy required to strip off a ligand from a receptor is likely different among mechanical perturbations applied from different directions. Figure 6 shows the disassembly of a complex in which a ligand, such as histone, is wrapped by a DNA (a receptor). In the first case (Figure 6, top panel), the disruption of the ligand-receptor complex is achieved by grabbing a DNA strand at two positions in the middle of the DNA strand, thereby unwinding the rest of the strand through a 'sliding' action (the 'slide open' or 'unwind open' event). In the second scenario (Figure 6, bottom panel), the ligand-bound DNA is pulled by grabbing the two termini of the DNA strand. In this geometry, the physical contact between a DNA and a ligand becomes tighter until the ligand is popped out from the top or bottom of the tightened DNA strand (the 'pop open' process) (Brower-Toland et al. 2002). From a perspective of mechanics, the former process generates less friction between the DNA and the ligand compared to the latter. Such a difference will be reflected in the unfolding work, which must overcome all friction before a ligand is released from a receptor. Because friction originates from a relative movement between a ligand and a receptor during unfolding, the dissipated heat accompanied by frictional is a function of force loading rate: The faster the loading rate, the more the frictional heat. As dissipated heat is an inherent component in $W_{affinity}$, this example clearly shows that $W_{affinity}$ is dynamic and has a localized feature.

Relationship between mechanical affinity and chemical affinity

Chemical affinity between a ligand and a receptor is attributed to intermolecular forces. From free-energy perspective, it is equivalent to the change in free energy of the binding between a ligand and a receptor ($\Delta G_{affinity} = \Delta G_{binding}$; it is a negative value). Using a Hess-like cycle (Koirala et al. 2011a), $\Delta G_{affinity}$ can be calculated as the difference between the change in free energy of unfolding a free receptor ($\Delta G_{receptor\text{-}unfold}$, a smaller positive value) and that of unfolding a ligand-receptor complex ($\Delta G_{complex\text{-}unfold}$, a bigger positive value),

$$\Delta G_{affinity} = \Delta G_{receptor\text{-}unfold} - \Delta G_{complex\text{-}unfold} \quad (3)$$

From mechanical energy perspective, the work to dissemble a ligand-receptor complex [$W_{affinity}$; see Eq. (2)] must overcome the intermolecular forces that constitute the chemical affinity ($-\Delta G_{affinity}$). In addition, $W_{affinity}$ should also cover the dissipated heat ($W_{friction}$) originated from the relative motion between a ligand and a receptor during the disassembly process. Thus, the mechanical affinity can be expressed by the following equation,

$$W_{affinity} = -\Delta G_{affinity} + W_{friction}, \quad (4)$$

which represents the first law of thermodynamics for ligand-receptor interactions from a mechanical perspective. For a reversible process in which the force loading rate is infinitely slow, the chemical affinity ($\Delta G_{affinity}$) and mechanical affinity ($W_{affinity}$) are equal ($\Delta G_{affinity} = -W_{affinity}$) because dissipated heat ($W_{friction}$) is negligible under such a situation.

It is noteworthy that $\Delta G_{affinity}$, as a state function like ΔG_{unfold}, should not vary with unfolding trajectories or loading rates. The isotropic nature of ΔG_{unfold} has been confirmed in the unfolding of a DNA G-quadruplex from different directions (Yu et al. 2012b, Dhakal et al. 2013). In another set of experiments, by varying the force loading rate during unfolding or refolding processes, ΔG_{unfold} can be retrieved from the work at which the unfolding and the refolding share the same probability (Crooks fluctuation theorem; Crooks 1999, Collin et al. 2005). Extending this trend, ΔG_{unfold} is equivalent to the work when work histograms of the unfolding and the refolding superimpose, which occurs when folding and

unfolding become reversible under infinitely slow force loading rate.

However, such a slow rate cannot be achieved in actual experiments within a reasonable time frame. In addition, mechanical unfolding of a bound complex is often not reversible as refolding of a receptor must occur prior to the binding of the ligand, whereas unfolding of a ligand-bound complex can happen cooperatively (simultaneously). Furthermore, refolding may not always be measured. For the slow refolding process, it occurs at a low-force region. The compromised signal-to-noise ratio in this region prevents a reliable measurement of the refolding force (F_{refold}) and refolding distance (Δx).

In practice, ΔG_{unfold} can be retrieved from the mechanical unfolding work by using Jarzynski equality theorem (Jarzynski 1997, Liphardt et al. 2002) for nonequilibrium systems. To do this, the hysteresis area between the stretching and relaxing *F-X* curves (see Figure 4), which is equivalent to the work done during the unfolding process, is calculated from hundreds of *F-X* curves. At the single-molecule level at which unfolding work is comparable to thermal energy ($k_B T$), there exists a small probability that contribution from thermal bath leads to an unfolding work smaller than ΔG_{unfold}. By increasing weighing factors of these smaller works with respect to other works, ΔG_{unfold} can be retrieved by the following equation (Jarzynski 1997, Liphardt et al. 2002),

$$\Delta G = -k_B T \ln \sum_{i=1}^{N} \frac{1}{N} \exp\left(-\frac{W_i}{k_B T}\right) \tag{5}$$

where N is the total number of *F-X* curves and W is the nonequilibrium work done to unfold a structure. In principle, chemical affinity, $\Delta G_{\text{affinity}}$, can be retrieved from mechanical affinity, W_{affinity}, using this Jarzynski approach.

Ligand screening based on mechanical properties

Currently, almost all drug screening is based on thermodynamic binding (or chemical affinity) between target receptors and small chemicals (ligands). Although this approach is highly effective to identify drug leads, it does not have the capability to reveal binding kinetics and completely ignores mechanical aspects of a binding.

Kinetics is a rather important aspect in receptor-ligand interactions. Many times, a transient binding between a ligand and a receptor is sufficient to lead to subsequent effects in cells. However, tight binding is not always desirable as it may cause unwanted long-term side effects due to the slow dissociation of a ligand-receptor complex. The mechanical property of a binding between a receptor and a ligand is a completely different concept in that it can profile kinetics as well as mechanics of the binding. Such a kinetic property is inherent for many critical processes orchestrated by motor enzymes such as RNA and DNA polymerases, ribosomes, and DNA/RNA helicases. It also plays a vital role in many biological processes such as attachment and rolling of the leukocytes in blood tubes during inflammation. For all these processes, mechanical properties of a receptor-ligand interaction are physiologically as important as thermodynamic features. In protein translation for example, there is a tug-of-war between the mechanical stability of RNA secondary structures and the stall force of ribosome, which represents a maximum load force as ribosome moves along the RNA template. Any potential secondary structures in mRNA must be resolved before a ribosome can continuously synthesize proteins. When a ligand binds to an RNA secondary structure, the mechanical affinity adds to the mechanical stability of a free RNA structure [Eq. (2)], increasing the mechanical work a ribosome must spend to clear the roadblocks.

However, depending on the change in the transition distance ($x^{\dagger}$) upon ligand binding, the F_{rupture} of the RNA-ligand complex may not exhibit a significant difference compared to those of the RNA structure without ligand. If this F_{rupture} is within reach of a ribosome, i.e., F_{rupture}<stall force of the ribosome, the RNA structure bound with ligand may not be effective to block the translation. Therefore, for the purpose of blocking protein translation, screening of ligands based on chemical or mechanical affinity may not always produce desirable effects. Instead, the screening based on the F_{rupture} of ligand-bound RNA secondary structures can be more effective.

One major advantage of using $F_{\text{rupture,}}$ instead of chemical affinity, in the drug screening lies in the fact that the method can identify ligands with weak chemical affinities or fast k_{off} rates. Current chemical-affinity-based screening prefers ligands with strong thermodynamic binding capability. However, experiments have shown that ligands with weak binding can have important physiological effects (Maerkl and Quake 2007). Moreover, in many surface-based heterogeneous screening, bindings with fast k_{off} rate are often overlooked (Maerkl and Quake 2007), even though they have high chemical affinities. The fast k_{off} issue can be addressed as mechanical screening has submicrosecond temporal resolution, and is homogeneous due to its single molecular nature. The weak binding can be evaluated by measuring the mechanical affinity, which can be readily obtained from

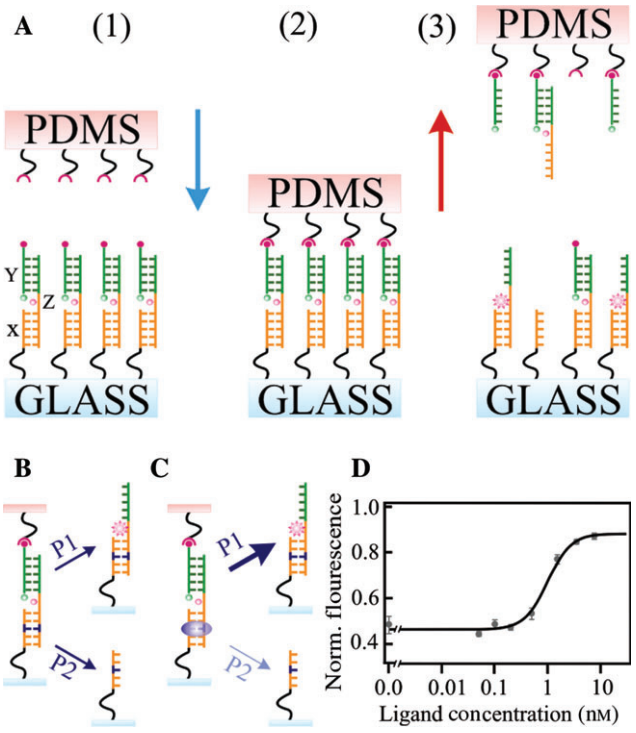

Figure 7 Schematic of the molecular force assay (MFA). From Severin et al. 2011. Reproduced by permission of The Royal Society of Chemistry. (A) Glass-surface-immobilized molecular force probes (MFPs) consist of three DNA strands (X, Y, and Z) in which XZ duplex and YZ duplex serve as the probe and the reference, respectively. A FRET pair is constituted by an acceptor fluorophore (green sphere) on the strand Y and a donor fluorophore (brown sphere) on the strand Z. In a typical experiment, the PDMS stamp covered by a receptor at the surface is moved into contact with a ligand that is tethered at the end of DNA strand Y (red circle, step 1). The PDMS stamp is then removed from the top (step 2). Owing to the different strengths between the following three interactions, the ligand and receptor, the Y and Z hybridization, and the X and Z hybridization, the fraction of FRET pair on the glass surface changes (step 3). This fraction can be probed by fluorescence signal of either the donor or the acceptor. (B) The probability of the donor only (P1) and that without any fluorophores (P2) remained on the glass surface after steps carried out in (A). C) When a ligand binds to the probe, P1 increases while P2 decreases. The ligand bound fraction can be obtained by normalized fluorescence intensity after comparison of (B) and (C). (D) A binding curve is constructed with different ligand concentrations.

the area underneath the rupture events as shown in the *F-X* curves (Figure 4). In fact, binding as weak as K_d=42 μM has been successfully measured recently (Koirala et al. 2011a). In the case where binding affinity is within a few k_BT, spontaneous rupture of an interaction becomes significant. To stabilize the binding, often times many such weak interactions are present. Whereas it is difficult for ensemble-based approaches to characterize these multiple events, single-molecule methods described here offer a unique opportunity to probe these interactions.

However, caution must be given to consider the change in $x^\dagger$ in the mechanical-affinity-based evaluation. As discussed above, if a ligand increases the transition distance upon binding, F_{rupture} may not show an obvious increase. In such a case, force loading rate can be varied to search for a particular $x^\dagger$ that can still yield a detectable change in F_{rupture} (Evans 2001). For binding processes that lead to the evolution of different conformations, comparison of both F_{ruptrue} and Δx between different populations could be useful to probe these successive events.

Another advantage of mechanical-property-based evaluation is that it can directly assess binding from mechanical perturbations applied from different directions. For physiologically relevant nucleic acid structures, mechanical perturbations from motor proteins mostly come from the 5′ to 3′ direction. However, when tertiary interaction exists between a protein and a nucleic acid structure, the perturbation is no longer necessarily from this direction. Using a click chemistry modification approach, the desired mechanical unfolding direction can be generated for biomacromolecules (Yu et al. 2012b, Dhakal et al. 2013). Such a direction-specific evaluation helps to evaluate the effect of a ligand on a receptor from physiologically important directions that are often blurred in chemical-affinity-based approaches due to their ensemble average nature.

A drawback for ligand screening using mechanical properties is the throughput of an assay. Optical tweezers and AFM can only evaluate one molecule a time. Although throughput can be increased by using microfluidic channels in which each channel can contain a different ligand (Koirala et al. 2011b), the point-by-point data collection still limits the throughput of the method. In AFM, it is possible to perform multiplexing screening by incorporation of multiple cantilevers (Fritz et al. 2000). Magnetic tweezes may provide an alternative approach with increased throughput (Danilowicz et al. 2009). However, the highest force it can apply may not be enough to dissemble a receptor-ligand complex.

Recently, Severin et al. (2011) employed an elegant strategy to evaluate DNA-binding ligands by placing DNA molecules between two planes followed by unfolding of the DNA-ligand complex after moving apart these two surfaces (Figure 7). By observing the fluorescence signal left at one of the surfaces, the effect of ligand binding can be quantified. This method does not require significant investment in instrumentation while providing a high-throughput capacity as multiple ligands can be evaluated in addressable wells on a chip.

Conclusions and outlook

With the invention of force-based single-molecule techniques such as optical tweezers and AFM, now it is possible to evaluate mechanical properties of the binding between a ligand and a receptor, which are largely overlooked in thermodynamic-based methods. We propose here that by using a universal parameter, mechanical affinity, binding can be conveniently evaluated from the unfolding work of a bound complex. The mechanical affinity is anisotropic and reflects the dynamic nature of the interaction between a ligand and a receptor. By exploiting mechanical properties such as rupture force, transition distance, and mechanical affinity, new ligand-screening methods can be designed to evaluate ligand-receptor complex from a specific unfolding direction. Compared to current screening approaches that are based on isotropic thermodynamic properties, these newly proposed methods are physiologically more relevant, especially in biological processes that involve motor proteins.

Acknowledgments: We thank the National Science Foundation (NSF) CHE-1026532 for financial support.

Received January 15, 2013; accepted April 27, 2013; previously published online July 17, 2013

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