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Single-Molecule Mechanoenzymatics

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Abstract

The ability of cellular signaling networks to sense, process, and respond to internal and external stimuli relies on their specific detection and transduction based on molecular recognition. The molecular mechanisms by which force is specifically sensed by mechanoenzymatic processes, translated into biochemical signals, and wired to cellular signaling networks recently became accessible with single-molecule force spectroscopy. By stretching such mechanobiochemical converters along their natural reaction coordinate, complex mechanical activation pathways and subsequent biochemical reactions may be measured in a dynamic and highly precise manner. The discovered mechanisms have in common well-tuned force-induced conformational changes that lead to exposure of active recognition sites. Newly developed strategies allow investigators to test different conformational states for activity and to elucidate mechanical architectures leading to highly specific mechanical activation pathways. Here, we discuss the advances in the new field of single-molecule mechanoenzymatics and highlight complementary examples studied in bulk and in vivo.

Contents

INTRODUCTION 498
MECHANICAL STABILITY OF STRUCTURAL ELEMENTS 499
Random Coils 499
Helical Elements 500
β-Sandwich Elements 501
MECHANISMS OF FORCE-SENSING AND THE DIFFERENT WAYS OF CONFORMATIONAL CHANGES 502
The Partial Unfolding Mechanism of Titin Kinase 503
Complete Unfolding of the von Willebrand factor A2 Domain 505
Binding of Vinculin to Force-Induced Conformations of Talin 505
MECHANICAL ARCHITECTURES AND SPECIFIC ACTIVATION PATHWAYS 506
COMBINATION OF SINGLE-MOLECULE FORCE SPECTROSCOPY AND SINGLE-MOLECULE FLUORESCENCE 509
ENSEMBLE AND IN VIVO FORCE MEASUREMENTS 509
OUTLOOK 512

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INTRODUCTION

A general concept describing and unifying biochemical and biophysical processes is the free energy landscape, which has various reaction coordinates defining a surface in a high-dimensional space (27). The dynamics of chemical reactions, protein folding and unfolding, and conformational changes through this landscape are described as diffusion driven by thermal energy. From this concept, whenever a change in a spatial reaction coordinate along a path is associated with a change in free energy, force is exerted or experienced by the molecule. Likewise, external forces bias a reaction pathway by tilting the energy landscape along their direction. Force is thus a physical quantity inherent to biological and biochemical processes in diverse ways (10) and presents a selection pressure during evolution. Nature created passive and active mechanisms to counteract and react to internally created and externally exerted forces. Passive mechanical diversity such as rigidity of bones or elasticity of muscles is equally present on a cellular level, where stiff filaments form the cytoskeleton or proteins act as passive springs. Active molecular mechanisms that sense force and translate it into neuronal or biochemical signals manifest themselves on a macroscopic level as touch sensation, muscle adaption, or hearing, but play a much more general role for cellular organization and adaption. How can force be sensed on a molecular level such that a mechanoenzymatic process translates the mechanical input into a biochemical signal? A meaningful way for a protein or an enzyme to measure forces is through a change in position against an increasing energy, i.e., through conformational changes. The activation force is tuned by the energy barrier and the specific biochemical response is associated with the new conformation and recognition sites.

Mechanoenzymatic process: a biochemical process that requires force to activate an enzyme or the substrate of an enzyme

SMFS: single-molecule force spectroscopy

Mechanical stability: the comparative ability of mechanical elements to resist force. A precise characterization of rupture forces would require a set of parameters such as loading rate, potential width, unfolding rate, and temperature

The endeavor of discovering and investigating the specific molecular mechanisms of mechanosensors or mechanoenzymes as depicted in **Figure 1** began only recently, mainly because force as a vectorial quantity is hard to control in ensemble experiments and because structural methods can hardly quantify dynamic behavior. The development of single-molecule force spectroscopy (SMFS) was required, which led to a basic understanding of mechanical stability and force-induced conformational changes of proteins. This knowledge, together with recent technical advances in automation of data recording, analysis, specific mechanical protocols, and the

combination of SMFS with single-molecule fluorescence, has opened the field of single-molecule mechanoenzymatics.

In this review we focus on mechanoenzymatic processes that have been deciphered with SMFS and in some cases could be compared to molecular dynamics (MD) simulations (54, 62). Reviews on the different types, advances, and applications of SMFS may be found elsewhere (1, 7, 37, 72, 74, 75, 85). In regard to mechanosensitive ion channels, we refer to other sources (59). We first discuss the mechanical stability of structural elements, which are to some extent mechanical building blocks of mechanoenzymes. By means of the three different examples depicted in **Figure 1**, we illustrate three classes of mechanisms that transform force into conformational changes, which in turn result in a specific biochemical reaction. Using titin kinase (TK) as an example, we show how single-molecule pump-and-probe protocols allow investigators to examine the different conformations along a mechanical activation pathway and to test them for activity. Because certain mechanoenzymatic processes require specific and well-defined mechanical activation pathways, we then describe how different mechanical architectures result in different mechanisms and how these architectures may be characterized. Single-molecule fluorescence microscopy offers an independent readout of enzymatic activity or binding events. The combination with SMFS is therefore promising for studying mechanoenzymatic processes, as we show by means of recent applications. In addition to investigating single and isolated proteins, it is important to compare the results to complementary experiments performed in the context of the cellular environment. We therefore briefly discuss other means to measure forces in vivo and provide an outlook on the difficulties and strategies to do so at the single-molecule level.

MD: molecular dynamics

TK: titin kinase

Mechanical activation pathway: the force-induced conformational transitions or parts of an unfolding pathway along the energy landscape leading to an active state

Mechanical architecture: the way in which mechanical elements are arranged and defined

Loading rate: the rate of force increase upon stretching a molecule

Mechanical element: a structural element or molecular interaction causing an energy barrier in the unfolding pathway

MECHANICAL STABILITY OF STRUCTURAL ELEMENTS

Mechanoenzymes or mechanoactivated substrates must be mechanically stable to withstand thermal fluctuation and random forces created by the environment in order to avoid noise in signaling. At the same time they must be reliably activated at a certain force. As the structure of a protein consists of structural elements, the mechanical properties are determined by mechanical elements, also referred to as unfoldons. Because force transmission through a protein is complex and dynamic, there is no unambiguous connection between structural and mechanical elements. However, it is fair to say that common structural elements that determine the mechanical stability of proteins (13, 17) also depend to a certain degree on the context such as the hydrophobic core (77) and the direction of force application (8, 12, 21). Theoretical work on how rupture forces depend on the loading rate and other parameters and how free energies of the barriers in the unfolding pathway may be obtained can be found elsewhere (22, 25, 45).

Random Coils

Random coils are flexible polypeptide chains that do not form a static structure. They connect structural elements within a protein and protein domains within a complex, or they form extended entropic springs, e.g., in the muscle protein titin (66, 92), providing it with its passive elasticity. The enthalpic parts of their interaction are small, such that their mechanical properties are dominated by entropic forces in the lower force regime. This results in the highly nonlinear force-extension profiles of unfolded proteins (**Figure 2**). Enthalpic contributions come into play only at higher forces where bond angles of the backbone are distorted and stretched (44, 67). Traditionally, force-extension curves are fitted with models that describe this polymer elasticity in order to determine the characteristic contour lengths or increments between barriers in the unfolding pathway. We developed a method to directly transform each data point of a

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force-extension trace into contour-length space without loss of information (84). Barrier positions are now represented in terms of the molecular coordinate contour length, allowing direct comparison of proteins by their unfolding pattern and automated recognition of molecular fingerprints.

Helical Elements

Studies of isolated helical structures and of helical structures in the context of larger proteins revealed that they form mechanically weaker elements. A common motif is the coiled-coil, in which two α-helices with a repetitive sequence of seven amino acids wrap around each other

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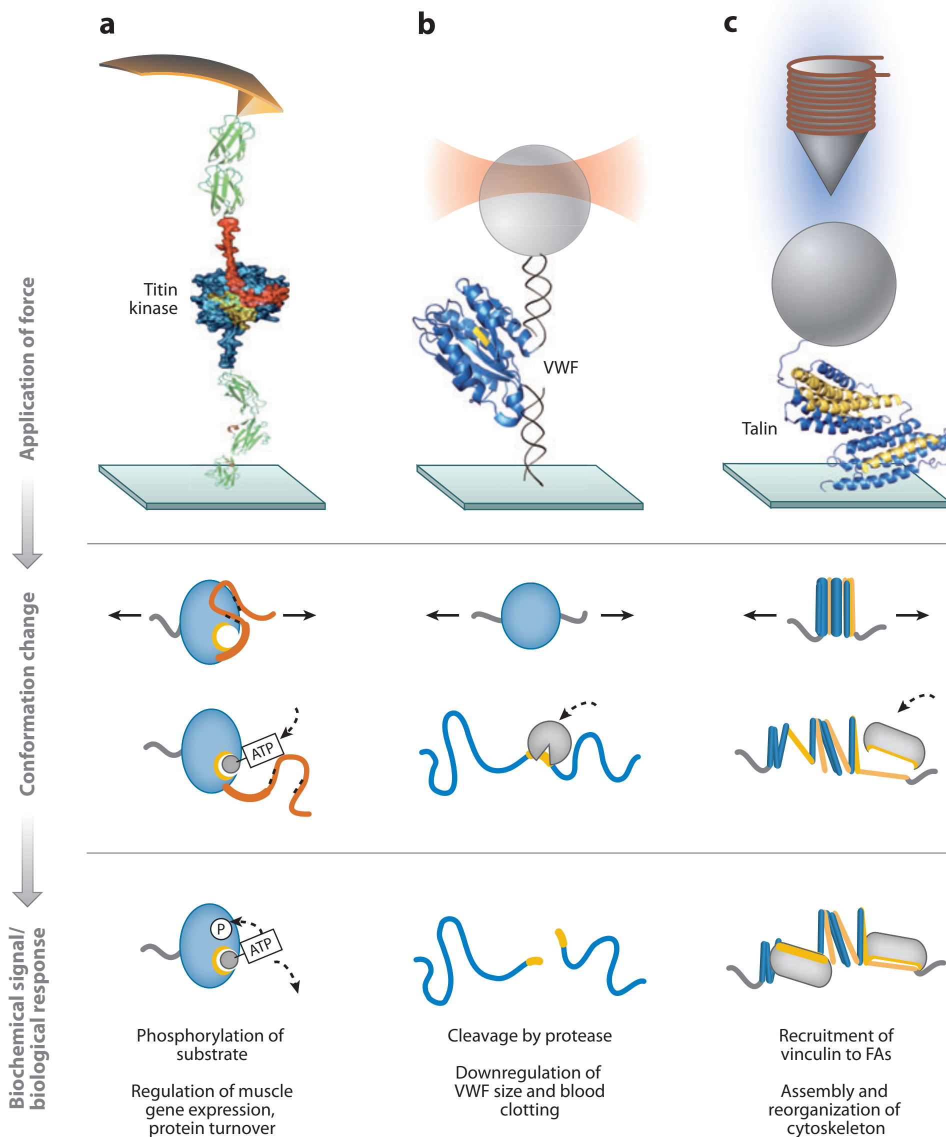

along their hydrophobic interface. If stretched in the so-called unzip geometry, the helices are sequentially separated (see **Figure 2**) and a force plateau between 9 and 15 pN is observed (6). In the more shear-like geometry of spectrin repeats consisting of three helices, cooperative unfolding with forces between 27 and 37 pN at 800 nm s^{-1} is measured (90). In the context of larger proteins, helical elements may appear in mixed geometries and act cooperatively. The T4 lysozyme, for instance, consists mostly of α-helical structures and unfolds at 50 pN at 400 nm s^{-1} (82).

AFM: atomic force microscopy

β-Sandwich Elements

The strongest and most extensively studied mechanical element is the β-sandwich topology. It consists of two or more β-sheets with strong hydrogen bonding loaded in shear geometry (see **Figure 2**). The immunoglobulin (Ig) and fibronectin (Fn) domains of titin, for instance, have been the first proteins investigated with atomic force microscopy (AFM)-based SMFS (88) and laser tweezers (56), and their mechanical stability shows a wide range. Unfolding and refolding of different titin domains has led to a detailed mechanical understanding (see Reference 65 for a review). In addition to Fn, whose domains unfold between 75 and 220 pN at 600 nm s^{-1} (78), many other β-sandwich polyproteins exhibit a similar range of unfolding forces (9, 12, 52, 105). The most stable domain reported so far is the cohesin 1 module c7A of scaffoldins, which unfolds at 480 pN at 400 nm s^{-1} (99). Valuable information is also gained by measuring the mechanical stability of engineered folds and domains (64). Shuffling different parts of the strong I32 Ig domain (300 pN at 400 nm s^{-1}) with the 100 pN weaker I27 domain, for instance, results in intermediate and lower stabilities ranging from 150 to 250 pN at 400 nm s^{-1} (2). Also, in the case of β-sheet topologies, the pulling geometry is crucial as shown for the protein E2lip3, which exhibits lower unfolding forces when its β-sheet structure is loaded in a zipper-like fashion (8).

Whereas all bonds above show as expected a reduced lifetime under force, an interesting class of receptor-ligand pairs called catch bonds exhibit the opposite behavior (see References 97 and 98 for a review). The examples discussed show that proteins contain a variety of structural elements that span a wide range of mechanical stability. Integrated into mechanoenzymatic processes, they fine-tune the respective physiological forces and the overall mechanical architecture that defines mechanical activation pathways as discussed below.

Figure 1

Mechanoenzymatic processes studied with SMFS. Mechanoenzymes or substrates respond to forces by conformational changes. In their force-induced active state, binding or recognition sites (*yellow*) become accessible. Subsequent conversion of force to a biochemical signal regulates a biological response. (*a*) The autoinhibited enzyme titin kinase was investigated with AFM-based SMFS (83). Upon application of force through a cantilever, the induced conformational changes are observed in force-extension traces. After two barriers, the hidden ATP binding site becomes accessible. Relief of autoinhibition results in autophosphorylation and phosphorylation of downstream substrates regulating muscle gene expression and protein turnover (PDB accession codes: 1TNM, 1TKI, 2NZI). (*b*) Mechanoenzymatic cleavage of the A2 domain of VWF was investigated with an optical trap (109). Attached to the bead through DNA handles, complete unfolding of the A2 domain over one major barrier with an intermediate and subsequent cleavage at a hidden site by a protease was investigated. The shear-dependent size control of VWF is important for regulating blood clotting (PDB accession code: 3GXB). (*c*) Force-induced binding of vinculin to hidden binding sites of talin was observed with a combination of magnetic tweezers and single-molecule fluorescence (19). After stretching talin, more fluorescently labeled vinculin was bound. Recruitment of vinculin is thought to strengthen focal adhesions and is involved in assembly and reorganization of the cytoskeleton (PDB accession code: 1XWX). Dimensions of proteins and force probes are schematic and not drawn to scale. Abbreviations: AFM, atomic force microscopy; FA, focal adhesion; SMFS, single-molecule force spectroscopy; VWF, von Willebrand factor.

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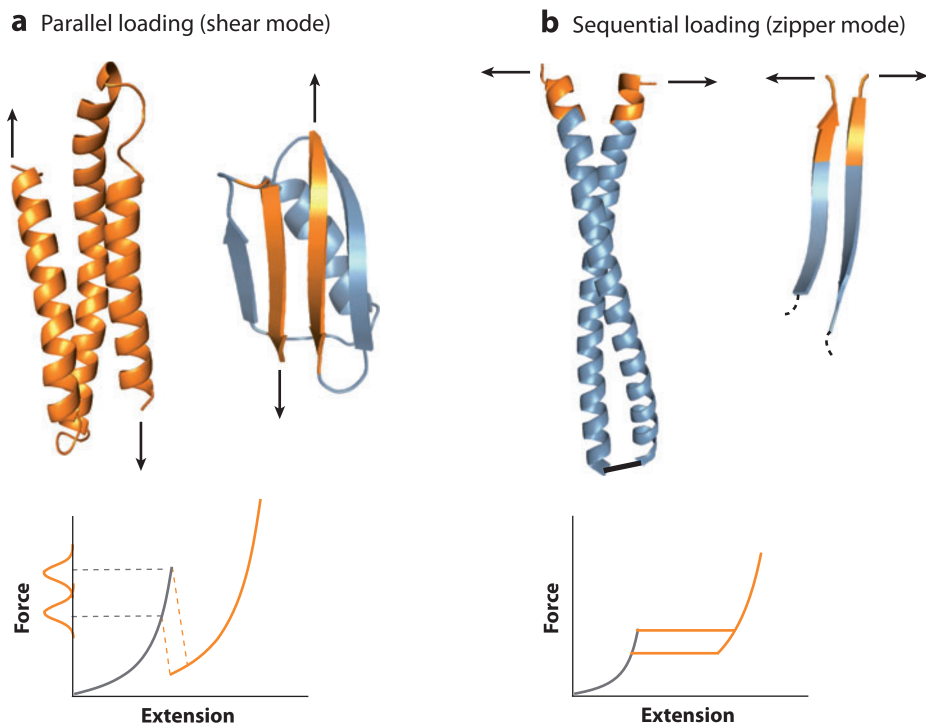

Figure 2

Mechanically stable structural elements in different pulling geometries. (*a*) When loaded in parallel (*orange*), α-helical or β-sheet structures such as spectrin (PDB accession code: 1AJ3) or GB1 (PDB accession code: 1PGA) unfold in an all-or-none fashion because all interactions break simultaneously. After the rupture, the unfolded polypeptide chain behaves as a random coil and shows the characteristic stretching behavior of an entropic spring. Different mechanical strengths result in different rupture forces and extensions. (*b*) If mechanical elements are loaded in a zipper-like fashion, the molecular interactions break sequentially (*orange*), which results in a force plateau as in the case of the GCN4-like leucine zipper (PDB accession code: 1GK6). The forces in this pulling geometry are typically much lower, because the forces are applied over a longer distance and may exhibit variations. Force-extension traces are schematic illustrations. Abbreviations: GCN4, General Control Nonderepressible 4.

MECHANISMS OF FORCE-SENSING AND THE DIFFERENT WAYS OF CONFORMATIONAL CHANGES

The mechanical elements of a protein act together in a mechanoenzymatic process and define the mechanical response, leading to force-induced conformational changes and the exposure of recognition sites. But how exactly do mechanical activation pathways follow the energy landscape, what are the specific mechanisms of converting force to a biochemical signal, and how can this be measured on a single-molecule level? In this section we discuss the three examples of **Figure 1**, which have been investigated by three major techniques: AFM-, optical tweezer–, and magnetic tweezer–based SMFS. These examples have been compared to MD simulations to gain deeper structural insights into and explanations of the conformational changes, and there is strong support that the mechanoenzymatic mechanisms are relevant in vivo. Furthermore, they highlight the diversity of mechanical activation pathways from partial conformational changes to complete

unfolding and of the subsequent biochemical reactions such as covalent modifications, cleavage, and exposure of co-catalytic or binding sites.

The Partial Unfolding Mechanism of Titin Kinase

The kinase domain of the muscle protein titin is embedded in the M-band of the sarcomere at an ideal position to sense forces arising from shear motions of the connected myosin motor filaments. More detailed reviews on titin and its role in mechanosensing and signaling can be found elsewhere (30, 31, 57, 101). The geometry is such that titin kinase (TK) with its surrounding Ig and Fn domains of the M-band titin is stretched between its N and C termini. Structural and biochemical investigations have shown that an autoinhibiting helix blocks the ATP binding site and that TK does not show catalytic activity in its native conformation (69). Truncation of the autoinhibiting element leads to subsequent autophosphorylation of Tyr-170 and an active form of TK that phosphorylates downstream elements controlling muscle gene expression and protein turnover (61). MD simulations show that the autoinhibiting element may be removed and unfolded by external forces such that the ATP binding site becomes accessible while the remaining structure stays intact (35, 83). Puchner et al. (83) investigated in great detail the mechanoenzymatics of TK by SMFS, MD simulations, and enzymatics (see **Figure 1*a***). Valuable information about the mechanical architecture and its force-induced activation mechanism was gained in the natural context of the surrounding protein domains. TK shows a well-ordered and reproducible unfolding pattern with seven distinct structural states (**Figure 3*a***) when stretched along its natural reaction coordinate, which highlights a unique and elaborate mechanical architecture. The mechanism of this pronounced order is addressed below. MD simulations allowed us to assign the structural transitions such as opening of the ATP binding site after the second experimentally observed barrier in the activation pathway. An additional force peak after this barrier shows ATP sensitivity at physiological concentrations with a confidence of 95% (83) (**Figure 3*a***). Furthermore, the appearance of this peak strongly anticorrelates with the pulling speed, which reflects different opening times of the ATP binding site. Together with the fact that MD simulations observe an additional interaction with ATP at this state, we concluded that the ATP- and speed-dependent peak reflects binding of ATP to the mechanically opened binding pocket. However, it cannot completely be ruled out from these experiments whether indirect or downstream effects of ATP binding such as autophosphorylation play an additional role. The measured activation forces of TK are below 50 pN at physiological speeds (mean forces 30–40 pN at 720 $\mu m\ s^{-1}$) and are well tuned to the stability of surrounding mechanical elements: Although forces already corresponding to an imbalance of 4–8 myosin motors may activate TK, the mechanically more stable surrounding Ig and Fn domains stay intact and serve as a mechanical scaffold.

In general it is not easy to elucidate in which state along an activation pathway the activity of mechanoenzymatic processes is actually reached—especially if the readout of the biochemical or binding reaction cannot be synchronized with the recorded force-extension trace by an independent signal such as fluorescence. Therefore, we developed a mechanical pump-and-probe protocol (86), analogous to laser spectroscopy or NMR, that exploits the conformational control over a protein by its end-to-end distance and the fact that the mechanoactivated reaction is triggered as soon as the active state is reached. The longer the state is kept active, the more likely the reaction will occur. In the first part of the protocol, the protein is brought at high speed to a certain conformational state, which is determined by the extension (see **Figure 3**). This state is then "pumped" by keeping the extension fixed for a certain time, allowing the conformation-sensitive reaction to occur. In the last part of the protocol, the protein is now completely unfolded at high speed and it is read out (i.e., probed) whether the reaction occurred or not. By varying the extension through

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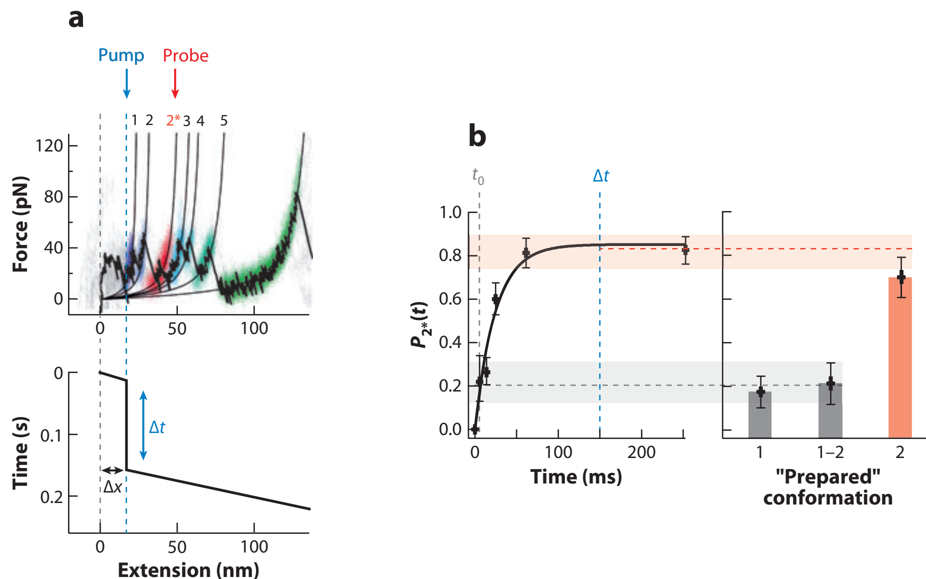

Figure 3

Identification of the active state by mechanical pump and probe. (*a*) Titin kinase (TK) is unfolded in the presence of ATP at high speed to a conformation determined by the extension Δx (*blue dashed line*). This extension is clamped for the time Δt, allowing a conformation-specific reaction to occur (here binding of ATP). TK is then further unfolded and it is "probed" if peak 2* (*red*) is present or not. The black pump-and-probe trace is plotted on top of colored force-extension traces depicted as semitransparent dots. (*b*) Histograms of 2* probabilities (*right*) in the different states show that only after barrier 2 the peak 2* occurs, with the saturation probability observed in Reference 83 at low pulling speeds or long opening times of the ATP binding pocket (*left*). In the preceding conformations only low values corresponding to the finite pulling speed are observed. Data adapted from Reference 86 with permission.

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the relevant range, all conformational states can be tested for activity, and only in the active state does the reaction occur with increased probability. In the case of TK, the readout is the appearance of the ATP-sensitive barrier 2*, but it can in principle be any other signal such as fluorescence or binding of reporters. By applying this protocol to TK, we found that the occurrence of barrier 2* reaches only its high equilibrium value when the conformational state after the second barrier is pumped. At all other preceding states, low probabilities of barrier 2* are observed, reflecting the finite pulling velocity (see **Figure 3*b***). Thus, the mechanically activated conformation modulating the occurrence of barrier 2* is reached after the second barrier in the unfolding pathway of TK. These results are consistent with the previously discussed model and the results from MD simulations, i.e., that the binding pocket of TK is mechanically opened at this conformation, leading to binding and interaction of ATP (35, 83). Because various studies have used AFM-based SMFS to detect ligand binding (4, 11, 53, 55, 76), the pump-and-probe protocol, which we have developed, may be useful in the future for investigating the multitude of other mechanoenzymatic processes.

The results from single-molecule experiments, simulations, and enzymatics discussed above reveal that the mechanoenzymatic mechanism of TK is based on a partial unfolding over two barriers with exposure of the ATP binding site. The remaining binding site and the catalytic lobe stay folded. In another study, SMFS experiments and MD simulations suggest that TTN-1

and twitchin kinase of *Caenorhabditis elegans* meet mechanical and structural requirements of a mechanoenzyme (36). It remains an intriguing goal to elucidate whether these and other similar cytoskeletal kinases (30) exhibit mechanoenzymatics like that of TK.

VWF: von Willebrand factor

FA: focal adhesion

Complete Unfolding of the von Willebrand factor A2 Domain

Whereas partial unfolding with intact catalytic or binding sites requires a sophisticated mechanical architecture, a complete unfolding with exposure of otherwise hidden peptide sequences serving as recognition sites is a simpler but nevertheless efficient mechanism for mechanosensing. A well-characterized example for such a process is the force-induced cleavage of the von Willebrand factor (VWF), a key player of shear sensing in hemostasis. Multimerized by specific disulfide bonds, the ultra large VWF (ULVWF) is secreted at sites of injury. Hydrodynamic forces in shear flow are thought to extend ULVWF bound to vessel walls and in solution. An important mechanism is the downregulation of the hemostatic potential that correlates with the length of ULVWF. According to a current model, size control is achieved by a shear-force-induced exposure of a recognition site hidden in the A2 domain (108) and by subsequent enzymatic cleavage by the protease ADAMTS13 (20, 91). This process was investigated in great detail with optical traps (109) (see **Figure 1*b***) and MD simulations (3). The A2 domain was specifically attached to DNA handles and repetitively unfolded and refolded. The A2 domain unfolds between 7 and 14 pN in the loading rate regime between 0.35 and 350 pN s^{-1}. A short-lived, partially unfolded state with 20% probability is observed. The measured contour-length increment corresponds well to the number of folded amino acids, indicating complete unfolding and providing, together with the unfolding forces, a clear fingerprint that one single domain is tethered and probed. The mechanoenzymatic cleavage by ADAMTS13 is observed by the following protocol: First, A2 is completely unfolded and then relaxed to a force of 5 pN. The lifetime of the unfolded state at 5 pN is larger than 140 s, which makes refolding unlikely during the timescale of the experiment. In this state, the time is measured until cleavage occurs and the force drops to zero. The revealed reaction kinetics and experiments at different enzyme concentrations show that the rupture process is enzyme specific. The fact that no cleavage is observed at 5 pN in the folded state is proof of its mechanoenzymatic nature. Further, the A2 domain unfolds in the context of its surrounding A1 and A3 domains (107).

Binding of Vinculin to Force-Induced Conformations of Talin

Talin and vinculin are important components of focal adhesions (FAs) (18). The talin rod mechanically couples integrins to the cytoskeleton and is involved in the mechanical connection between the inside and outside of the cell. Upon application of force, vinculin is recruited to FAs and strengthens them (29). Interestingly, talin contains vinculin binding sites (33) buried by hydrophobic interactions of the helix bundles. The force-induced structural transitions of the fragment H1-H12 containing five vinculin binding sites were investigated by MD simulations in two different geometries (46). When stretched at their N and C termini, the first and last helices unravel turn by turn. The breakdown of the remaining helix bundle into three fragments is followed by independent and complete unfolding. In the other geometry a more physiological binding interface is mimicked by distributing the tensile force along the whole N- and C-terminal helices instead of applying it to the N- and C-terminal atoms. Here, the breakdown of the talin rod into two fragments requires higher forces and is followed by fragmentation into three parts until complete unfolding occurs. During this process the five vinculin binding sites become sequentially

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exposed, which could lead to binding of vinculin. Complete unfolding of the helices might prevent binding, but it is suggested that binding to still-intact interfaces could stabilize the helices.

This force-induced process was investigated in an innovative sequential combination of magnetic tweezers and single-molecule fluorescence, which allows parallel exertion of force and readout of binding (19) (see **Figure 1*c***). The talin rod was attached by its N and C termini through a histidine and biotin tag to the surface and to the magnetic beads while forces of 2 and 12 pN were applied for 1 h. During that time, fluorescently labeled vinculin was allowed to bind. This method cannot explicitly rule out multiple attachments per bead or give information about the unfolding state because no time-extension traces have been recorded, but the subsequent single-molecule fluorescence readout gives a sharp and self-consistent picture. When no force is applied, bleaching of only one molecule is observed, and in the control experiment with a dimeric talin, bleaching of two molecules is observed. When 12 pN is applied, up to three molecules are distributed and in the case of the dimer, up to six. In the initial state, one of the five vinculin binding sites is already exposed, and application of force exposes two more intact binding sites. Why only three of the five expected binding sites are accessible and intact remains to be elucidated. On the one hand, the extrapolated unfolding rates from force-clamp experiments suggest that each talin molecule per bead should be completely unfolded so that some binding sites may be unfolded and no longer functional. On the other hand, high refolding rates at these low forces could result in limited access to some of the binding sites.

Similar to TK, the talin fragment shows a regular unfolding pattern with clearly distinct conformational states in force-extension traces. Together with the investigated force-induced exposure of binding sites, this finding makes talin a promising protein for further mechanistic investigations at the single-molecule level to find general mechanical design principles of mechanoenzymatic processes. Interesting questions for further exploration include whether binding of vinculin may be mechanically detected, in which states binding sites become exposed and become functional, and whether the sequence of unfolding events is topologically determined as in the case of TK.

Despite the diversity of the discussed mechanisms, the mechanoenzymatic processes share the basic property that the active state is hidden behind one or more barriers in the activation pathway. This principle can also be exploited to probe other enzymatic reactions under force such as disulfide-bond reduction by thioredoxin (104). It is enlightening for chemical or enzymatically catalyzed reactions to track the different states of a reaction pathway, and the same is true for mechanical activation pathways. How the active state is reached is indicative of the specificity of an activation pathway as we discuss in the following section.

MECHANICAL ARCHITECTURES AND SPECIFIC ACTIVATION PATHWAYS

From the examples of mechanoenzymatic processes discussed above, it is obvious that structural elements with a range of mechanical stability create barriers in the activation pathway that are overcome until the active state is reached. The way the active state is reached is determined by the mechanical architecture, i.e., the arrangement and connection of mechanical elements. The architecture determines whether mechanical activation pathways or parts thereof are disordered, offer alternative pathways, open up in nondeterministic ways, or are strictly ordered with a successive sequence of unfolding events. Whereas some proteins and enzymes with no mechanical function possess different unfolding pathways in parallel with a disordered sequence of events such as green fluorescent protein (70) or T4 lysozyme (82), an ordered sequential pathway may be of high value for mechanoenzymatic processes. If the biochemical events that follow force-induced activation

require structurally intact catalytic or binding sites, a specific mechanical activation pathway may even be required for proper function.

Two general mechanisms exist in such a pathway that cause two barriers to always be overcome in the same order. In the first scenario the two corresponding mechanical elements are loaded in parallel, with one element mechanically much more stable than the other. This case of mechanical hierarchy was observed most obviously for members from the large family of multidomain proteins such as titin (89) or fibronectin (78), which consist of independently unfolding domains of different strength (see **Figure 4*a***). For a protein with several internal barriers in the unfolding pathway, the situation is not obvious anymore. Here, two barriers may be overcome in sequence because of mechanical hierarchy or structural topology, meaning that the mechanical elements are not connected in parallel but in series and shield each other. Such a case was artificially engineered (81) by inserting the mechanically labile T4 lysozyme (T4L) into a loop of the strong β-sandwich protein GL5 (see **Figure 4*a***). T4L unfolds at about 50 pN, whereas GL5 unfolds at 130 pN at a pulling speed of 400 nm s^{-1}. Therefore, when GL5 domains are fused to the N and C termini of T4L, the unfolding shows clear mechanical hierarchy in which T4L unfolds first followed by the stronger GL5 domains. However, if T4L is fused into a loop of GL5, it is shielded by the force-bearing β-sheet of GL5 and not subjected to force as long as the β-sheet of GL5 stays folded. Consequently, the stronger GL5 domain unfolds before the weaker T4L. This approach of engineering mechanical architectures is promising for the design of other novel functionalities such as artificial force sensors.

How can one discriminate the two types of mechanical architecture? In the case of the T4L-GL5 insertion it would be obvious because the first unfolding event shows much higher forces. But if sequential force peaks have comparable heights or exhibit even increasing mechanical stability, a discrimination based on force-extension traces becomes difficult or even impossible (**Figure 4*b***). The key to distinguishing between the two arrangements is through the unfolding kinetics under constant force (96) using single-molecule force-clamp spectroscopy (79). If several barriers are loaded in parallel, then the unfolding times for each barrier are distributed exponentially. However, if mechanical elements are connected in series and shield each other, the differential equations describing unfolding become coupled and the unfolding times follow a peaked distribution.

Both cases were demonstrated for TK with its neighboring Fn domain (96). The Fn3 domain mostly unfolds after TK but exhibits an exponential distribution of unfolding times, which demonstrates that it is loaded in parallel to TK. For TK itself, the unfolding time of the last state shows a clearly peaked distribution, proving that mechanical elements shield each other (**Figure 4*b***). These experiments reveal that the remarkable mechanical architecture and the specific activation pathway of TK are based on structural topology. This property is of high relevance for proper function: Although two barriers have to be overcome by physiological forces to open the ATP binding site, the binding site itself and the catalytic core are shielded to assure a functionally intact structure. Shielding the active state behind two sequential barriers might even be a sophisticated mechanism to suppress noise in signaling and allow for activation at relatively low forces. Each of the two initial barriers with corresponding free energy has a certain probability to be overcome by thermal fluctuations and forces. Splitting the total change in free energy between these two barriers instead of keeping it in one maintains the total change in free energy, and thus the probability of random activation, while allowing for lower activation forces.

This strategy is widely applicable and may prove to be helpful for the investigation of the mechanical architecture of other mechanoenzymatic processes. It remains to be elucidated whether structural topology is a more general mechanism leading to specific activation of mechanosensing processes.

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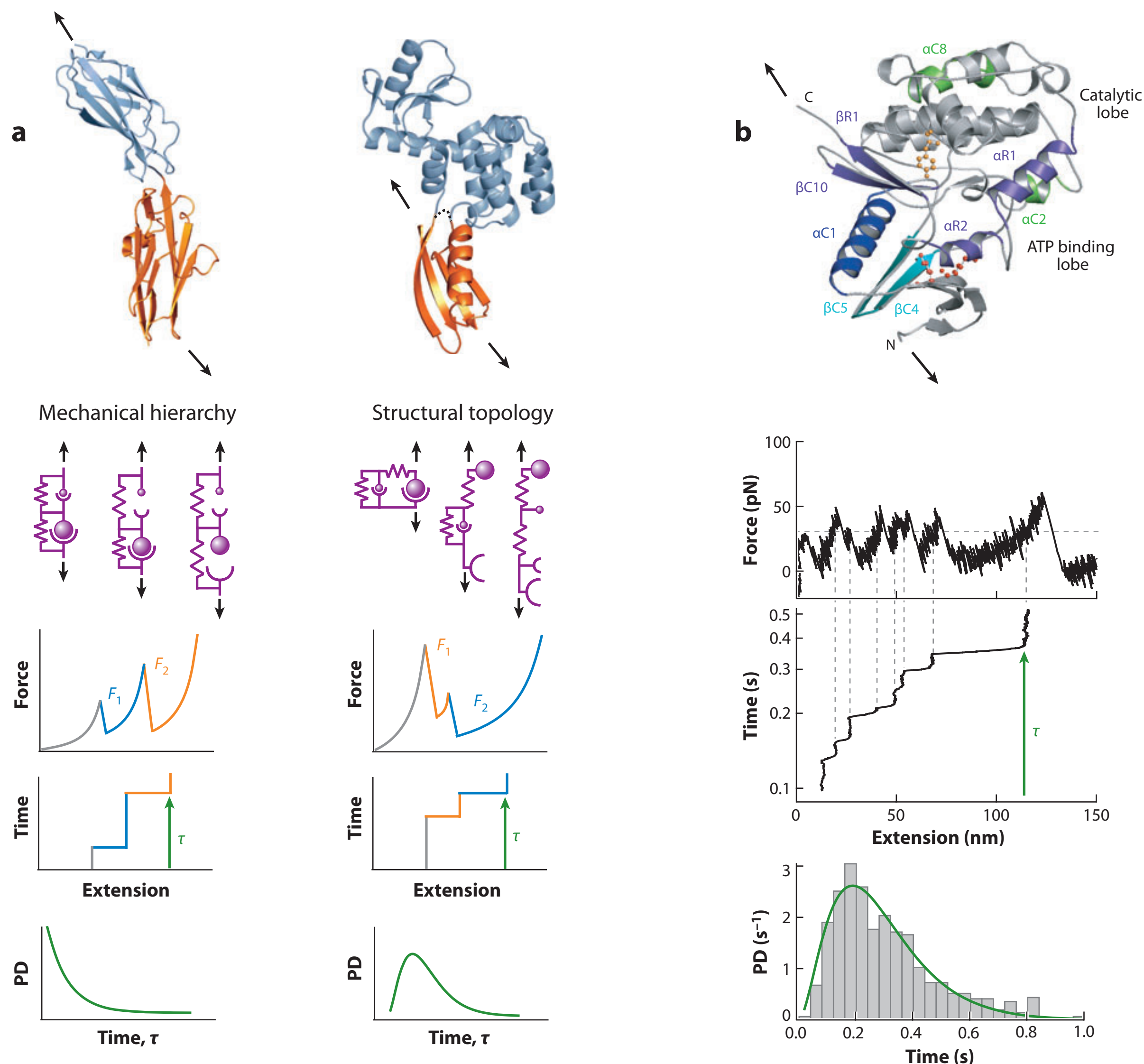

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Figure 4

Mechanical architectures of multibarrier proteins. (*a*, *left*) If two elements such as a FnIII and an Ig domain (PDB accession code: 2NZI) are connected in parallel, force acts on both simultaneously so that a weaker element unfolds before the stronger element (highlighted blue and orange in the ribbon structure and as a small ball and large ball in the mechanical diagram). In force-extension traces the different unfolding forces would be measured, whereas in a force-clamp trace the extension would show steps in time. The lifetime τ of both elements (the element with the larger step is in orange) shows an exponential distribution. (*a*, *right*) Connection of two mechanical elements in series depicted as the synthetic construct (81) in which the weak T4 lysozyme (*blue*; PDB accession code: 1L63) was inserted into a loop of the stronger GL5 domain (*orange*; PDB accession code: 1PGA). The unfolding sequence is topologically predetermined even if the first unfolding event shows higher forces or longer lifetimes. Because unfolding of the second element depends on the first one, its lifetime τ would follow a peaked distribution. (*b*) In the case of a complex protein with many barriers such as titin kinase (PDB accession code: 1TKI), it is neither obvious from the structure nor directly evident from force-extension traces how mechanical elements are arranged and shielded. However, from the unfolding kinetics under constant force it was shown that the unfolding time of the last and largest increment in force-extension traces, corresponding to the largest step size in a force-clamp trace (correspondence shown as gray dashed lines), follows a peaked distribution. Data adapted from Reference 96. Abbreviation: PD, probability density.

COMBINATION OF SINGLE-MOLECULE FORCE SPECTROSCOPY AND SINGLE-MOLECULE FLUORESCENCE

Single-molecule fluorescence microscopy is a versatile and complementary approach to investigate biomolecular processes (28, 71, 94). The developments of zero-mode waveguides (63) reviewed by Zhu & Craighead (110), for instance, expanded applications by confining the excitation volume to a zeptoliter regime, allowing enzymes with labeled substrates to be investigated in the micromolar range. In the field of single-molecule enzymology, the measurement of single turnover rates of fluorogenic substrates led to interesting discoveries, such as static or dynamic disorder, that are hidden in ensemble experiments (24, 26, 41, 68, 100). These phenomena are attributed to either static conformational differences of single enzymes or dynamic conversions between states with different rates. Because it can directly manipulate the conformation of enzymes, SMFS is thus an ideal technique to be combined with single-molecule fluorescence. Such combinations with atomic force microscopes (39, 40, 80) or optical traps (49, 60) have led to a broad range of applications ranging from investigations of molecular processes (42) to the pick-up and assembly of single DNA oligomers into highly precise supramolecular complexes (58). The ability to arrange functional units such as nanoparticles (87) in this defined manner will be useful for studying the interplay and synergies of enzymatic cascades. These combinations are promising, especially in the field of mechanoenzymatics, as fluorescence as an independent readout for binding or activity may be correlated with force-induced conformational changes.

TIRF: total internal reflection fluorescence

We recently demonstrated this approach with the *Candida antarctica* lipase B (CalB) (38). The enzyme was covalently and specifically immobilized at low concentrations to a coverslip to observe the activity of spatially well-separated single enzymes through the fluorogenic substrate CFDA (carboxyfluorescein diacetate) in TIRF (total internal reflection fluorescence) excitation (see **Figure 5**). The conformation of CalB was not directly manipulated with the tip of the AFM cantilever but instead with an attached antibody-coated agarose bead, which selectively binds the GCN4 peptide fused to the N terminus of CalB. Owing to parallel force-exertion, no single-molecule force-extension traces can be recorded; the antibody-peptide interaction acting as a mechanical fuse was characterized instead by AFM-based SMFS. In one pulling cycle, the force acting on each enzyme increases until the antibody-peptide interaction yields at approximately 60 pN and is free for the next cycle. The correlation of averaged fluorescence traces from many pulling cycles shows that enzymatic activity begins with low values directly after the release of force, rises to a peak after 1.7 s, and drops again to the low basal value. Because CalB fluctuates between active and inactive conformations in the absence of force, the rise of activity after release of force is interpreted as a multistep relaxation of CalB from a force-inactivated conformation over the active conformation until the equilibrium distribution is reached again. Modeling this process gives a total number of nine steps, with a lower limit for the total free energy change ΔG of 8 $k_B T$.

The combination of SMFS with single-molecule fluorescence is a powerful tool to investigate enzymes in general and will be of great importance for future mechanoenzymatic studies.

ENSEMBLE AND IN VIVO FORCE MEASUREMENTS

Single-molecule experiments allow mechanoenzymatic processes to be investigated at a fundamental and detailed level and reveal the mechanisms of force activation. These experiments are performed in vitro with isolated and known components. It is advantageous to investigate such a minimal mechanoenzymatic set and to map conformational transitions along the activation pathway. However, given the complexity of cellular signaling networks and the dynamic nature of cell

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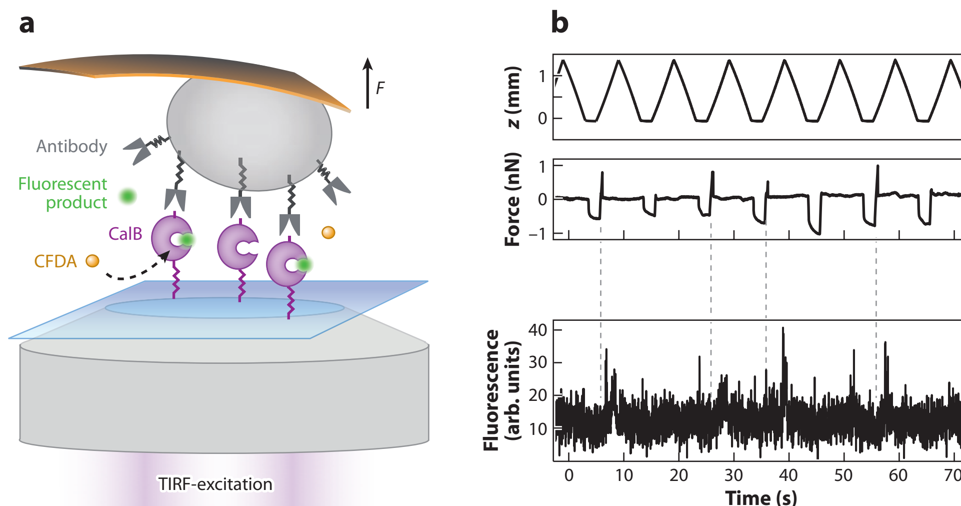

Figure 5

Combination of AFM-based force spectroscopy and single-molecule fluorescence. (*a*) CalB (*purple*) is specifically and covalently immobilized to a coverslip, which is mounted on a TIRF microscope. Enzymatic activity is observed by turnover of the fluorogenic substrate CFDA. An agarose bead is attached to the cantilever of the atomic force microscope and coated with antibody fragments against the N-terminal GCN4 peptide fused to CalB. Once in contact with the surface, the antibodies bind to CalB, and upon retraction the force rises until the interactions rupture. (*b*) Force-time traces show rupture forces up to several hundreds of picoNewtons, indicating multiple parallel interactions (*upper traces*). By correlating rupture events with the single-molecule fluorescence of individual CalB enzymes (*lower trace*), the impact of force on enzymatic activity may be investigated. Data adapted from Reference 38. Abbreviations: AFM, atomic force microscopy; CalB, *Candida antarctica* lipase B; CFDA, carboxyfluorescein diacetate; TIRF, total internal reflection fluorescence.

morphology, it is equally important to study in vivo the action and effects of mechanoenzymatic processes. Especially in the field of cell adhesion, in which cells probe their mechanical environment by exerting, transducing, and sensing force, many different components and mechanisms that are involved have been identified. A detailed discussion of the field may be found elsewhere (14, 32, 47, 48, 73, 102, 103). However, we want to highlight some examples in which forces directly result in a biochemical response due to conformational changes and in which further SMFS experiments are promising to gain deeper insight into the mechanical mechanisms.

Common approaches to exert forces on cells or to measure the created forces include stretching cells on elastic substrates and measuring their distortion. Typical readouts are biochemical analysis of the cell components or optical detection, which allows real-time observation with spatial resolution across the cell (see **Figure 6**).

In a recent study the force activation of the c-Src kinase substrate Cas was demonstrated in vivo and in vitro (93). Having two flanking domains that are both involved in recruitment to FA, Cas could be extended by stretch and in this way act as a mechanosensor that activates the small GTPase Rap1. Cas phosphorylation increases significantly upon cell stretching without changing the activity of the kinase, suggesting that forces induce a conformational change of Cas itself. By immobilizing purified Cas either on both termini or as a control on one terminus on a latex membrane, it was further demonstrated that stretching Cas indeed increases phosphorylation proportional to the membrane stretch. Complementation of split yellow fluorescent protein that becomes nonfluorescent when separated through stretch but gains fluorescence again

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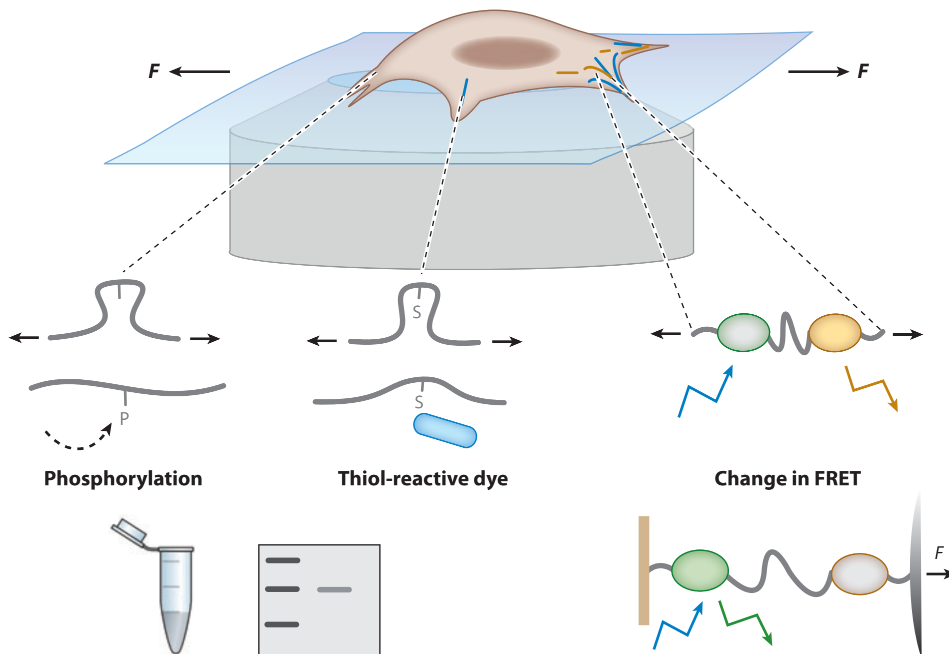

Figure 6

In vivo investigation of mechanoenzymatic processes. Cells are either stretched on flexible substrates or the created forces are observed through distortion of the substrate. The subsequent force-induced reactions such as phosphorylation can be revealed by biochemical analysis. Fluorescence microscopy techniques in combination with FRET allow forces to be measured in real time and in live cells. Abbreviation: FRET, Förster resonance energy transfer.

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when complementing halves are allowed to bind proved the extension of the Cas domain upon stretching. Together these experiments suggest that a force-induced conformational change extends Cas in vivo and primes it for phosphorylation and subsequent activation of downstream processes. Because the molecular mechanisms of force activation remain unknown, it would be beneficial to investigate this mechanoenzymatic process with SMFS to address questions regarding mechanical stability and architecture and force-induced conformational changes along the mechanical activation pathway.

To gain more proteome-wide information about force-induced conformational changes, a cysteine-shotgun approach was developed to detect cysteines that are buried in tertiary or quaternary structures and become exposed upon application of forces on whole cells (51). Cells are loaded with Cys-reactive dyes and stretched in shear flow. A sequential staining with distinguishable dyes before and after stretching allows a differential detection of cysteines that become exposed. Analysis of the cells by fluorescence imaging and biochemical analysis of the samples by SDS-PAGE, chromatography, and mass spectrometry reveal increased labeling with stretch and time and allow the corresponding proteins to be identified. This method is a complementary approach to screen force-bearing proteins for further mechanistic investigations or to demonstrate that proteins investigated in vitro actually unfold in vivo.

In addition to the possibility of observing binding events, optical methods allow distances between specifically labeled components to be measured within a cell. If a fluorescent reporter pair such as two green fluorescent proteins, which exhibit a distance-dependent spectral shift (50),

FRET: Förster resonance energy transfer

or a Förster resonance energy transfer (FRET) pair are connected by a flexible element, changes in distance correspond to changes in force. In general, such measurements require extensive and well-designed control experiments to discriminate between inter- and intramolecular distance changes. Grashoff et al. (34) designed and calibrated such a FRET force sensor at the single-molecule level with optical tweezers. In this way, average forces acting on vinculin could be monitored in vivo. Together with careful controls, these experiments reveal that the highest forces are associated with assembly and growth of FAs. Experiments with a different FRET geometry that reports on a conformational change of vinculin to an actin binding state (15) show that the stabilization of FA goes along with recruitment of vinculin and transmission of force across vinculin but that these two processes are independently controlled.

The discussed examples and approaches show the richness and diversity of possibilities for in vivo experiments to investigate mechanoenzymatic processes and force-induced conformational changes. Up to now, they complement single-molecule studies or help screen for proteins that are subjected to force.

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OUTLOOK

The development of AFM and other SMFS techniques led to a systematic and fundamental understanding of the mechanical properties of proteins. This knowledge, together with further technical developments and in combination with single-molecule fluorescence techniques, paved the way to answer more complex questions on how force can be translated into biochemical signals through conformational changes. Different mechanoenzymatic mechanisms and the underlying mechanical architectures allowing such specific responses have been elucidated in great detail, and comparisons with MD simulations have given an atomistic picture about the structural transitions involved. Approaches to study mechanoenzymatic processes in vivo by optical and biochemical methods put this mechanistic understanding into the larger and more complex context of cellular signaling. The adventure of discoveries in this field is only beginning, and many processes remain to be investigated with existing techniques. However, it would be ideal if future approaches could overcome current experimental limitations. On the one hand, SMFS probes are large and cannot be brought into living cells. On the other hand, current optical techniques to measure forces are ensemble measurements that average out the properties of single molecules.

An intriguing future goal would be to merge single-molecule and in vivo techniques (23) so that mechanoenzymatic processes could be studied at the single-molecule level in a living cell. The advances in the field of single-molecule super-resolution imaging (5, 43, 95) are promising for future combinations, for example, with FRET. Individual FRET pairs could be read out by stochastically switching donors in the fluorescent state. By learning from nature, other approaches could exploit force-induced conformational changes that expose binding sites and activate reporter molecules compatible with single-molecule readout.

Finally, we believe that investigated mechanoenzymatic mechanisms could become a useful tool for synthetic biology (106). Force-sensing signaling pathways in cells could be rewired or hooked to artificial inputs to create a designed biological response. Given the importance of cellular mechanics for cell differentiation (16) or its role in diseases, such strategies could become helpful to biomedical research.

SUMMARY POINTS

1. Forces play a pivotal role at the cellular and molecular levels and are specifically sensed and converted to biochemical signals by mechanoenzymatic processes.

2. SMFS provides superb sensitivity to measure and exert molecular forces and to follow and control force-induced conformational changes of mechanoenzymes.
3. A general mechanism of mechanoenzymatics to convert force to a biochemical signal is the exposure of recognition sites after force-induced conformational changes over well-tuned barriers in the activation pathway.
4. Important examples for single-molecule investigations thus far include the force-induced opening of the ATP binding site of TK, the exposure of a protease cleavage site in the VWF A2 domain, and the force-induced binding of vinculin to talin.
5. The mechanical architecture of a protein defines how the active state is reached. A highly specific activation pathway of TK is achieved by structural topology, i.e., shielding of subsequent barriers.
6. Combinations of SMFS with single-molecule fluorescence have led to new applications and discoveries in the field of single-molecule bioscience and nanoscience and are promising for studying mechanoenzymatic processes and enzymes in general.
7. Complementary in vivo investigations enable researchers to study the action and effects of mechanoenzymatic processes in the context of the cellular structure and signaling network. However, because these investigations currently cannot give detailed insights into single-molecule mechanisms, the merging of in vivo and single-molecule force measurements would be desirable.
8. A multitude of mechanoenzymatic processes are waiting to be investigated by existing SMFS techniques. The future will show whether the discovered mechanisms of force-induced conformational changes and specific activation pathways are more general design principles of mechanoenzymatics.

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DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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96. Reveals a peaked and nonexponential unfolding time distribution of TK and shows that its remarkable mechanical architecture is based on mechanical elements shielding each other.

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Contents

How Should We Think About the Ribosome?
Peter B. Moore 1

Mechanisms of Sec61/SecY-Mediated Protein Translocation Across Membranes
Eunyong Park and Tom A. Rapoport 21

Racemic Protein Crystallography
Todd O. Yeates and Stephen B.H. Kent 41

Disulfide Bonding in Protein Biophysics
Deborah Fass 63

Prokaryotic Diacylglycerol Kinase and Undecaprenol Kinase
Wade D. van Horn and Charles R. Sanders 81

Allostery and the Monod-Wyman-Changeux Model After 50 Years
Jean-Pierre Changeux 103

Protein Structure in Membrane Domains
Arianna Rath and Charles M. Deber 135

Bacterial Mechanosensitive Channels—MscS: Evolution's Solution to Creating Sensitivity in Function
James H. Naismith and Ian R. Booth 157

Cooperativity in Cellular Biochemical Processes: Noise-Enhanced Sensitivity, Fluctuating Enzyme, Bistability with Nonlinear Feedback, and Other Mechanisms for Sigmoidal Responses
Hong Qian 179

Network-Based Models as Tools Hinting at Nonevident Protein Functionality
Canan Atilgan, Osman Burak Okan, and Ali Rana Atilgan 205

Filamins in Mechanosensing and Signaling
Ziba Razinia, Toni Mäkelä, Jari Ylänne, and David A. Calderwood 227

Annu. Rev. Biophys. 2012.41:497-518. Downloaded from www.annualreviews.org
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ATP Utilization and RNA Conformational Rearrangement by DEAD-Box Proteins
Arnon Henn, Michael J. Bradley, and Enrique M. De La Cruz 247

Zero-Mode Waveguides for Single-Molecule Analysis
Paul Zhu and Harold G. Craighead 269

Single-Molecule Views of Protein Movement on Single-Stranded DNA
Taekjip Ha, Alexander G. Kozlov, and Timothy M. Lohman 295

Extending Microscopic Resolution with Single-Molecule Imaging and Active Control
Michael A. Thompson, Matthew D. Lew, and W.E. Moerner 321

Metabolite Recognition Principles and Molecular Mechanisms Underlying Riboswitch Function
Alexander Serganov and Dinshaw J. Patel 343

Biophysical Dynamics in Disorderly Environments
David R. Nelson 371

Radical Use of Rossmann and TIM Barrel Architectures for Controlling Coenzyme B_{12} Chemistry
Daniel P. Dowling, Anna K. Croft, and Catherine L. Drennan 403

Biomolecular Simulation: A Computational Microscope for Molecular Biology
Ron O. Dror, Robert M. Dirks, J.P. Grossman, Huafeng Xu, and David E. Shaw ... 429

Recent Advances in Magnetic Tweezers
Iwijn De Vlaminck and Cees Dekker 453

Virus Maturation
David Veesler and John E. Johnson 473

Single-Molecule Mechanoenzymatics
Elias M. Puchner and Hermann E. Gaub 497

Forcing Stem Cells to Behave: A Biophysical Perspective of the Cellular Microenvironment
Yubing Sun, Christopher S. Chen, and Jianping Fu 519

Receptor Signaling Clusters in the Immune Synapse
Michael L. Dustin and Jay T. Groves 543

Functional Architecture of the Nuclear Pore Complex
Einat Grossman, Ohad Medalia, and Monika Zwerger 557

Structural and Energetic Basis of Allostery
Vincent J. Hilser, James O. Wrabl, and Hesam N. Motlagh 585

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