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Perspective

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Force-dependent binding constants.
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Abstract
Life is an emergent property of transient interactions between biomolecules and other organic
and inorganic molecules that somehow leads to harmony and order. Measurement and
quantitation of these biological interactions is of value to scientists, and is a major goal of
biochemistry, as affinities provide insight into biological processes. In an organism these
interactions occur in the context of forces and the need for a consideration of binding affinities
in the context of a changing mechanical landscape necessitates a new way to consider the
biochemistry of protein-protein interactions. In the last few decades the field of
Mechanobiology has exploded, as both the appreciation, and the technical advances required
to facilitate the study, of how forces impact on biological processes has become evident.

The aim of this review is to introduce the concept of force-dependence of biomolecular
interactions, and the requirement to be able to measure force-dependent binding constants.
The focus of this discussion will be on the mechanotransduction that occurs at the integrin-
mediated adhesions with the extracellular matrix, and the major mechanosensors talin and
vinculin. However, the approaches that the cell uses to sense and respond to forces are
applicable to other systems, and therefore provides a general discussion of the force-
dependence of biomolecule interactions.

Introduction
The development of life has evolved in the context of physical forces acting on biological
systems. From the individual molecules, to the organelles, to the cells, the tissues, the organs,
every part of every organism is exposed to, and experiences forces. These forces, generated
or experienced, impact on every aspect of physiology1-3; every cell interprets “classical”
signalling pathways (growth factors, hormones etc.) in the context of its physical environment5-8.

The purpose of this review is to provide a brief introduction to the concept of force-dependent
binding constants and we will introduce the study of how forces can impact on biomolecular
interactions. This review will be divided into three sections, the first section will be a general
discussion of biomolecular interactions and their importance in biological processes, in
particular this will focus on the protein interactions involved in mechanotransduction leading
to the appreciation that many of these protein interactions have a force-dependent component.
In the second section we will discuss the modifications to the theory of binding constants
required to enable force-dependence to be considered. Finally, in the third section we will
discuss some of the novel approaches that are emerging, and/or required to enable force-
dependent binding constants to be measured.

Biomolecular interactions.
For the purposes of this review we focus on interactions with proteins and DNA, although the
concepts and principles are applicable to other systems. Interactions between proteins and
other proteins, DNA, lipid membranes, inorganic metal ions, etc. are mediated by compatible
interacting residues and surfaces, i.e. a surface on the substrate protein that has the optimal
shape to recognise its ligand (a moiety that forms a complex with that biomolecule to serve a biologically relevant purpose). The ligand can be any biomolecule, or non-organic molecule that interacts with the biomolecule in a meaningful way. Interacting surfaces that have important biological functions tend to be highly conserved through evolution, to preserve and maintain the interaction.

The equilibrium dissociation constant, $K_d$.

The binding affinity of an interaction describes the strength of the binding between a target molecule and its ligand. This binding affinity is usually reported as the equilibrium dissociation constant, $K_d$. This quantity is defined as the ratio between the off-rate, $k_{off}$ typically in units of $s^{-1}$ and the on-rate, $k_{on}$ typically in units of $M^{-1}s^{-1}$, where $M$ is Molar and $s$ is second. Therefore, the dissociation constant, $K_d = \frac{k_{off}}{k_{on}}$, has a dimension of concentration typically expressed in Molar concentration. $K_d$ can also be expressed by the equilibrium ratio of the fractions of bound target ($a_{on}$) and that of the unbound target ($a_{off} = 1 - a_{on}$) molecules, $\frac{a_{on}}{a_{off}} = \frac{c}{K_d}$, where $c$ is the concentration of free ligand molecules.

Recent rapid development of single-molecule technologies has made it possible to investigate binding of ligands to a single target molecule, thus, enabling determination of the binding affinity $K_d$ with single-molecule level of accuracy. Such quantification of $K_d$ is mainly achieved either by measuring the on- ($k_{on}$) and off-rates ($k_{off}$) through $K_d = \frac{k_{off}}{k_{on}}$, or by measuring the equilibrium probabilities of the bound ($p_{on}$) and unbound states ($p_{off} = 1 - p_{on}$) of the target molecule through $\frac{p_{on}}{1-p_{on}} = \frac{c}{K_d}$. At equilibrium, the ratio of the bound probability over the unbound probability should follow the Boltzmann distribution, $\frac{p_{on}}{1-p_{on}} = e^{\beta \Delta g_0}$, where $\beta = \frac{1}{k_B T}$ and $\Delta g_0$ is the free energy difference between the unbound and bound states, which is related to the dissociation constant by $K_d = ce^{-\beta \Delta g_0}$.

Measurement and quantitation of biological interactions is of value to scientists, and is a major goal of biochemistry, as affinities provide insight into biological processes. In drug discovery measuring affinities is important to aid the design of drugs with higher affinity for the target and thus higher efficacy. If there are two potential ligands available to bind to a single target molecule, at similar concentrations then the relative affinities of each for the biomolecule will dictate which binds preferentially. If one has a higher affinity (lower $K_d$) then it will bind preferentially. Modulation of the affinities for the two ligands (via post-translational modification including alteration of conformation via mechanical force) can alter the complexes that form.

Forces in biology

Life that lives under the sea has very different forces acting on it than those on land, or those that take to the skies, and changes in mechanical signalling that arise from these different physical environments enable the stunningly beautiful diversity of creatures. Strikingly, despite this incredible diversity, the adhesive structures holding the cells in place, via contact with neighbouring cells and to the surrounding extracellular matrix are largely made of the same building blocks. The appreciation of forces in biology has been the subject of many excellent reviews\textsuperscript{10-13}, and these all provide excellent accounts that we refer the reader to. The focus here is how these forces can be sensed by the cell and how they can alter signalling outcomes.

Forces on biomolecules

Many forces exist in cells, arising from collisions, flow (both retrograde flow of proteins inside the cell, and flow of blood past proteins on the surface of cells), force generation machinery and motor proteins (myosins, kinesins etc.), as well as forces exerted from the outside world,
gravity, pressure, friction etc. All of these forces, are sensed by mechanosensors in the cell and used to control cell behaviour. As these forces are ubiquitous it seems safe to assume that many biomolecules in the cell will experience forces, and as such that the binding constants of interactions involving these molecules will have a force-dependent component.

Actomyosin contraction.
Motor proteins harness the energy from ATP hydrolysis to generate mechanical energy that drives conformational changes that act on other cellular structures (reviewed in 16, 20). In the case of myosin, its interaction against actin filaments enables motion, either with the myosin moving along the filament, or pulling the actin filament towards it 21, 22. These forces generated by myosin motors pulling on the actin filament, is dubbed actomyosin contraction, and is a major method the cell utilises to generate and maintain forces. Much of the discussion herein will focus on the mechanisms with which the cell responds to this internally generated force.

For a force to act on a biomolecule it requires the force generation machinery to couple to the protein. This coupling can either be direct, if the protein directly binds to the actin filament, or indirect if the actomyosin pulls on another protein that acts on the protein. If the biomolecule is bound just to the force generator then this will pull the biomolecule towards the force. Trafficking of proteins can occur in this manner, for instance MyosinX can couple to cargo proteins and drag them along actin tracks to the tips of filopodia 23, 24. In this scenario the biomolecule is dragged along (and will experience the forces associated with drag).

However, if a protein couples to the force generation machinery but is also tethered to a second less mobile system, then the forces “pull” on the tethered protein and the forces are exerted on all proteins constituting the force-transmission molecular linkage. An example of this tethered system is seen for talin, bound to the integrin:ECM complexes at the plasma membrane 18, 25 (Fig. 1). Here when actomyosin contractions act on talin, as the talin is tethered, the forces are exerted on the length of the molecule, and as these forces exceed force thresholds of stability for any talin domains, they can trigger domain unfolding (see next section for the discussion of the consequences of domain unfolding).

This action of actomyosin contraction pulling on a tethered protein is not exclusive to adhesion, any protein/DNA/membrane that experiences forces has the potential to undergo changes in its shape and thus its function. So, any consideration of the mechanobiology of biomolecules needs to consider where the force is originating, how it is acting on the biomolecule, and how the biomolecule will react, which will depend on its mobility (can it move? is it tethered?).

Structural Mechanobiology.
One exciting, though not altogether unsurprising, aspect of the mechanosensitive events identified to date is that knowledge of the structural basis of the interaction provides an atomic basis of the mechanosensitive mechanism of the interaction. For instance, in the case of talin it is possible to identify the exact amino acids that render the molecule mechanically sensitive. A striking example of this is the R3 domain of talin 25-27, this domain has reduced mechanical stability due to a cluster of 4 threonine residues in the central hydrophobic core, which due to their more polar nature destabilise the domain. Using this precise structural information it is possible to modulate mechanosensing via targeted point mutations that alter mechanosensing. In the case of R3, modification of the 4 threonine residues to isoleucine and valine residues (a so-called “IVVI mutant”) results in stabilisation of the R3 domain 25-27 shifting its mechanical stability from 5 pN to 8 pN. Therefore, a comprehensive appreciation of mechanobiology at the atomic level requires atomic resolution structural biology.

Types of Force-dependent binding.
There are many ways that force can impact on binding, and these effects can quickly stack to give diverse responses. Here we offer a non-exhaustive summary of some of the common
force-dependencies. Protein:protein interactions can change their affinity by orders of magnitude under different force constraints, and as a result biochemistry done in bulk solution in vitro only captures part of the picture, and lacks consideration of the mechanical regulation of the interaction. As a consequence it is necessary to consider the force-dependence of biomolecular interactions.

The focus of this review is on equilibrium binding constants under mechanical force, $K_d(F)$. Discussion of the $K_d(F)$ will facilitate the description of forces impacting on the affinity of binary interactions, autoinhibition and the interplay of these factors. This will enable the description of two common force-dependent processes that regulate mechanosensitivity through talin, namely exposure of cryptic binding sites, whereby force exposes hidden binding sites, a type of autoinhibition, and disruption of binding sites, where the binding site is accessible to ligand, but force results in the domain unfolding and destroying the binding site. The talin rod contains 13 rod domains, R1-R13, which all combine both of these processes together, to create a series of mechanochemical switches (Fig. 1).

**Figure 1.** Talin serves as a force-dependent mechanochemical switch. Talin (grey) is shown bound to integrin and to F-actin. The 13 rod domains are shown arranged like beads on a string. The switch behavior of the R3 domain is shown. Left panel: Talin-RIAM-Rap1 linkage. Inset shows the schematics of RIAM binding to folded talin R3. Right panel: Talin-Vinculin-F-actin linkage. Inset shows the schematics of full-length vinculin bound to exposed VBS in unfolded talin R3.

**Mechanochemical switches.**

Tension-sensitive conformational change is a very rapid way for proteins to respond to mechanical force. In many ways this can be considered as a post-translational modification, force alters the conformation of a protein and if this change in shape elicits a change in biochemical function then force can be converted into biological signals. If the conformational change is reversible, that is when force is released the domain reverts back to its low force condition, then this provides incredible plasticity, and can enable rapid and dynamic changes in signalling outcomes.

The theoretical basis of these processes will be discussed in the next section, but to illustrate this concept of force-dependent binding constants, consider the interactions of a talin rod domain (here the example is R3) with three different ligands,

i) **Conventional protein interactions.**

Here two proteins interact in a “classical” manner; whereby one ligand binds to a binding site on a folded domain (i.e. RIAM binding to the folded talin rod domain R3, as demonstrated in the left panel in Fig. 1). In the absence of force the talin domains are folded, and so the interaction can occur. In this scenario, binding is highest at low force and so the $K_d$ is lowest. If the domain is unfolded by mechanical force then the binding surface on the domain is
ii) Cryptic binding sites that in the absence of force are inaccessible to binding.

In i) force results in the loss of ligands binding to folded rod domains, however, 8 of the 13 talin rod domains contain vinculin binding sites (VBS), amphipathic helices where the vinculin binding epitope is buried inside the domain. At low force the affinity of the VBS interaction with vinculin is weak (it has a high $K_d$ as the binding site is not accessible to vinculin). Here, the $K_d$ profile with force is different, at low force, the $K_d$ is high as binding is not possible, at forces above the unfolding threshold, then the $K_d$ is low (exposed VBS bind tightly to vinculin head, with nanomolar $K_d$ (although as we will see the vinculin itself is also regulated by forces acting on vinculin)). However, the force dependence is complicated as at high forces the VBS helix can unfold and lead to loss of vinculin binding.

iii) Cryptic binding sites that are only exposed when all secondary structure is destroyed.

Whilst no such ligand has been identified yet for talin, there is also the third scenario where a fully extended talin polypeptide creates linear epitopes that bind ligands. Here the affinity will be the highest at high force when the domain is completely unfolded.

Autoinhibition

Autoinhibition is not always considered to be mechanosensitive, but where proteins that are regulated by autoinhibition form part of the mechanical linkages in the cell then the affinity of the protein with its ligands is directly correlated to the force on the system. The cryptic nature of VBS in talin is also a type of autoinhibition\(^{25}\), and talin is further autoinhibited by the molecule folding up into a tight globular compact structure\(^{29-31}\). Vinculin is also regulated by a head-tail interaction, where the binding sites for talin and actin are rendered cryptic\(^{32,33}\). All of these layers of autoinhibition have a strong force-dependent component, as the affinity of autoinhibition is controlled by whether the protein is under force (if the autoinhibitory domains are held apart by mechanical force then the protein is maintained in an activated state)\(^{27,34,35}\). What is emerging is that autoinhibition of proteins in force-transmitting pathways represents a major force-dependent mechanism to enable mechanotransduction.

Force-dependent kinetic changes in binding.

Another key force-dependence of interactions arises from scenarios where forces alter the kinetics of the interactions. As the binding affinity is determined by the ratio of the dissociation rate over the association rate, the influence of force on the binding affinity must be through force-mediated changes of the dissociation and association rates. Whilst, a detailed discussion of these is beyond the scope of this review it is worthwhile to mention these effects as they impact on the mechanical functioning of force-dependent interactions. The force-dependent dissociation rate is of particular interest, as it defines the average lifetime of force-bearing molecular complexes once formed. The two most well characterised occurrences of force-dependent dissociation kinetics affecting biomolecular interactions, are the slip bond and catch bond.

Slip bonds

The slip bond refers to the phenomenon that the rate of dissociation of a molecular complex increases as the applied force increases. It indicates that a protein interaction is weakened under force, as the force pulls the two interacting components apart.

Catch bonds

The catch-bond refers to an anti-intuitive phenomenon where the molecular lifetime of an interaction increases as force applied to the interacting molecules increases\(^{36-39}\), which plays...
Critical roles in cell-matrix and cell-cell adhesions\textsuperscript{40-42}. Interestingly, catch-bond kinetics can have a geometric component, where the interaction exhibits catch bond behaviour only when force is exerted in a particular pulling geometry. This leads to directionally asymmetric catch bonds as are seen for the interaction between vinculin and F-actin\textsuperscript{43}. Directional asymmetry can be rationalised at the atomic level by looking at the geometry of the force vectors onto the interacting proteins (Fig. 2). Here the two extremes are “unzipping” and “shearing” geometries\textsuperscript{44-47}. Unzipping geometry typically exhibits slip bond kinetics, while in contrast the shearing geometry often exhibits catch bond kinetics. At the same force, the dissociation rate is often faster in the unzipping force geometry than for the shearing force geometry. We refer the readers to our recent publications\textsuperscript{44,46} for the physical principles underlying the effects of pulling geometry on the force-dependent dissociation kinetics.

Intriguingly, many force-bearing protein-protein interactions, such as integrin-talin connection, talin-vinculin connection and vinculin-actin connection, are under shearing force geometry\textsuperscript{43,48-50}. These protein-protein interactions form the interfaces in various force-transmission supramolecular linkages in cells, to enable mechanosensing. Perhaps these linkages evolved to achieve high mechanical stability for their functions through making shearing-force connections. In contrast, both force geometries occur frequently in force-dependent unfolding of protein domains. For example, when force is exerted through the N- and C-termini of proteins, Ig domains and $\alpha$-helix bundles consisting of odd number of $\beta$-strands/$\alpha$-helices are typically subjected to shearing force-geometry, while domains with even number of $\beta$-strands/$\alpha$-helices are under the unzipping force geometry, as illustrated in Fig. 2B.

Whilst important to our understanding of mechanobiology these two force-dependent kinetic phenomena will be the subject of a subsequent review and will not be considered further here.

**Force-dependent binding affinity, $K_d(F)$**

The theoretical description of the dissociation constant defined in the previous section can be extended to include the effects of force-dependence.

**Two-state binary interactions**

The force-dependent affinity of binary interactions has been discussed previously for simple two-state interactions\textsuperscript{51}, where a molecule can exist in either an unbound state or a bound state. When force is applied to this target molecule, each state is associated with a force-induced free energy, which is additional to the free energy change associated with its ligand. Therefore, the applied force can cause an additional change in the free energy from the bound to the unbound states, $\Delta g(F) = \Delta g_0 + \Delta \phi(F)$, where $\Delta g_0$ is binding energy (i.e., the free energy cost of unbinding) at zero force, and $\Delta \phi(F)$ is the force-dependent conformational free energy difference between the unbound and the bound states. Based on the Boltzmann distribution
of the states and the definition of the dissociation constant, \( \frac{p_{\text{on}}}{p_{\text{off}}} = e^{\Delta \phi(F)} = \frac{e}{K_d(F)} \), it is straightforward to see that, for a simple two-state binary interaction, \( K_d(F) = K_0^o e^{-\beta \Delta \phi(F)} \), where \( K_0^o = c e^{-\beta \phi_0} \) is the dissociation constant in the absence of force. Depending on the sign of \( \Delta \phi \) (F), force may increase or decrease the value of the dissociation constant.

Therefore, the force-dependent affinity of such simple two-state binary interactions is solely determined by \( \Delta \phi(F) \), which can be calculated by \( \Delta \phi(F) = -\int_0^F \Delta x(f) df \). Here \( \Delta x(F) = x_{\text{off}}(F) - x_{\text{on}}(F) \) is the extension difference between the unbound state and the bound state of the target molecule at the same applied force\(^{52-54} \). Over more than a decade of single-molecule manipulation studies, the force-extension curves of many interesting molecules, such as double stranded DNA (dsDNA), single stranded DNA (ssDNA), folded protein domains, and unfolded protein peptide chains, have been investigated. Therefore, the \( K_d(F) \) of two-state binary interactions can be considered well understood. Fig. 3 shows three test-case examples of force-dependent dissociation constant calculated based on the well characterized force-extension curves of DNA molecules. In each case, a ligand binding causes extension changes of the target DNA molecule at the same applied force (Fig. 3-B, E, and H), which leads to the force-dependent binding affinity \( K_d(F) \) (Fig. 3-C, F, and I). In each of these scenarios the strength of the interaction behaves markedly differently when force is exerted.

**Binary interactions involving autoinhibition**

Many proteins can adopt multiple conformational states, where the binding site(s) in these proteins are exposed at different levels of force. Autoinhibition is where the native state (i.e. the conformational state with the lowest energy), results in suppression of the accessibility of the binding site. In this scenario, binding can be discussed based on a three-state model, which involves two unbound states (the closed state "off,1" and the exposed unbound state "off,2") and one bound state (the exposed bound state "on")

\[ \frac{p_{\text{on}}}{p_{\text{off}}} = \frac{e^{-\beta \phi_{\text{on}}}}{e^{-\beta \phi_{\text{off},1}} + e^{-\beta \phi_{\text{off},2}}} \]

it can be shown that \( K_0^o = K_{d,o}^o(1 + e^{\beta \mu_c}) \), where \( K_0^o \) is the zero-force dissociation constant of the ligand molecule binding to the target molecule, and \( K_{d,o}^o \) is the zero-force dissociation constant of the exposed binding site in a constitutively open conformation of the target molecule, respectively. \( \mu_c = g_{\text{off},2} - g_{\text{off},1} \) is the autoinhibition energy, which is the chemical potential energy difference between the open conformation and the closed conformation of the target molecule. This reveals that the dissociation constant approximately increases exponentially as the autoinhibition energy increases.

Autoinhibition can be relieved via a number of mechanisms, that reduce the value of \( \mu_c \), including biochemical processes such as phosphorylation or binding of an activating molecule\(^{55-58} \). For force-bearing mechanosensing proteins, mechanical stretching provides another possible means to release autoinhibition, which has not been extensively studied in the field. Relief of autoinhibition by mutation is one way to study these processes, as this shifts the autoinhibition dynamics towards a more open conformation, effectively maintaining the protein in an open conformational state. By reducing the value of \( \mu_c \) this enables the life time of the open conformation to be extended as if the protein is under force. This provides an effective way to study protein dynamics that would normally be observed for the wild-type protein only when under force.
Here we provide a succinct discussion of force-dependent release of autoinhibition and its impact on molecular interactions.

A binary interaction involving autoinhibition can still be understood based on the aforementioned three-state model. The only difference from the zero-force binding case is that, each state now contains an additional force-dependent conformational free energy. Similar derivation based on leads to an expression of the force-dependent dissociation constant of ligand binding to autoinhibited target molecule:

$$K_d(F) = K_{d,0}^0 (1 + e^{\beta \mu_c - \beta \Delta \phi_{1,2}(F)}/(e^{-\beta \Delta \phi_{on,2}})),$$

where $\Delta \phi_{1,2}(F) = \phi_{off,1}(F) - \phi_{off,2}(F)$ and $\Delta \phi_{on,2}(F) = \phi_{on}(F) - \phi_{off,2}(F)$.

Figure 3. Force-dependent dissociation constants, $K_d(F)$ for three examples of two-state binary interactions. (A-C) Test case 1 - DNA annealing: DNA annealing causes a ssDNA to be paired with the complementary ssDNA to form a dsDNA. The change in the force-dependent conformational free energy $\Delta \phi(F)$ can be explained by the distinct force-extension curves of naked ssDNA and dsDNA, which leads to the force-dependent interaction affinity of DNA annealing. (D-F) Test case 2 - DNA-stiffening protein binding to dsDNA: force-extension curves of naked dsDNA and the dsDNA bound by a stiffening protein (e.g. H-NS$^4$) which causes the increase in persistence length of dsDNA from 53 nm to 174 nm. (G-I) Test case 3 - DNA-bending protein binding to DNA: force-extension curves of naked dsDNA and dsDNA bound by a bending protein (e.g. IHF$^9$) which causes an effective decrease in persistence length of dsDNA from 53 nm to 30 nm. (C), (F), and (I) show the fold change of force-dependent $K_d(F)$ relative to $K_d^0$ for the DNA annealing, the binding of DNA-stiffening protein to dsDNA, and the binding of DNA-bending protein to dsDNA. As each interaction shown in (B), (E) and (H) results in different effects on the DNA force-extension curves, the force dependence of the binding constant is markedly different.
Test-case 4: ssDNA binding to an autoinhibited region in a dsDNA hairpin

We demonstrate the application of this equation using a "simple" model system of autoinhibition. Here, the annealing of a short single-stranded DNA (ssDNA) oligo, acting as the "ligand", binds to a 10-nucleotide complementary region which is buried, cryptic, inside a 20-base pair (bp) double-stranded DNA (dsDNA) hairpin (Fig. 4A). The dsDNA hairpin can exist in two distinct unbound states: a closed hairpin state (state "off,1" in Fig. 4A) and an open unzipped state (state "off,2" in Fig. 4A). Considering the bound state (state "on" in Fig. 4A) where the ligand ssDNA binds to the complementary region of the dsDNA hairpin, there are in total three states that need to be considered. In this case, Eq. 1 can be directly applied to calculate the force-dependent dissociation constant of ssDNA binding, where the autoinhibition energy, $\mu_c$ is the base pairing energy in the hairpin. In physiological conditions, a base pair energy is in the range of 1-4 $k_B T$ depending on the nearest-neighboring di-nucleotide sequences\(^{59, 60}\). Assuming an average 2 $k_B T$ per bp, for a 20-bp DNA hairpin, $\mu_c$ is around 40 $k_B T$, which completely inhibits binding of the ligand ssDNA. However, such strong autoinhibition can be easily released by forces. Based on $\mu_c = 40 k_B T$ and the force-extension curves of ssDNA and dsDNA, it is found that forces around 15 pN can decrease the dissociation constant (i.e., increase the binding affinity) by more than 10\(^{15}\) fold (Fig. 4B).

Interestingly, the predicted $K_d(F)$ has a biphasic force-dependence, which can be divided into two regions: a monotonically decreasing function at forces below 15 pN due to the force-dependent release of autoinhibition, maximal binding affinity at 15 pN where the binding region is no longer autoinhibited, and a monotonically increasing function at forces above 15 pN due to force-dependent destabilization of the DNA duplex\(^{52}\). Even in this simple model system, the force-dependence on the $K_d$ is complex.

Test case 5: vinculin D1 domain binding to talin

Another important example of autoinhibition affecting the $K_d(F)$ is the binding of vinculin D1 domain (Vd1) to a vinculin binding site (VBS) buried in a talin rod $\alpha$-helical bundle. In the absence of force, the VBS is cryptic in the folded rod domains. In contrast to a dsDNA hairpin which can only exist in two distinct unbound states, an $\alpha$-helical bundle can exist in three distinct unbound states: an autoinhibited folded state (state "off,1" in Fig. 4C), an unfolded state where the VBS exists in an $\alpha$-helical conformation (state "off,2" in Fig. 4C), and an unfolded state where the VBS becomes an unstructured peptide polymer (state "off,3" in Fig. 4C). Considering the bound state (state "on" in Fig. 4C) where the Vd1 binds to the $\alpha$-helical conformation of VBS, there are in total four states that need to be considered. Denoting $\epsilon$ as the chemical potential energy between the unstructured and $\alpha$-helical conformations of the VBS (broadly equivalent to the stability of one $\alpha$-helix) and $K_{d,0}$ the zero-force dissociation constant of Vd1 to the exposed $\alpha$-helical conformation of VBS, based on similar analysis of the force-dependent energies of the four states, it can be shown that:

$$K_d(F) = K_{d,0}^3(1 + e^{\beta \mu_c} e^{-\beta \Delta \phi_{1,2}(F)} + e^{-\beta \epsilon} e^{-\beta \Delta \phi_{3,2}(F)}).$$

Here $\Delta \phi_{1,2}(F) = \phi_{\text{off},1}(F) - \phi_{\text{off},2}(F)$ and $\Delta \phi_{3,2}(F) = \phi_{\text{off},3}(F) - \phi_{\text{off},2}(F)$, which can be computed based on the force-extension curves of the states. Using a fixed value of $\mu_c$ of 11 $k_B T$, the fold change of $K_d(F)$ relative to $K_{d,0}$ can be calculated for several values of $\epsilon$ (Fig. 4D). At these parameter values, a force of ~5 pN can decrease the dissociation constant (i.e., increase the binding affinity) of Vd1 to the talin rod $\alpha$-helical bundle by more than 10,000 folds. In other words, the high autoinhibition energy $\mu_c = 11 k_B T$ which limits vinculin binding in the absence of force, can be released by a small force around 5 pN.

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The predicted $K_d(F)$ exhibits an overall biphasic profile, which can be divided into three regions: a monotonically decreasing function at forces below 5 pN resulted from force-dependent release of autoinhibition energy ($\mu_c = 11 k_BT$), an almost force-independent basin region, followed by a monotonically increasing function due to force-dependent destabilization of the $\alpha$-helical conformation and thus the bound complex. In contrast to the sharp switch from the decreasing profile of $K_d(F)$ to the increasing profile of $K_d(F)$ at ~ 15 pN in the case of ssDNA binding to an autoinhibited region in a dsDNA hairpin (Fig. 4B), the switch is much less sharp in the case of Vd1 binding to a VBS-containing domain (Fig. 4D). Here, after the domain unfolds and autoinhibition is relieved, the VBS binding helix is exposed and has maximal affinity for Vd1 all the while the VBS helix is folded. The more stable the VBS helix is, the greater the force range over which binding affinity is maximal. As a result maximal affinity is present over a range of force, as seen by the force-independent basin region in Fig. 4D with the force range of the basin determined by the stability, $\varepsilon$, of the VBS alpha helix.

Mutually exclusive binary interactions involving autoinhibition

The previous examples describe the scenarios of the complex force-dependence on the $K_d(F)$ where the applied force can drastically increase the binding affinity between the target molecule and its ligand by releasing the autoinhibition of the target molecule thus exposing the binding site(s) for its ligand, and where the applied force can also then decrease the binding affinity by destabilizing the conformation of the bound state.

In the case of talin rod domains, these force-dependent components can be multiplexed, to create force-dependent switching of binding partners. The force-dependent switching of binding partners on talin rod domain 3 (R3) provides an example of this (Fig 1). At low forces, the Rap1-effector RIAM binds the $\alpha$-helical bundle form of R3 where the VBSs are cryptic (Fig 1, and upper panel in Fig. 5A), whereas at high forces, the autoinhibition of VBSs in R3 is released and exposure of the VBSs significantly increases the binding affinity to Vd1 (Fig. 1, Fig. 4C-D and lower panel in Fig. 5A). As such, force drives a change in binding partners.
To be specific, the force-dependent binding constants $K_d(F)$ of R3-RIAM interaction and R3-Vd1 interaction can be derived based on similar analysis of the force-dependent energies of all the states involved. By analyzing the four states involved in the R3-RIAM interaction (illustrated in the upper panel in Fig. 5A), $K_d(F)$ of R3-RIAM interaction can be derived to be:

$$K_d(F) = K_d^0(1 + e^{-\beta \mu_c - \beta \Delta \phi_{2,1}(F)} + e^{-\beta (4\varepsilon + \mu_c) - \beta \Delta \phi_{3,1}(F)}),$$

(3)

where $K_d^0$ is the zero-force dissociation constant of RIAM to the $\alpha$-helical bundle form of R3, $\Delta \phi_{2,1}(F) = \phi_{off,2}(F) - \phi_{off,1}(F)$ and $\Delta \phi_{3,1}(F) = \phi_{off,3}(F) - \phi_{off,1}(F)$.

Regarding the R3-Vd1 interaction, there are 2 VBSs in talin R3 and in such scenario a complete description needs to consider the multiple bound states and the effect of volume exclusion. For the simplicity of demonstrating the idea of force-dependent switching of binding partners, here we only consider binding to one VBS in R3. As such, there is one bound state and three unbound states (illustrated in the lower panel in Fig. 5A), and the force-dependent dissociation constant $K_d(F)$ of R3-Vd1 interaction can be directly calculated by Eq. 2.

In both Eq. 2 and Eq. 3, $\mu_c$ is the chemical potential energy between the $\alpha$-helical bundle form of R3 and its extended $\alpha$-helices chain, and $\varepsilon$ is the chemical potential energy between the unstructured and $\alpha$-helical conformations of one $\alpha$-helix. Using fixed values of $\mu_c = 11 k_BT$ and $\varepsilon = 5 k_BT$ for demonstration purpose, the force-dependent switching between R3-RIAM and R3-Vd1 interaction can be shown in Fig. 5B.

Figure 5. Force-dependent switching of Vd1 and RIAM binding to talin R3. (A) Schematics of states involved in R3-RIAM interaction and R3-Vd1 interaction. (B) The fold change of $K_d(F)$ of talin R3-RIAM interaction (yellow curve shows the ratio of $K_d(F)$ to $K_d^0$ in Eq. 3) and talin R3-Vd1 interaction (red curve shows the ratio of $K_d(F)$ to $K_d^{0,0}$ in Eq. 2).

Measurement of the $K_d(F)$ in experiments
To further explore the impact of forces on the affinity of binary interactions, direct measurement of $K_d(F)$ in experiments will give a straightforward understanding on the force-dependency of binding affinity. This section is devoted to providing a brief discussion on the measurement of $K_d(F)$ in experiments, which includes i) the measurement of zero-force dissociation constant $K_d(0) = K_d^0$ by bulk technology, ii) the measurement of dissociation constant under force $K_d(F)$ by single-molecule manipulation technology.
Bulk technology to measure the $K_d(0)$

There are many methods to measure zero-force dissociation constant biochemically, which are generally based on two approaches.

The first way to quantify the $K_d(0)$ is based on the measurement of the bound ($\alpha_{on}$) or unbound fraction ($\alpha_{off}$) of target molecules through $K_d = \frac{\alpha_{off}}{\alpha_{on}}c$ (where $c$ is the concentration of ligand). For example, the electrophoretic mobility shift assay (EMSA) can be used to quantify protein-DNA interaction. Here, fluorescence DNA dyes are usually used to label the DNA molecules which present the DNA targets in the bound and unbound states as two bands migrating with different speeds in agarose or polyacrylamide gel\textsuperscript{62, 63}. The fractions of the bound and unbound fractions are indirectly estimated based on the intensity ratio of the bands, under an assumption that the intensity is proportional to the amount of target molecules in the corresponding bands. Such methods require an additional assumption that the bound and unbound fractions of the target molecules remain fixed during the gel shift assay.

The other commonly applied way to quantify the binding affinity is based on the measurement of the association ($k_{on}$) and dissociation rates ($k_{off}$) through $K_d = \frac{k_{off}}{k_{on}}$. The surface plasmon resonance (SPR) technology is a representative example, which detects binding of ligands to target molecules tethered on a gold surface based on the binding-induced shift in the resonant oscillation of conduction electrons (Fig. 6A)\textsuperscript{64, 65}. In typical experiments (Fig. 6B), SPR measures the resonance signal evolution after flowing ligand-containing solution until it reaches equilibrium. Based on the most commonly used Langmuir model\textsuperscript{66, 67}, this time evolution follows a single-exponential relation, $R(\Delta t_a) = R_{eq}[1 - e^{-(c k_{on} + k_{off})\Delta t_a}]$, where $R_{eq}$ is the resonance signal when the binding and unbinding of ligands reach equilibrium, $c$ is the ligand concentration, and $\Delta t_a$ is the time duration after flowing in the ligand-containing solution. After the removal of the ligand from solution, the bound ligands dissociate, resulting in an SPR signal time evolution: $R(\Delta t_d) = R_{eq}e^{-k_{off}\Delta t_d}$, where $\Delta t_d$ is the time duration after the removal of the ligand from solution. By fitting the association and dissociation SPR data with the two equations, one can obtain the values of association and dissociation rates and thus $K_d$.

Besides EMSA and SPR assays, a number of other methods have been developed to quantify the binding affinity of molecules based on either measurement of the bound and unbound fraction of target molecules, or based on measurement of the kinetic rates, which include (but are not limited to) Fluorescence, Fluorescence Polarisation (FP), Nuclear Magnetic Resonance (NMR), Isothermal Titration Calorimetry (ITC), Stopped flow kinetics, etc. and these are discussed elsewhere\textsuperscript{68-70}. However, these biochemical assays are not applicable for the study of the force-dependence of molecular interactions, because they do not apply mechanical constraints to molecules.

![Figure 6. Quantification of $K_d$ based on measuring kinetic rates in SPR experiments. (A) Schematics of the association and dissociation phase in $K_d$ measurement. (B) Typical SPR signal. The kinetic rates ($k_{on}$ and $k_{off}$) and binding affinity ($K_d$) can be determined by fitting the sensorgram data to an appropriate interaction model.](image)
Single-molecule technology to measure the $K_d(F)$

As mentioned earlier, single-molecule technologies can also be used to determine the dissociation constant, $K_d$, either by quantifying the equilibrium binding probability $p_{on}$ through $K_d = \frac{1 - p_{on}}{p_{on}c}$ (where $c$ is the concentration of ligand), or by quantifying the association $k_{on}$ and the dissociation rate $k_{off}$ through $K_d = \frac{k_{off}}{k_{on}}$. Most of the single-molecule measurements of $K_d$ to date have been done using technologies such as single-molecule fluorescence imaging\textsuperscript{71-73} and single-molecule mechanical manipulation\textsuperscript{74-77}. For measurement of $K_d(F)$, single-molecule mechanical manipulation is necessary to apply force to a target molecule and measure its force-dependent interactions with ligand. Therefore, the following section will focus on the measurement of $K_d(F)$ using single-molecule mechanical manipulation technology.

Single-molecule mechanical manipulation technologies\textsuperscript{78} can be categorized into two groups based on the types of mechanical constraint they apply to a molecule. In one group represented by optical tweezers (OT)\textsuperscript{79} and atomic force microscopy (AFM)\textsuperscript{80}, an external Hookean spring is attached to one end of the tethered molecule and its distance $R$ from the other end of the molecule is controlled (Fig. 7, upper panel). In the other group, represented by magnetic tweezers (MT)\textsuperscript{81}, centrifuge tweezers\textsuperscript{82} and acoustic tweezers\textsuperscript{83}, an external force is applied to a bead attached to tethered molecule and the level of the force is controlled (Fig. 7, lower panel). Through a force-clamping feedback control, OT and AFM can also apply an external force control to molecules\textsuperscript{84, 85}. In order to measure the force-dependent dissociation constant $K_d(F)$, it is most convenient to measure the interaction across a range of constant forces.

Since the measurement is performed on a single target molecule, it is desirable to record repetitive binding and unbinding events over a long duration of measurement. This imposes strong requirements on the stability of the instrument over long durations. Magnetic tweezers can make measurements over a time course of hours to days with negligible spatial and force drifts\textsuperscript{86, 87}. The force-dependent binding to, and unbinding from, the mechanically manipulated target molecule can in principle be detected based on integration of single-molecule fluorescence imaging with single-molecule manipulation\textsuperscript{88}. However, such integration has a drawback of photobleaching, which impairs the long-duration measurement for interactions with slow kinetics.

Hence, it is important to develop label-free measurement approaches for detection and quantitation of single-molecular interactions under force. In a force-constraint single-molecule manipulation experiment, the target molecule is tethered between a coverslip surface at one end and a bead at the other end\textsuperscript{89, 90}. A well-controlled stretching force, which can be calibrated at a sub-pico Newton (pN) resolution, is applied to the target molecule through the bead, meanwhile the bead height from surface can be measured at a nanometer (nm) resolution. Hence, it can detect molecular extension changes of a few nm. Utilizing this spatial resolution, label-free measurement of single-molecular interaction under force can be based on detecting; i) binding induced deformation of the target molecule (Fig. 8A-B), or ii) binding induced delay....
to a structural transition (Fig. 8C-D). These two label-free detection approaches, discussed in the next section, have been applied in a number of recent studies to detect DNA-protein\textsuperscript{76, 77, 91, 92} and protein-protein interactions\textsuperscript{27, 34}. More recently, these detection approaches have been further developed to quantify the dissociation constant, $K_d(F)$\textsuperscript{76, 77}.

Detection based on target molecule deformation
Ligand binding can be probed based on the changes in the end-to-end extension resulting from binding-induced structure transition or deformation of the binding site (Fig. 8A-B). This approach is suitable for ligands that induce a detectable change in the extension of the tethered molecule. An example of this is the interaction between Vd1 and the mechanically exposed VBS in talin R3\textsuperscript{27} and $\alpha$-catenin\textsuperscript{34}. At forces higher than 15 pN, the exposed VBS exists in a randomly coiled peptide conformation. Binding of Vd1 induces formation of the $\alpha$-helical conformation of the VBS, resulting in a detectable stepwise extension decrease of 2-3 nm dependent on the applied force. Similarly, when Vd1 dissociates, it will be accompanied with a 2-3 nm stepwise extension increase (Fig. 8A-B). This 2-3 nm step provides a visible and quantifiable readout of the ligand binding and unbinding. From the time trace of such two-state stepwise extension fluctuation, both the association and dissociation rates can be determined by obtaining the dwell time in bound and unbound states; therefore, the force-dependent dissociation constant can be determined by $K_d(F) = \frac{k_{off}(F)}{k_{on}(F)}$.

Detection based on delayed structural transitions
A bound ligand on a target molecule can also be detected if binding results in a delayed structure transition. This can be i) delayed refolding if the ligand is bound on an unfolded structure via an exposed binding site (Fig. 8C) and ii) delayed unfolding if the ligand is bound on a folded structure (Fig. 8D).

An example of delayed protein refolding due to ligand binding is the interaction between Vd1 and VBS in talin rod and $\alpha$-catenin domains\textsuperscript{27, 34, 35}. Previous works from our group have shown that Vd1 bound on the mechanically exposed VBS in the domains can keep the domains in the unfolded conformation for a longer duration after force is released, compared with in the absence of Vd1. This resulting longer extension (Fig. 8C) can be detected and quantified.

A ligand bound on a folded structure can be detected similarly (Fig. 8D). As the structural unfolding of the target molecule can only happen after the ligand dissociates, ligand
binding can stabilise the target which often results in a slower unfolding transition. Thus, if the ligand results in a detectable delay in unfolding transition after jumping to a higher force, it becomes a readout on whether the target molecule is bound by a ligand or not right before the force jump. While the delayed unfolding has mainly been applied to quantify protein-DNA interactions\textsuperscript{77, 91, 92}, the same principle can be utilized to detect ligand binding to a protein domain (e.g., RIAM binding to talin R3) and so quantify the force-dependent dissociation constant.

Based on these delayed structural transitions, the bound and unbound states of the target molecule can be determined, which enables the determination of the equilibrium binding probability $p_{\text{on}}(F)$ using a force-jump assay. In such an assay (Fig. 8C-D), many cycles of force-jump between two force levels are used: i) a binding-force ($F$) at which the target binding site is stable for a duration of $T_b \gg \frac{1}{c k_{\text{on}} + k_{\text{off}}}$ to ensure binding equilibrium, and ii) a detecting-force at which the binding-induced delayed structural transition can be observed. The probability of binding is then determined by the ratio of the number of cycles where binding is detected ($M$), to the total number of cycles ($N$), $p_{\text{on}}(F) = \frac{M}{N}$. From this, the force-dependent dissociation constant can be determined by $K_d(F) = \frac{1 - p_{\text{on}}(F)}{p_{\text{on}}(F)} c$, where $c$ is the ligand concentration.

**Discussion**

In this short review, we have sought to provide a brief discussion of some of the force-dependent considerations at the heart of mechanobiology, and outline some of the strategies to measure and study these. The requirement to consider the force-dependence of binding constants in biology necessitates development of the existing mathematical descriptions of binding constants to include mechanical descriptors. Further, we discuss the novel experimental approaches required to measure these.

The examples of force-dependent binding events illustrated in Fig. 3-5 highlight some of the diverse and ingenious ways that biological systems sense, and respond to, forces. Even in these simplified *in vitro* systems it is evident that the ways binding constants in each scenario are affected by force is complex, and often biphasic. The consequence of these complex force dependencies is that the binding affinities between two ligands can change, either increasing or decreasing, by 1000 fold or greater dynamically over a physiological force range. This creates incredible complexity in these mechanical linkages, with the same components assembling differently in different force environments.

**Multivalent interaction**

Our review on the force-dependent affinity has been developed based on single-site binary interactions. In many cases, such as antibody-antigen interactions, however, multivalent interactions play a crucial role in biological functions. In such cases, the binding strength of multivalent interactions cannot be formulated based on a two-state model. The binding strength or the functional activity of such multivalent interactions is often referred to as “avidity”. Such multivalent interactions are also implicated in mechanosensing reactions. For example, talin contains 11 VBS\textsuperscript{35}. When multiple VBSs in talin are mechanically exposed for binding to vinculin, the functional activity of force-dependent talin-vinculin interaction is expected to be further boosted.

**Multiplexing force-dependent factors**

A hint at the immense amount of information that can be encoded by such mechanosensitive complexes is evident when you consider these principles in a simple two component system, and the multiplexing of the multiple force-dependent contributions that can emerge. Take, for
example the mechanosensitive interactions between talin and vinculin at the core of the focal adhesion. There is the well documented force-dependence of the exposure of cryptic VBS from within the core of the folded talin bundles discussed earlier in this review. Above a certain threshold talin bundles unfold exposing cryptic VBS and allowing vinculin to bind. The description in Fig. 4 describes a single VBS binding a single vinculin. However, talin contains 11 VBS and the mechanical response of talin is complex⁵⁵, with diverse force thresholds governing exposure of each VBS. This set up is further impacted by additional force-dependent considerations.

For instance, both talin and vinculin are further regulated by autoinhibition, and in both cases the affinity of the autoinhibition (which is a head tail interaction) is reduced when the protein is under force; here the effective binding constant of the autoinhibition is massively increased as the two interacting domains (the head and the tail) are physically held apart from each other (Fig. 1). As such there exist layers upon layers of inhibition on these molecules all exhibiting force dependence⁹³. The forces exerted on talin change constantly and if the force decreases, then the talin rod domain and the head tail autoinhibition will have a higher effective affinity (they will no longer be held apart from each other) and can be trying to revert back to their closed inactive conformations.

There is also considerable hysteresis on talin domain refolding²⁵, ³⁵, a domain that unfolds at 15 pN will not refold when the force drops to <15 pN, instead it requires forces less than 3 pN to refold on a reasonable timescale. When refolding of the talin domains occur it will affect the exposure and thus the affinity of the talin-vinculin interactions. With up to 11 VBS in talin, many force-linkages can be coupled.

However, additional force-dependence is introduced by the connectivity of vinculin with the actin filaments. If vinculin engages talin via its head domain, and couples to actin via its tail domain, then this will exert force onto the talin-vinculin interaction which will affect the affinity, and as the geometry of this force on the VBS-vinculin interaction is likely to be shearing force geometry it means that force will strengthen this interaction (with catch bond kinetics). Further, tethering vinculin to talin and actin also restricts vinculin autoinhibition and so the effective \( K_d \) of vinculin autoinhibition is also greatly increased which will further enhance the VBS-vinculin interaction.

This description is complex but still does not include potential force dependence of unfolding of vinculin domains, the strength of the talin linkages with the integrin and actin, nor the recruitment, or displacement, of factors as a result of domain unfolding that might enhance or decrease contractility. All of these will further augment the mechanical connections. So, even within this simplified description of the system, there are almost endless possibilities for diverse outcomes. When you layer on the myriad of other mechanoeffectors and regulators that assemble on this hyper-plastic framework, it becomes apparent that there is a huge capacity in these linkages to encode vast amounts of data²⁵.

**Future perspective**

This review is centred around the molecular interactions involved in mechanosensing, with a focus on how force applied to a molecule may influence the affinity with its binding partners. In addition, the review also briefly discusses how force applied to interacting partners may affect the lifetime of the complex.

What has been missed in these discussions is how force applied to one molecule affects the binding and unbinding rates of its binding partners in solution. Since force can drastically change the affinity of the interaction, it should have a significant influence on the binding rate, or unbinding rate, or both. Compared to force-dependent affinity, the force-dependent kinetics
of binary interactions have not been as extensively studied and remain less well understood. A deeper understanding of the force-dependent interaction kinetics is required because in cells many interactions do not reach equilibrium. The force-dependent reaction rates will provide crucial insights into how non-equilibrium molecular interactions under mechanical force in cells can be understood.

Another interesting topic, which is not included in the present review, is how other types of mechanical constraints may affect interactions. Cytoskeleton filaments, such as actin filaments, are often subjected not only to tensile force but also to rotation constraint. The latter will result in torque in the filament, which is transmitted to the proteins, such as formin or other actin capping proteins, linked to the end of the actin filaments. Another example is that DNA in many topologically isolated chromatin domains is supercoiled, which also results in torque applied to the DNA. How torque may affect the affinity and the kinetics of the molecular interactions is currently poorly understood, which should be another interesting future direction.

The rapid advances in the field of mechanobiology are making great strides in advancing our understanding of these complex mechanosensing signalling systems.
**Box 1**  
**Model and parameters of force-extension curves of target molecules**

**Two-state binary interactions**

- **ssDNA** – described by the worm-like chain (WLC) polymer model that contains two parameters, the bending persistence length $A_{ssDNA}$ and the contour length $L = n_{nt}l_{nt}$, where $n_{nt}$ is the number of nucleotides. ssDNA has a persistence length of $A_{ssDNA} \approx 0.7 \text{ nm}$ and a contour length per nucleotide of $l_{nt} \approx 0.7 \text{ nm}^{98}$. Based on the WLC model, the force-extension curve of ssDNA $x_{ssDNA}(F)$ can be obtained by solving the inverse function of the Marko–Siggia formula$^{99}$:

$$F A_k B_T = x L + \frac{1}{4(1-x/L)^2} - \frac{1}{4}.$$  

- **dsDNA** – described by WLC model where the dsDNA has a persistence length of $A_{dsDNA} \approx 50 \text{ nm}$ and a contour length per base pair of $100 \text{ nm}$. Similarly, the force-extension curve of dsDNA $x_{dsDNA}(F)$ can be obtained by solving the inverse function of the Marko–Siggia formula.

- **stiffening protein-dsDNA/bending protein-dsDNA** – both described by WLC model where the only difference compared with the naked dsDNA is the bending persistence length. In Fig. 3E-F, the bending persistence length of stiffening protein-dsDNA is taken $A_{sf} - dsDNA \approx 174 \text{ nm}$ which is adapted from the case of H-NS binding$^4$. In Fig. 3H-I, the bending persistence length of bending protein-dsDNA is taken $A_{bd} - dsDNA \approx 30 \text{ nm}$ which is adapted from the case of IHF binding$^9$.

**Binary interactions involving autoinhibition**

- **dsDNA hairpin** – described by the rigid body with a size of $L_{hp} \approx 2 \text{ nm}$ which is the width of dsDNA$^{101}$. Its force-extension curve is $x_{hp}(F) = L_{hp}[\coth \left( \frac{FL_{hp}}{k_B T} \right) - \frac{k_B T}{FL_{hp}}]$.

- **α-helical bundle** – described by the rigid body with a size of $L_{bd} \approx 3.4 \text{ nm}$ which is the N-C terminus distance obtained from talin R10 (PDB ID: 2KVP)$^{102}$.

- **α-helical chain** – described by the freely-jointed chain (FJC) polymer model with a contour length of $L_{ahc} = n_{ah}L_{ah}$, where $n_{ah}$ is the number of α-helix and $L_{ah}$ is the size of each α-helix. In Fig. 4D and 5B, fixed values of $n_{ah}$ and $L_{ah}$ are taken to be $n_{ah} = 4$ and $L_{ah} = 4.8 \text{ nm}^{102}$. Its force-extension curve is $x_{ahc}(F) = L_{ahc}[\coth \left( \frac{FL_{ahc}}{k_B T} \right) - \frac{k_B T}{FL_{ahc}}]$.

- **α-helix form of VBS** – described by the rigid body with a size of $L_{ah,VBS} = N_{VBS}l_{ah,aa}$, where $N_{VBS} = 25$ is the number of amino acids in VBS$^{103}$ and $l_{ah,aa} = 0.15 \text{ nm}$ is the extension per amino acid in the form of α-helix$^{104,105}$. Its force-extension curve is $x_{ah,VBS}(F) = N_{VBS}[\coth \left( \frac{FL_{ah,VBS}}{k_B T} \right) - \frac{k_B T}{FL_{ah,VBS}}]$.

- **unstructured peptide chain** – described by WLC model where the peptide chain has a persistence length of $A_{pt} \approx 0.8 \text{ nm}$ and a contour length per amino acid of $l_{aa} \approx 0.38 \text{ nm}^{106}$. Similarly, the force-extension curve of peptide chain $x_{pt}(F)$ can be obtained by solving the inverse function of the Marko–Siggia formula.
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