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Atomic Force Microscopy

Exploring the Conformation-Regulated Function of Titin Kinase by Mechanical Pump and Probe Experiments with Single Molecules**

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Protein function such as catalytic activity or molecular recognition is tightly coupled to the conformation and dynamics. Since protein conformations may be controlled by forces, diverse active and passive mechanisms have evolved that allow biological systems to respond to mechanical signals. However, forces act in a predetermined direction on these biomolecules and are so minute that the investigation of the underlying mechanisms is difficult to achieve in ensemble experiments and has to be performed on the level of individual molecules. Atomic force microscopy (AFM) based[1] single-molecule force spectroscopy (SMFS)[2] has evolved into a powerful tool for the investigation of such biomolecules in their natural parameter space of force and extension. In addition, it provides the necessary resolution in the submolecular nanometer and pico-Newton range to control or detect intermolecular conformational changes and their related functions[3,4] and to investigate protein folding[5–7] and unfolding.[8–10]

Here, we develop a new experimental AFM-based single-molecule protocol to successively drive proteins along their natural unfolding pathway into different conformations and afterwards allow the corresponding function to be read out. The rationale behind this type of experiment is analogous to pump and probe protocols, which are already well established in the fields of NMR and ultrashort laser spectroscopy: first, a certain state of the molecules is prepared by means of a laser or radio-frequency pulse. This state is then allowed to evolve for a certain time, after which the molecules are probed by a second pulse. In the case of mechanical, single-molecule pump and probe techniques, the individual molecules are investigated one after the other, and repetition of the experiment results in an ensemble of traces from which values with high statistical confidence can be determined.

First, the protein is contacted and stretched between the surface and the tip of an AFM cantilever to give a certain conformational state, which is predetermined by the extension of the protein. This conformation is then maintained for a certain time by keeping the extension fixed by means of a simple control loop. During this time, the protein has the chance to fulfil the function of the probed conformation, such as specific binding of ligands[4,11,12] or catalytic reactions.[9,13] After this reaction time, the protein is further unfolded so that mechanically detectable and reaction-induced changes in the energy landscape of the protein can be read out. These changes include additional barriers and changes in the barrier height or position. The spatial precision of AFM allows all the different conformational states along the pulling direction to be selectively prepared and to test their competence for the corresponding function. Furthermore, by varying the preparation time, the non-equilibrium kinetics of the reaction with corresponding forward and reverse rates may be deduced. In the following we apply this protocol to investigate the function of the different conformations of the force sensor titin kinase (TK) along its natural mechanical activation pathway and in its natural molecular environment. Our experimental results show that the activated conformation, which is competent for the binding of the cosubstrate adenosine triphosphate (ATP), is hidden behind two strictly ordered barriers that are overcome by physiological forces.

The autoinhibited muscle enzyme titin kinase is embedded in the M-band structure of the sarcomere[14] at an ideal position to sense imbalances of force generation.[15] Recently, it was shown through a combination of single-molecule force spectroscopy, molecular dynamics simulations, and enzymatics that the binding pocket for the cosubstrate ATP is mechanically activated through a force-induced conformational change.[16] These findings suggest, together with the investigated signaling pathway, that TK acts as a force sensor to regulate muscle gene expression and protein turnover.[17] However, up to now there has been no experimental possibility to investigate which of the force-induced conformations along the complex mechanical activation pathway is the one with an activated binding pocket. Here we employ the AFM-based single-molecule pump-and-probe protocol to mechanically prepare predefined conformations of TK for a certain time and with high precision. Afterwards we read out whether the ATP bound by means of the additional barrier in the force spectrum resulting from the interaction of ATP with the binding pocket. As shown in Figure 1a, the natural

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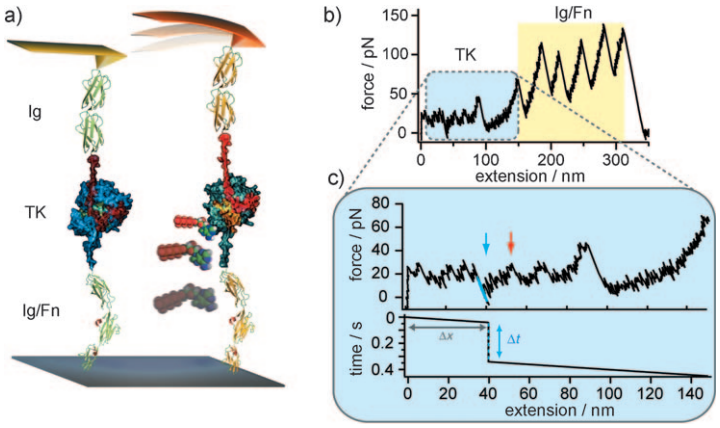

Figure 1. Single-molecule mechanical pump and probe. a) In one experimental cycle, the natural protein construct consisting of titin kinase and its surrounding Ig/Fn domains is stretched between the tip of an AFM cantilever and the surface. b) The recorded force–extension traces show a strict hierarchy: first TK unfolds at forces below 50 pN and at the end the five Ig/Fn domains leave their fingerprint. c) In one pump-and-probe cycle, TK is first brought into a certain conformation, which is determined by the extension Δx (blue arrow). This conformation is maintained for a time span Δt during which ATP has the possibility to bind. Afterwards TK is further unfolded to probe whether ATP had bound or not. Successful ATP binding causes an additional barrier (red arrow), which is detected with high accuracy.

segment of the M-band structure, which consists of TK and its surrounding immunoglobulin (Ig) and fibronectin (Fn) domains, is stretched between the tip of an AFM cantilever and a gold surface in the presence of 2 mM ATP (sample preparation, data recording, and selection of traces was performed according to Ref. [16]). The force–extension traces, such as in Figure 1b, show a strict hierarchy and contain an internal fingerprint[18] which allows identification of the trace section in which TK was sequentially activated: at the end, five contour length increments of about 30 nm are visible, which are caused by the five independently unfolding Ig/Fn domains, whereas the initial low-force part is caused by the sequential unfolding and activation of TK. If ATP does not bind to an activated binding pocket, five distinct barriers with contour length increments of 9.1, 28.6, 7.3, 18.0, and 57.9 nm are observed. The binding of ATP results in an additional barrier so that the increments change to 9.1, 19.4, 10.1, 7.5, 16.4, and 58.3 nm ±2% (for details see Ref. [16]).

In one pump-and-probe cycle, TK is first unfolded to a certain conformation, which is determined by the distance between its two ends. This conformation is then maintained for 300 ms by keeping the end-to-end distance fixed. In the meantime, ATP has the chance to bind to the prepared conformation and to reach its equilibrium binding constant. After this time, TK is further unfolded, to determine by means of the ATP barrier whether the ATP bound to this conformation or not (Figure 1c). As shown in Figure 2, all conformations can be tested for their competence for binding ATP by varying the position at which TK is prepared. Repeating these experiments many times and counting the number of binding events for each conformation allows the corresponding affinities to be determined. The results shown in Figure 3 reveal that the equilibrium value of ATP binding is only measured if the conformation after barrier two is generated. The values observed for the conformations before the first barrier and between the first two barriers do not correspond to a background signal, but are due to the finite pulling speed. Even if a closed conformation is prepared for 300 ms, the binding pocket of TK is opened for a short period of time during the sequential unfolding pathway so that the corresponding non-equilibrium value is observed (for comparison, the non-equilibrium kinetics measured in Ref. [16] are shown in Figure 3). Therefore, these results show that the conformations before barrier two have no affinity to ATP, whereas the conformation after barrier two can bind ATP with an equilibrium affinity of about 350 μM.

Our experimental findings agree well with molecular dynamics simulations performed on TK and their comparison with SMFS experiments, and support the structural explanation of the force-induced activation pathway shown in Figure 3b. Thus, titin kinase exhibits a dual and sequential mechanical autoinhibition, which is a very clever mechanism used by nature to avoid errors in the signaling pathway and at the same time to

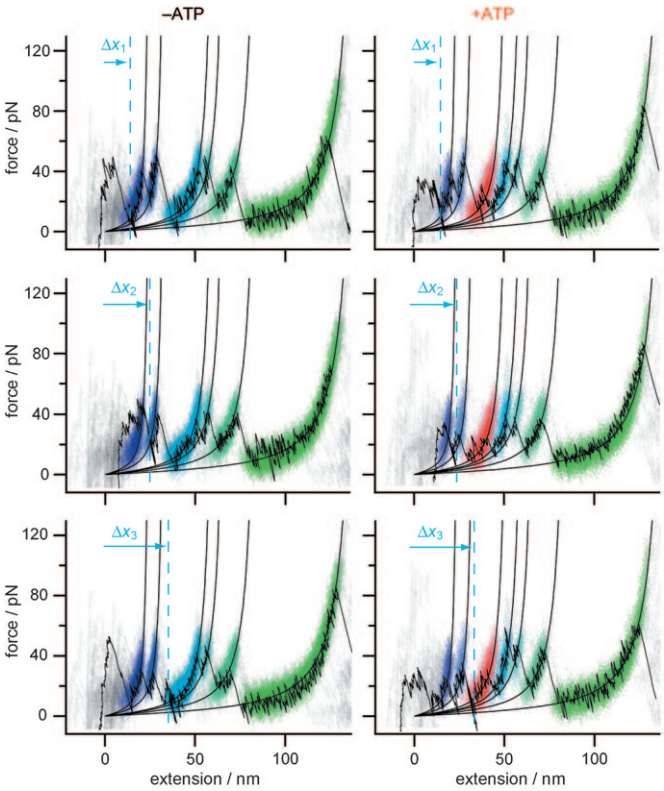

Figure 2. Testing all conformations for their competence to bind ATP. The colored superposition of traces show the five sequential barriers of TK in the absence of ATP and the additional barrier (red) caused by the interaction of ATP with the binding pocket. Black traces are examples of pump-and-probe cycles, in which the conformation before the first barrier, between the first two barriers, and after the second barrier were maintained for 300 ms. In all cases a certain fraction of traces is detected in which the ATP had bound.

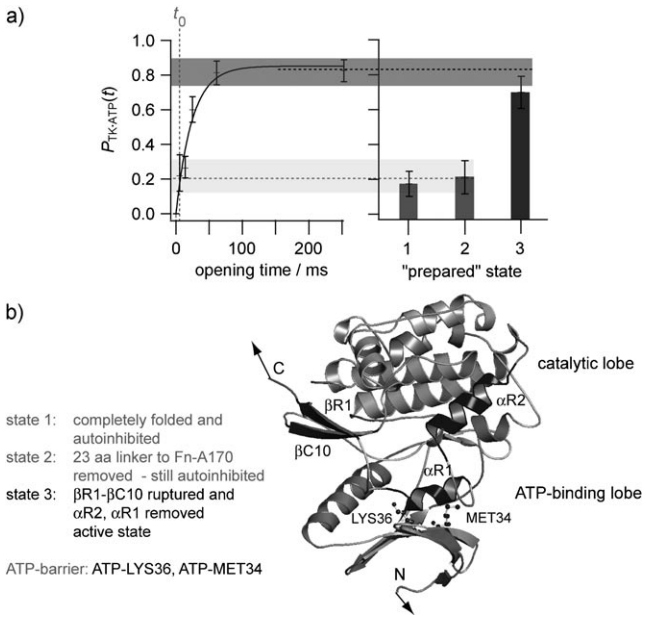

Figure 3. Identification of the active state of TK and structural explanation. a) Left: the non-equilibrium binding kinetics published in Ref. [16]. At high pulling speeds, the binding pocket is opened for a short time and, therefore, only a low probability of ATP binding is observed, whereas saturation at the equilibrium value is observed at long opening times. Right: Binding values of the different conformations, which have been prepared. The value for ATP binding only reaches within the experimental error of the equilibrium if the conformation after barrier two is generated (Δx_3 in Figure 2). The conformations before the first and between the first and second barrier only exhibit the value arising from the finite pulling speed and therefore show no detectable affinity for ATP. The dataset includes 273 individual pump-and-probe traces and the error bars represent the 95% confidence interval. b) These findings agree well with the structural explanation of molecular dynamics simulations: before the first barrier, TK is completely folded and autoinhibited and after the first barrier a 23 amino acid (aa) long linker is removed. At barrier two, the initial β sheet ruptures so that the autoinhibiting element of TK gets removed (pdb:1TKI).

Experimental Section

The TK protein construct A168M2 (867 amino acids, from position 24422 to 25288 in human cardiac N2-B titin, accession number NP 003310.3) was kindly provided by M. Gautel (King's College London). Expression and purification is described in the Supporting Information of Ref. [16]. Protein solution (20 μL; 1 mg mL^{-1}) was incubated for 20 min on a gold surface and washed three times with the measurement buffer consisting of 40 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (Hepes)/KOH, 2 mM $MgCl_2$, 2 mM dithiothreitol, and 2 mM ATP (pH 7.2). SMFS experiments were performed with Biolevers A (Olympus) and a custom built AFM,[26] which can be combined with single-molecule fluorescence studies.[27] The pump-and-probe protocol and data analysis procedures were programmed with Igor Pro 5.0 (Wavemetrics) and executed by the MFP3D controller (Asylum Research). Two datasets where combined in Figure 3a) to enlarge the database and reduce the statistical errors: one with a pulling speed of 1 μm s^{-1} (sampling rate 2 kHz, 161 traces) and one with 2 μm s^{-1} (sampling rate 4 kHz, 112 traces). Both pulling speeds result in much shorter opening times (18 and 9 ms assuming a length of 18 nm), far from equilibrium binding of ATP, compared to the preparation time of 300 ms. Since the difference beween the opening times of 9 and 18 ms is small compared to the preparation time of 300 ms, both datasets may be combined without falsifying the measured difference between the equilibrium and non-equilibrium binding of ATP. After a dwell time of 0.5 s at the surface, the three different conformations were prepared at an extension of 20, 25, and 45 nm above the surface for 300 ms, which is far from equilibrium for the binding of ATP (see Figure 3a). Traces showing the complete unfolding of TK were automatically selected by a pattern-recognition method described in Ref. [18]. Since the protein construct may be contacted at different positions, the traces are shifted by the different lengths of the folded polypeptide chain (corrected in Figure 2). Therefore, the predetermined extensions only maximize the probability for probing the corresponding conformation and it is found that, for example, state 1 or state 2 is prepared at an extension of 25 nm. However, the clear fingerprint allows each recorded trace to be reliably classified and to assign the proper conformation (prepared" state in Figure 3a).

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be sensitive to forces. On the one hand, the barriers should not be overcome by thermal fluctuations, while on the other hand, the barriers should be low enough so that the physiological forces acting on the M-band structure of the muscle sarcomere are sufficient for mechanical activation. Hiding the active state behind two sequential barriers with low enough forces is actually a good solution, since the strict order makes it even more unlikely that both barriers are overcome.

This single-molecule approach for the measurement of non-equilibrium binding kinetics to mechanically prepared conformational states is a very general and useful method for the investigation of other force- and conformation-controlled processes[19] such as in the case of shielded binding sites,[20,21] "catch bonds",[22] or motor proteins.[23] Future experiments with other autoinhibited kinases which do not have a mechanical function[24,25] may give insight into the evolution of force-sensing mechanisms.

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