Mechanoenzymatics of titin kinase

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Biology and muscle mechanics require strain-sensing molecules, whose mechanically induced conformational changes are relayed to signaling cascades mediating changes in cell and tissue properties. In vertebrate muscle, the giant elastic protein titin is involved in strain sensing via its C-terminal kinase domain (TK) at the sarcomeric M-band and contributes to the adaptation of muscle in response to changes in mechanical strain. TK is regulated in a unique dual autoinhibition mechanism by a C-terminal regulatory tail, blocking the ATP binding site, and tyrosine autoinhibition of the catalytic base. For access to the ATP binding site and phosphorylation of the autoinhibitory tyrosine, the C-terminal autoinhibitory tail needs to be removed. Here, we use AFM-based single-molecule force spectroscopy, molecular dynamics simulations, and enzymatics to study the conformational changes during strain-induced activation of human TK. We show that mechanical strain activates ATP binding before unfolding of the structural titin domains, and that TK can thus act as a biological force sensor. Furthermore, we identify the steps in which the autoinhibition of TK is mechanically relieved at low forces, leading to binding of the cosubstrate ATP and priming the enzyme for subsequent auto-phosphorylation and substrate turnover.

Mechanical activity and adaptive responses to changes in load in muscle are tightly linked, but the mechanosensors triggering the sweeping adaptive changes seen in vivo are as yet poorly understood on the molecular level. In the vertebrate muscle sarcomere, titin serves as a molecular ruler for sarcomere assembly and is responsible for resting elasticity of muscle (1, 2) (Fig. L4). At the M-band, titin contains a serine/threonine protein kinase domain (TK) (Fig. LB) (3, 4). TK is regulated in a dual autoinhibition mechanism by a C-terminal regulatory tail, blocking the ATP binding site, and tyrosine autoinhibition of the catalytic base by tyrosine-170 (5). For access to the ATP binding site and the autoinhibitory tyrosine, the C-terminal autoinhibitory tail must be removed. In most autoinhibited kinases, the relief of intramolecular autoinhibition is essentially a partial unfolding event of the autoinhibited conformation, driven by ligand binding or post-translational modification. Although TK activity can be modestly stimulated by calmodulin when tyrosine phosphorylation is mimicked, calmodulin or other calcium binding proteins are unable on their own to activate it (5). Because titin is firmly embedded in the contractile machinery (Fig. L4), its conformation and function can readily be affected by mechanical forces (1, 6). The M-band, being much more compliant than the Z-disk (7, 8), is ideally placed as a strain sensor (9, 10). Because the M-band lattice is deformed only during active contraction, it is optimal for detecting the actual workload on the myofibril (10). Force-probe molecular dynamics simulations of the mechanical properties of TK suggested that kinase activation might be possible by mechanical forces (11). Indeed, a mechanosensitive signaling complex (signalosome) was identified that interacts with an open conformation of TK, and by controlling protein turnover and muscle gene transcription (12) seems to contribute to the adaptation of muscle in response to changes in mechanical strain. The importance of TK in maintaining the turnover of muscle proteins is highlighted by a point mutation in the human kinase domain that causes a myopathy with failure of load-dependent protein turnover (12).

Two recent reports on single-molecule force spectroscopy of titin kinase and C. elegans giant muscle protein kinase (13, 14) showed that these giant muscle protein kinases can unfold in a stepwise fashion, as predicted (11). To be strain regulated, association of the cytoskeletal lattice with the N- and C-terminal ends of the kinase domain is required. Titin is firmly integrated into the M-band lattice by interactions with obscurin, obscurin-like 1, and myomesin, which form a ternary complex at its C terminus (15). Because of their I-band (16) or broad A-band localization (17), the nematode giant muscle protein kinases have been implicated in contraction regulation. It is therefore as yet unclear whether the invertebrate giant protein kinases are similarly integrated into the cytoskeleton, follow the same activation pathways, and serve analogous functions as the M-band-associated TK.

However, experimental proof of direct mechanical activation, rather than simply partial unfolding, is lacking not only for titin kinase, but for all biological force sensors in muscle (2). Can mechanical force really induce a catalytically competent kinase conformation that will be able to bind substrates? The complex protein composition of the sarcomere M-band precludes an unequivocal experimental answer. Studying single molecules in isolation, however, can unravel their intrinsic properties in molecular detail and allow these to be compared with the known properties of intact sarcomeres and measurable enzymatic properties. We therefore used atomic force microscopy (AFM)-based single-molecule force spectroscopy, molecular dynamics simulations, and enzymatics to investigate the molecular details of mechanical TK activation.

Results

Sequential Unfolding of TK at Low Forces. We expressed a TK construct A168-M2, encompassing the human kinase domain flanked by its naturally surrounding Ig/Fn domains [Fig. 1B and C; see details in supporting information (SI) SI Text (section 1), Fig. S1, and Table S1]. The TK construct was attached to an atomic force microscopy | force–probe molecular dynamics simulation | muscle signaling | protein kinase regulation | single-molecular force spectroscopy

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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AFM cantilever [see SI Text (sections 4 and 5) and Fig. S2] and stretched with nanometer accuracy. The resulting force, recorded with piconewton precision (Fig. 1D), showed a characteristic saw-tooth appearance as TK was gradually stretched and unfolded, mimicking the mechanical stress in muscle. (In a very simple comparison, the slowest experimental pulling speed per folded protein length amounts to 300 nm/s/25 nm ~ 12 s and is close to physiological rates. A rabbit sarcomere of 2 μm length can contract with 6 μm/s, yielding a contraction rate of 3/8.) Typically, a series of five initial low-force peaks below 50 pN was followed by up to five distinct saw-tooth-shaped high-force peaks that correlated exactly with the number and contour lengths of the flanking Ig/Fn-domains (18, 19) [Fig. 1; and see SI Text (sections 6–8) and Figs. S3–S5, and Table S2]. Therefore, the low-force peaks, occurring before Ig/Fn unfolding, derive from unfolding events within the kinase domain (see Fig. S2B for a schematic). These low-force unfolding events are strictly ordered, although their height is similar. In contrast to the independently unfolding Ig/Fn-domains, their fixed sequence is not determined by mechanical stability but rather by topology. The forces required even for complete unfolding of the kinase do not exceed 50 pN at 23°C, or 30 pN at 37°C [see SI Text (section 9) and Fig. S6] and at pulling speeds of 1 μm/s. Such low forces were also predicted from force probe simulations (11). The fact that the mechanically more stable Ig/Fn domains always unfold after the kinase domain shows that the force acts on all domains in series, and that the protein construct is therefore completely stretched in the beginning of a retraction cycle.

**Mechanically Activated ATP Binding Detected by AFM.** Mechanical activation of TK must at an early stage uncover its ATP binding site while leaving the active site intact. Subsequent ATP binding alters the stability of the enzyme (Fig. 2A) and should therefore give rise to ATP-dependent changes in the TK unfolding profile (20). In the absence of ATP, five energy barriers separated by 9.1, 28.6, 7.3, 18.0, and 57.9 nm in contour length are observed (Fig. 2B). In the presence of Mg$^{2+}$-ATP at physiological concentrations (2 mM), a certain fraction of the traces shows an additional well pronounced peak (peak 2*) at 51.6 nm (Fig. 2C). This peak denotes an early interaction with ATP during the sequential unfolding of the kinase and thus demonstrates the initial opening of the active site.

The probability of observing the ATP-dependent peak should depend on the likelihood for ATP binding during the time span between the opening of the binding site and the moment when the ATP barrier (peak 2*) is probed and, therefore, on the ATP concentration (Fig. 2D). More interestingly, because this time span is controlled by the pulling speed, we gain direct experimental access to the ATP binding kinetics (see SI Text, section 10).
Table 1. Kinetic parameters for ATP of autoinhibited titin kinase (WT and K36A) and the constitutively activated WT-kin3 measured by AFM or in solution

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experiment can be seen as a mechanical pump–probe experiment: first the binding pocket of the TK is “pumped” open, and after a certain time ATP binding is probed. Variation of this time window provides the kinetic constants. The calibration of the time axis was estimated by the ratio of the MD-determined extension, during which the binding pocket is open but not deformed, and by the pulling speed. This mechanical pump–probe experiment showed saturation after ~100 ms (Fig. 2E). Toward higher pulling rates, the probability of ATP binding decreased strongly and approached zero (Fig. 2E). This time dependence demonstrates that ATP binding is mechanically induced and perfectly agrees with the absence of catalytic activity of autoinhibited TK in solution (Table 1). Furthermore, this experiment allows estimates of the apparent on and off rates and resulting dissociation constant, which compare with affinity values observed for titin (Table 1) and other kinases in solution (21).

Following a suggestion from our MD simulations, we mutated lysine-36 to alanine (K36A), a highly conserved residue equivalent to lysine-72 interacting with the αβ phosphate of ATP in cAMP-dependent protein kinase (22, 23). This mutation abolishes kinase activity in TK (5). Now the ATP affinity of TK was dramatically reduced, with a >6-fold increase of $k_{off}$ and a concomitant decrease of $K_a$ to millimolar values (Fig. 2D, SI Text, section 10 and Table 1). These results localize mechanically induced ATP binding to the canonical site in TK and confirm that the conserved lysine residue, known to play a crucial part in ATP binding in homologous protein kinases, is also a key residue in the TK binding pocket.

**Molecular Mechanism of TK Activation by Force.** We used force–probe MD simulations (24, 25) to characterize the force-induced unfolding of TK at the atomic level and to correlate the structural states with the energy barriers observed by the single-molecule force spectroscopy experiments. Force–probe molecular dynamics simulations (24, 25) used the TK x-ray structure [Protein Data Bank entry 1TKI (5)] as the starting structure, with the autoinhibitory tail partially removed [see SI Text (sections 11–16) and Tables S3 and S4]. Two sets of simulations (five each) were carried out for this truncated TK. One set with an empty binding pocket, and one set with an ATP molecule and magnesium ions inserted into the (closed) binding pocket. As a control, the autoinhibited complete TK was also subjected to force–probe MD simulations (see SI Text, section 17). As in the experiment, the two force profiles obtained from the simulations of the truncated TK (Fig. 3B Top and Middle) are largely similar. A notable exception is the more pronounced force peak seen in the presence of ATP (see Fig. 3B Inset) at the position of the measured force peak 2*. To allow direct comparison of the unfolding pathways between experiment and simulation, we transformed the force extension traces of Fig. 2 into barrier position histograms (14) and derived the same from our simulations (see SI Text, section 18). The two histograms agree well both in the presence and absence of ATP [Fig. 4 (dashed lines) and Fig. S7], allowing the conclusion that the main unfolding events are correctly described by the simulations.

Next, we investigated which molecular interactions determine the observed force peaks. For the ATP peak 2*, two strong interactions are seen, a salt bridge from lysine-36 to the α-phosphate group of ATP, and a contact between methionine-34 and the adenine moiety of ATP (Fig. 3C). Both interactions break irreversibly upon βC3–βC4 rupture, giving rise to the significantly larger force peak of 270 ± 39 pN in the simulations with bound ATP as compared with 188 ± 13 pN without ATP (Fig. 3B Inset and Movie S1). Notably, in the AFM experiment, the contour length of 51.6 nm for the ATP peak position (Fig. 4, peak 2*) also points to a residue close to lysine-36. An additional peak is seen at 18 nm for the simulation with ATP present (plus sign in Fig. 3B Top). Here, a force-induced deformation of the N-terminal domain triggers the transient rupture and reformation of the methionine-34–ATP and lysine-36–ATP interactions.

Closer structural analysis of our simulations suggests the following sequence of events (colors in Fig. 3A and B, and Fig. 4). Peak 1 (Fig. 4) is caused by unfolding of the 23-residue linker at the N terminus of TK, which is not present in the simulations (see SI Text for details). At peak 2, the autoinhibitory tail is unfolded and removed, rendering the ATP binding site accessible (region shaded in gray in Fig. 3B Bottom). Subsequently, N-terminal β-sheets βC1–βC2 and βC2–βC3 rupture (regions B and C). For these events, no force peak is seen in the experiment, because it would fall into the lag time after force peak 2. Peak 2* described above is dominated by interactions of ATP with the binding pocket. The truncated construct necessarily lacks part of the autoinhibitory tail stabilizing the adjacent C-terminal α-helix αR1 in the full-length TK. Accordingly, αR1 unfolds first in the truncated kinase (Fig. 3B Top and Middle, region A) but after βR1 and αR2 in the autoinhibited kinase (Fig. 3B Bottom). Hence, and in agreement with the complete TK unfolding simulations (Fig. 3B Bottom), peaks 3 and 4 are assigned to unfolding of αC1 and βC4–βC5, respectively (regions D and E). Finally, peak 5 arises from the combined effect of αC2 and αC8 rupture (Fig. 3B Bottom). At peak 6, the complete TK is unfolded and stretched. Taking the diameter of the folded TK into account (5.5 nm), the contour length increment to peak 1 (121 nm) corresponds to (5.5 + 121 ± 2) nm/0.365 nm = 346 ± 5 residues, in agreement with the 344 aa of TK including its N-terminal linker (see SI Text, section 7).

**Autophosphorylation of TK.** Our simulations show that the open ATP binding site does not relieve autoinhibition of the catalytic base aspartate-127 by tyrosine-170. However, our model of the ATP-bound state of TK suggests that this semi-opened state might autophosphorylate, in agreement with previous predictions of the open apo-enzyme (11). We tested this notion by assaying recombinant TK with its ATP binding site released (TK-kin3, mimicking the mechanically induced open state after peak 2), and found that the release of ATP binding not only activates kinase activity toward substrates like telethonin, but also allows tyrosine autophosphorylation (see SI Text, sections 1 and 2, for experimental details). As shown in Fig. 5, although low levels of phosphotyrosine are detected by the 4G10 antibody before incubation with ATP, tyrosine phosphorylation is strongly stimulated by ATP, with a preference toward Mn²⁺, similar to other enzymes (26, 27).
Discussion

Our results show that mechanical stress is able to activate titin kinase by releasing the active site for ATP binding, and they unravel the first step of this mechanical signaling pathway. We also show that mechanical release of the ATP binding site allows a second step in TK activation by triggering autophosphorylation on the inhibitory tyrosine. That TK can thus indeed act as a biological force sensor is supported by the fact that the forces activating ATP binding are within the physiological range and, importantly, lower than the ones unfolding the surrounding structural titin domains. Small force imbalances of four to eight myosin motor domains, equivalent to \( \frac{3}{10} \) of the 147 myosin molecules (1) between adjacent thick filaments could thus translate into a physiologically significant signal by activation of the TK mechanosensor. Because of the necessarily much faster pulling rates of 0.8 m/s used for the simulations, larger unfolding forces are seen, which can be related to the experimental loading rates (11). In the force–probe MD simulations, harmonic springs were attached to the protein and retracted with constant velocity (lower schematic, ATP shown as red spheres). 

Once the ATP binding site is opened by mechanical force, not only does the enzyme bind ATP, but it actually undergoes the next necessary step for full activation, the phosphorylation of the autoinhibitory tyrosine-170. Rather than leading to a dead end, mechanical activation of ATP binding thus activates the full catalytic activity of TK. This mechanism may be particularly relevant when the sarcomere is extended while generating active tension due to an opposing force greater than that generated by the muscle, also called eccentric exercise (9). Under such conditions, large changes in M-band structure are observed (30). Eccentric exercise is a strong stimulator for muscle growth and repair (31–34), and the interaction of titin kinase with ubiquitin-associated scaffold proteins with links to multiple signaling pathways controlling muscle gene expression and protein turnover (12) supports plausibly that titin kinase can act as a force sensor in the activated sarcomere. Unlike myosin filaments on the order of 10 nm (28, 29), which would translate into shear strain on the M-band. Both the forces and displacements required for mechanical TK activation are therefore within the ranges observed in muscle.

Fig. 3. Molecular dynamics (MD) simulations of the force-induced unfolding of titin kinase (TK). (A) Representative unfolding intermediates with the unfolding secondary structure elements colored according to the scheme in Fig. 1D; the \( \beta \)-strands unfold pairwise, and colors refer to the respective N-terminal strand of each pair. (B) Unfolding forces of truncated TK with ATP (Top), without ATP (Middle), and of the complete TK (Bottom). For the complete TK, two independent 90-ns simulations were carried out (solid and dashed lines). Starting from a partially unfolded structure at \(-19 \) nm, five 26-ns trajectories (thin gray lines) were averaged for both sets of simulations (thick lines in Top and Middle). Color-shaded areas indicate main unfolding events, which correspond to the colors used in A and in Fig. 1D. An additional force peak in the presence of ATP is predicted (plus sign and pink-shaded area in Top). This force peak (Inset) is higher for bound ATP (270 pN) than for an empty binding pocket (188 pN). Because of the necessarily much faster pulling rates of 0.8 m/s used for the simulations, larger unfolding forces are seen, which can be related to the experimental loading rates (11). (C) In the force–probe MD simulations, harmonic springs were attached to the protein and retracted with constant velocity (lower schematic, ATP shown as red spheres). (C Insets) Representative structures shortly before (Left) and after (Right) the ATP force peak, ATP and the two key residues methionine-34 and lysine-36 are shown in ball-and-stick representation, and the rupture of molecular interactions is indicated by dotted lines.
the homologous nematode kinases, which retain catalytic activity in their inhibited form (13), we show that TK is completely inactive in its autoinhibited form. Mechanical switching of its ATP-binding site thus confers a significant signal between active and inactive kinase, as expected for a signal that modulates energy-costly processes like protein breakdown and transcriptional activity (12).

Our surprising observation that a protein kinase can be activated by local protein unfolding induced by mechanical force may find analogies in the small GTPase Rab8, whose activation by the nucleotide exchange factor MSS4 also involves local protein unfolding (35). The mechanoenzymatic sensor found in titin kinase may therefore be paradigmatic also for other members of the family of cytoskeletal autoregulated protein kinases, a branch of the calcium-calmodulin-regulated enzymes of the human kinome (36), containing myosin light-chain kinase and obscurin kinases. These enzymes share with titin the N- and C-terminal cytoskeletal association (37) or specific residues involved in autoinhibition (38) and may thus bear features of mechanical modulation. Furthermore, other autoregulated cytoskeletal signaling domains, like GDP-GTP exchange factor domains, may be similarly activated. Our single-molecule approach will therefore be useful for investigating the mechanochemistry of many cellular systems that may share similar mechanosensitive regulation mechanisms.

Materials and Methods

Titin kinase expression was carried out in sf9 insect cells by using a recombinant baculovirus system essentially as described in ref. 5. Purification and enzymatic assays were performed essentially as described in refs. 5 and 12 (for details, see SI Text). Atomic force microscopy using a custom-built instrument, and analysis of the data were carried out essentially as described (14, 19); for details see the SI. Force–probe molecular dynamics simulations (24, 25) used the TK x-ray structure [Protein Data Bank entry 1TK1 (5)] as the starting structure, with the autoinhibitory tail partly removed. Two sets of simulations (five each) were carried out for this truncated TK, one set with an empty binding pocket, and one with an ATP molecule and magnesium ions inserted into the (closed) binding pocket. As a control, the autoinhibited complete TK was also subjected to force–probe MD simulations. For further details, see SI Text.

ACKNOWLEDGMENTS. We thank Thorsten Kampmann for help with the ATP force field and Carsten Kutzner for help with the GROMACS force probe code. U.H. and L.V.S. were supported by the Deutsche Forschungsgemeinschaft (research training group 782 and SFB Grant 755). L.V.S. was supported by the Boehringer Ingelheim Fonds and by the European Union. This work was supported by the Center for Integrated Protein Science Munich and the Medical Research Council of the United Arab Emirates.

Fig. 4. Contour length histograms obtained from single-molecule force spectroscopy experiments (transformation with QM-WLC and P = 0.8 nm) and from MD simulations (Inset). The folded kinase construct has a length of 25 nm. The peak positions with (red) and without (black) ATP are similar in both histograms (dashed lines), except for one additional peak in the presence of ATP (red peak at ~51.6 nm). The experimentally determined contour length increments are, in the absence of ATP, 9.1, 28.6, 7.3, 18.0, 57.9 nm; and, in the presence of ATP, 9.1, 19.4, 10.1, 16.4, 58.3 nm— with an estimated error of ±2%. The position of the initial peak (24 nm) reflects the mean length of the TK construct with completely folded domains.

Fig. 5. Autophosphorylation of TK on tyrosine. Incubation of the highly purified TK-kin3 enzyme in the absence (−) and presence (+) of ATP and Mn2+ ions leads to tyrosine phosphorylation detected by Western blot, using the phosphotyrosine antibody 4G10. The autoinhibited kinase construct A168-M2 (WT) shows no appreciable phosphotyrosine incorporation under any tested condition. Lower blot: loading control, detection with anti-titin kinase antibody (α-TK).

Correction

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Supporting Information

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SI Text

1. Expression and Purification of Titin Kinase. Three titin kinase constructs were expressed for this study: A168-M2 (867 aa, from 24422 to 25288 in human cardiac N2-B titin, accession number NP_003310.3); A168-M2 K36A, where lysine 24783 is mutated to alanine; and TK-kin3 (314 aa, from 24725 to 25038 in NP_003310.3), where the autoregulatory tail is partially removed to expose the ATP binding site (2). The boundaries of TK-kin3 mimic the mechanically opened state where the autoinhibitory tail is removed apart from the mechanostable αR1 helix (see molecular dynamics section for details). The numbering of the constructs can be converted to that of the amino acids in the titin kinase structure [Protein Data Bank (PDB) entry 1TKI] by subtracting 24747 from the amino acid numbers in N2B titin NP_003310.3, respectively, by adding 24747 to the numbers in 1TKI. A conversion table for residues discussed in the manuscript is given in Table S1. The cDNAs encoding these constructs were cloned into a modified baculovirus shuttle plasmid and N-terminally fused to a tag sequence encoding, from the N-terminus, a hexa-histidine tag, HA-tag, and TEV cleavage site. Constructs were verified by DNA sequencing. After generation of recombinant virus, expression was carried out in suspension cultures of Spodoptera frugiperda sI9 cells essentially as described (3). Soluble protein was purified at a yield of 6 mg/liter of culture for A168-M2, and 20 μg/liter for TK-kin3 and purified by sequential nickel-affinity chromatography, ion-exchange chromatography on a Mono Q column, and size-exclusion chromatography on a Superdex 75 column (GE Healthcare). For some experiments, the His-tag sequence was cleaved off by TEV protease, but the presence or absence of the tag had no detectable influence on kinetic parameters or unfolding characteristics. Final fractions were >99.5% pure as judged from overloaded gels (Fig. S1). Protein was stored in small aliquots at a concentration of 1 mg/ml in 20 mM 2-[4-(2-hydroxyethyl)-1-piperazine]ethanesulfonic acid (Hepes)/KOH (pH 7), 2 mM MgCl2, 50 mM NaCl, 2 mM DTT, and 10% glycerol in liquid nitrogen. Identity of the proteins was ascertained by gel electrophoresis, analytical gel filtration, Western blots using anti-titin kinase antibodies (4), and mass spectrometry (MS). MS confirmed not only the correct molecular weight for TK-kin3, but also detected two bound magnesium ions in this open form of the kinase, indicating that protein kinases bind Mg2+ independently of ATP. Kinase assays with recombinant telethonin showed that only kin3, but not the long forms of the kinase, showed protein kinase activity in vitro.

2. Kinase Assays. Michaelis–Menten kinetics of TK was performed similarly as described for phosphorylase kinase (5) but using telethonin as substrate at a constant concentration of 10 μM in assay buffer [20 mM Hepes/KOH (pH 7), 5 mM MgCl2, 5 mM DTT]. ATP concentrations varied from 165 nM to 800 μM for kinetic analysis with a constant 165 nM [γ-33P]ATP label. Samples were taken from replicate assays at regular intervals from 20-μl reaction mixtures, spotted on nitrocellulose, and immediately quenched in 5% ice-cold trichloroacetic acid (TCA). After several washes with TCA, the filters were dried and protein-incorporated radioactivity determined by liquid scintillation counting. After correction for background (label and telethonin, no enzyme), the averaged protein phosphorylation rate expressed as μmol/minute was fitted to the Michaelis–Menten equation by using Mathematica, and KM was derived. Results are summarized in Table 1.

3. Autophosphorylation of Titin Kinase. Highly purified TK kin3 (see above) was incubated at 30°C in assay buffer supplemented with 0.1 mM sodium orthovanadate in the absence and presence of ATP, with either 5 mM Mg2+ or Mn2+ as counterion (6). The reaction was stopped after 20 min by addition of Laemmli sample buffer, and the samples were electrophoresed on 14% SDS-polyacrylamide gels. After Western blotting of the gels by standard procedures, phosphorimaging was detected by using the 4G10 monoclonal antibody (Upstate Biotechnology) and a site-specific anti-titin kinase antibody raised against the peptide APEYY(PO3)3APE, which specifically reacts with titin kinase phosphorylated on tyrosine 170.

Both antibodies detect the formation of phosphorytrosine under these conditions, and autoradiographs of assays in the presence of [γ-33P]ATP demonstrate label incorporation. Similar assays were performed at several enzyme concentrations (0.5, 1, 2, and 5 μM) in the presence of 165 nM [γ-33P]ATP and 100 μM total ATP, and TK-incorporated radiolabel was determined as above to test for concentration dependence of phosphate incorporation. The correlation of label incorporation was linear with enzyme concentration, suggesting that the phosphorylation occurred intramolecularly (7).

Atomic Force Microscopy (AFM) Measurements

4. AFM Sample Preparation. Microscope slides were washed with denatured ethanol and H2O2ad in a supersonic bath. After drying, a layer of 4-nm Cr/Ni followed by a layer of 40-nm Au was evaporated onto the surface. Twenty microliters of the protein solution (1 mg/ml) was incubated for 20 min on the gold surface and washed three times with the measurement buffer, consisting of 40 mM Hepes/KOH, 2 mM MgCl2, 2 mM DTT (pH 7.2), and, depending on the experiment, 2 mM ATP.

5. AFM Measurements. All force spectroscopy measurements were performed with a custom-built and completely automated AFM (Fig. S2). The instrument is controlled with an Asylum Research MFP3D controller and Igor Pro 5.0 (WaveMetrics). The software was written such that it automatically adjusts the height of the AFM and the position of the photodiode to compensate for instrument and cantilever drift. If an experiment is performed at several pulling speeds, the software periodically changes the speed after each force extension trace to allow a better comparison. In this way, force extension traces can be recorded for several days with the same sample and the same cantilever. To avoid evaporation of the buffer, a fluid cell consisting of Teflon and a latex membrane seals the probe volume during the experiment.

After calibration of the cantilevers (Olympus biolevers A) using the equipartition theorem (8), the automated measurement was started as described above. Because the surface is not homogeneously covered with the non-specifically adsorbed protein constructs, the x-y scanner of the AFM moves the sample by 20 nm after each trace. In this way, the number of traces showing unfolding of the protein construct is enhanced and in each trace a new molecule is picked up that was not unfolded before.

6. Selection of Traces. In single-molecule force spectroscopy experiments, the density of molecules attached to the surface has to be low enough to address individuals. Therefore, in ∼95% of measurement cycles, the approach of the cantilever to the surface does not result in a contact with a molecule. Furthermore, the protein construct is contacted at random positions
resulting in traces that do not show unfolding of the complete titin kinase. Because the protein construct contains three domains at the N terminus of the titin kinase and two on its C terminus, all traces showing more than three domains must contain the unfolding of the complete enzyme. Because of the small tip radius of the AFM cantilever (30 nm), traces showing more than one protein construct in parallel are very rare. They can be identified by the higher forces and by the superposition of their unfolding patterns that are in most of the cases shifted with respect to each other. In total, \(\sim 1\%\) of the recorded traces show the unfolding of one individual titin kinase molecule and are therefore selected for further analysis.

Because of the large amount of traces recorded for this study, and the low efficiency of complete unfolding traces due to the reasons mentioned above, we developed a method that allows automated selection of traces and recognition of unfolding patterns (1). In a first step, force extension traces are transformed into contour length space by using the QM-WLC model (9). In the next step, contour length histograms are obtained that directly reflect the barriers of the unfolding potential. This fingerprint is independent of fluctuations and variable experimental parameters and therefore can be used to screen for characteristic patterns. In this study, we adjusted the parameters such that traces were selected showing more than three Ig-domains with a barrier spacing of \(\sim 30\) nm. Because the folded protein construct is contacted at random positions, the initial part of a force extension varies in length. This variable offset can be determined choosing the rupture of the first Ig-domain as the point of reference. This has to be taken into account in superpositions of force extension traces or averaged barrier position histograms (Fig. S3). Once we identified the complete unfolding pattern of the titin kinase, we also took traces into account that show less than four Ig-domains but contain the complete unfolding pattern of the enzyme. In these cases, the protein construct was either picked up at its ends but desorbed before all domains were unfolded, or it was contacted at the surrounding domains so that complete unfolding of the titin kinase took place.

**Overview of the number of recorder traces.** Recorded total traces, 392,000; total traces with some captured “bait,” \(\sim 30,000\); total complete kinase traces, 2,100. Therefore, in \(\sim 95\%\) of measurement cycles, the approach of the cantilever to the surface does not result in a contact with a molecule. Because of the random attachment of the molecules, a substantial number of these traces are incomplete and contain mostly just some Ig/Fn domain unfolding signatures.

Traces with complete kinase signature used for analysis were as follows: kinetics with WT, 812 complete kinase traces; kinetics with K72A mutant, 962 complete kinase traces; concentration series with WT, 275 complete kinase traces; concentration series with K72A mutant, 348 complete kinase traces.

7. Molecular Calibration by Using Ig/Fn Domains. To confirm the proper calibration of the AFM, we compare the experimental contour length increments of Ig/Fn domain unfolding with the expected ones. The theoretical values are obtained in the following way. First, the number of folded amino acids is multiplied by 0.365 nm (corresponding to the separation of the following way. First, the number of folded amino acids is expected ones. The theoretical values are obtained in the contour length increments of Ig/Fn domain unfolding with the proper calibration of the AFM, we compare the experimental folded conformation, then the distance between the attachment points corresponds with the diameter of the folded domain. Because this conformation is the starting point for the length measurement, the domain diameter is missing and has therefore to be subtracted from the complete contour length if compared with the experimental one (Table S2). The theoretical mean contour length of 30.86 nm deviates from the experimental one, determined in Fig. S4 to be 30.45 nm, by only 1%. 8. Influence of Ig Domains on the Unfolding Pattern. The use of spacer domains that provide their fingerprint for the selection of force extension traces has become a widespread approach (10). Although the unfolding pattern of these spacer domains is well studied, we verified the interpretation of force extension traces by performing experiments with TK protein constructs containing three and five spacer domains. As can be seen in Fig. S5, the maximum number of equal intervals corresponding to a contour length increment of \(\sim 30\) nm perfectly correlates with the number of spacer domains. Although the initial part of titin kinase unfolding is not so clearly resolved with *Escherichia coli*-expressed protein constructs (most likely because of folding differences, in agreement with *E. coli*-derived protein being catalytically inactive), this experiment justifies the interpretation that allows to separate unfolding of the titin kinase from unfolding of spacer domains.

9. Unfolding Behavior of the Titin Kinase at 37°C. Although also the titin of cold-blooded animals contains the titin kinase domain (e.g., frogs and fish), we investigated the unfolding pattern of the TK protein construct at 37°C. For these measurements, the whole AFM setup was put into a temperature-controlled oven, where force extension traces were recorded. We found that the unfolding forces of the titin kinase are smaller (\(\sim 30\) pN) than at room temperature (\(\sim 50\) pN) because the energy barriers are overcome more easily due to the higher thermal energy. This observation is consistent with other studies (11, 12). However, the unfolding pattern was found to be the same as the one observed at room temperature (Fig. S6). Therefore, we conclude that the structure and function of titin kinase is the same at both temperatures, allowing its investigation under the experimentally more stable conditions.

10. Detection of Force-Induced ATP Binding and Determination of the Reaction Kinetics. As shown, binding of ATP to the titin kinase causes an additional energy barrier in the unfolding pathway. By counting the traces showing this barrier, the relative frequency of ATP binding can be determined. Because the activation is mechanically triggered, the binding process takes place in chemical nonequilibrium and can be described as follows. Let TK* be the activated enzyme with accessible binding pocket and TK*·ATP the titin kinase–ATP complex. Then, the probability \(P_{TK^*·ATP}(t)\) of finding the complex after a reaction time \(t\) is given by the simple differential equation

\[
\frac{dP_{TK^*·ATP}(t)}{dt} = k_{on}[ATP] - k_{off}P_{TK^*·ATP}(t),
\]

where \(k_{on}\) and \(k_{off}\) are the association and the dissociation rates of ATP. Solving this equation yields

\[
P_{TK^*·ATP}(t) = \frac{k_{on}}{k_{on} + k_{off}} (1 - e^{-k_{off}t}).
\]

This model perfectly describes our experimental data and allows us to determine the kinetic parameters of ATP binding. The experimental time scale is given by the inverse ratio of the pulling speed and the extension increase, during which the ATP binding pocket is accessible and still correctly folded. This extension increase was determined with MD simulations and values 18 nm. The errors of the experimentally determined kinetic parameters could be estimated by

\[Puchner et al. www.pnas.org/cgi/content/short/0805034105\]
are mainly due to the fact that a limited number of random samples is available. Therefore, the errors are calculated by using the beta function and assuming a 95% confidence interval to be certain.

11. Molecular Dynamics (MD) Simulations. All simulations were carried out with the GROMACS simulation suite (13, 14), using the OPLS all-atom force field (15) and periodic boundary conditions. NpT ensembles were simulated, with the protein and solvent coupled separately to a 300-K heat bath (τp = 0.1 ps) (16). The systems were isotropically coupled to a pressure bath at 1 bar (τp = 1.0 ps) (16). Application of the Lincs (17) and Settle (18) algorithms allowed for an integration time step of 2 fs. Short-range electrostatic and Lennard–Jones interactions were calculated within a cut-off of 1.0 nm, and the neighbor list was updated every 10 steps. The particle mesh Ewald (PME) method was used for the long-range electrostatic interactions (19), with a grid spacing of ≈0.12 nm.

12. ATP Force Field. The atomic partial charges of ATP used along with the OPLS force field were derived from quantum chemical calculations. The charges were obtained from B3LYP/6–31+G* calculations using the CHelpG electrostatic potential fitting scheme (20). The quantum chemical calculations were carried out with Gaussian 03 (21). All nonbonded parameters are given in Tables S3 and S4. Bonded parameters were taken from ref. 22.

13. Generation of Starting Structure. The simulations of the full-length titin kinase (TK) were set up as described in ref. 23, with the exception that, here, the OPLS force field was used (see above).

The starting structures of the truncated TK were obtained from the TK crystal structure (PDB entry 1TKI) (3) in three steps detailed below. First, 33 residues at the C terminus comprising the βR1 and αR2 motives of the autoinhibitory tail (ai tail) were removed from the structure, because they block the ATP binding site. Second, the ATP ligand was docked into the active site. Third, the ligand-induced conformational closure of the protein structure was enforced by MD.

14. Docking of ATP. ATP was docked into the active site of TK by using the protein kinase A (PKA) (PDB entry 1Q24) (24) as a homology model. After aligning of the two protein structures, the ATP and one Mg2+ ion were adopted from PKA. The second Mg2+ ion was added by using the phosphorylase kinase structure as a template (PDB entry 2PHK) (25), because it was not resolved in the PKA structure.

15. Energy Minimization and Equilibration of the Solvent. Before the free MD simulations, the systems were solvated with TIP4P water within a cubic box of 8.5 nm length. Sodium and chloride ions were added (c ≈ 0.15 mol/liter), and the systems were energy-minimized for 1,000 steps by using steepest descent. The solvent was then equilibrated for 500 ps with positional restraints on the protein heavy atoms (force constant 1,000 kJ·mol⁻¹·nm⁻²).

16. Closure of the Active Site. The ligand-induced conformational closure of the protein structure was enforced by means of essential-dynamics MD (26). To this end, the closed conformation of the PKA (1Q24) was used as the target structure. First, the two protein structures (TK and PKA) were aligned. Then, the N-terminal β-hairpin (residues 13–21 in TK) was selected, and a principal component analysis (PCA) was carried out, yielding one eigenvector that describes the closing motion. Essential dynamics sampling was then performed, during which motion along the eigenvector toward the target structure was enforced [radcon option in EDsampling module (27) of GRO-

MACS]. To allow the protein to relax along the enforced closing motion, the maximal step size along the eigenvector was restricted to a maximum of 0.05 nm per step, leading to a closed structure within ≈1 ns. Subsequently, the closed structure was simulated for 1 ns with positional restraints on the Cα atoms (force constant 1,000 kJ·mol⁻¹·nm⁻²). Finally, a 1-ns free MD simulation was carried out, during which no opening motion was observed in the presence of ATP. As a control, the closed structure was simulated also in the absence of ATP. As expected, significant reopening motion was observed. The starting configuration for the force-probe MD simulations were taken from the final free MD simulations after 1 ns.

In the resulting structure with ATP bound, a salt bridge between Lys-36 and the α-phosphate group of ATP, and interaction between Met-34 and the adenine moiety, respectively, were formed. For the latter, two types of interactions with ATP were observed. For the hydrogen bond between the sulfur atom and the NH2 group of adenine, and, alternatively, a stacked conformation of the S-CH3 group and the adenine 6-ring. Additional hydrogen bonds were formed between the β-phosphate of ATP and the N-H backbone of Glu-17, as well as between the hydroxyl groups of the ribose and the carbonyl backbone of Arg-15.

17. Force–Probe MD Simulations. To mimic the AFM experiments, force–probe MD simulations (FPMD) were carried out (28, 29). In these simulations, a harmonic spring was attached to the carbon atom of the carboxyl C terminus and one to the nitrogen atom of the amino N terminus, each described by a spring potential,

\[ V_{\text{spring}}(t) = \frac{k_0}{2} (z_i - z_{\text{spring}}(t))^2, \]  \[ \text{[3]} \]

which was included within the force field. In Eq. 3, \( k_0 = 500 \) kJ·mol⁻¹·nm⁻² is the force constant of the spring, \( z_i \) is the position of the pulled atom \( i \), and \( z_{\text{spring}} \) is the position of the spring that is attached to atom \( i \). The springs were then moved with constant velocity \( v = 0.4 \) m/s in opposite directions, \( z_{\text{spring}}(t) = z_i(0) \pm vt \). Because of the moving springs, the pulled atoms experienced an additional force

\[ F_i = k_0 (z_i - z_{\text{spring}}(t)). \]  \[ \text{[4]} \]

The average force at the two springs was monitored during the simulations, yielding the force profiles shown in Fig. 3B.

Before the FPMD simulations, the protein was aligned along the pulling direction (z axis), and the simulation box was extended along the z axis to ≈20 nm, allowing for the accommodation of an elongated conformation. Further, water, sodium, and chloride ions (c ≈ 0.15 mol/liter) were added, and the system was energy-minimized, followed by equilibration for 200 ps with positional restraints on the protein heavy atoms (force constant 1,000 kJ·mol⁻¹·nm⁻²).

To generate statistically independent unfolding trajectories (five trajectories with and without ATP, respectively), a partially unfolded conformation was selected after 19.2 and 20.2 ns of FPMD simulation time with and without ATP, respectively. These conformations were chosen because they correspond to minima in the force profiles. The nitrogen atom of the N terminus and the carbon atom of the C terminus, respectively, were kept fixed with a positional restraint (force constant 1,000 kJ·mol⁻¹·nm⁻²), and the systems were equilibrated for 1 ns with free MD. From this trajectory, five equidistant frames (\( \Delta t = 200 \) ps) were chosen as starting structures for the additional force–probe simulations.

Simulations were interrupted before any of the springs had crossed the box boundary. Here, care was taken that, because of the applied periodic boundary conditions, the pulled termini did
here does not interact with each other. At this point, fully unfolded residues at both termini were removed, and new termini, water, and ions were added. The FPMD simulations were then continued after equilibration of the solvent with positional restraints on the protein heavy atoms (force constant 1,000 kJ mol⁻¹ nm⁻²) for 200 ps.

The simulated systems comprised ~180,000 atoms. The total simulation time was ~500 ns.

18. Contour Length Plots. The plotted contour lengths aim at quantitative comparison between the simulations and the experiments. Such comparison is complicated by the fact that (i) different pulling speeds are used and, (ii) in the simulations, a stiffer spring has to be used then in the AFM experiments. To account for these differences, we derived contour length plots from the force profiles obtained from the MD simulations in three steps. First, prominent force peaks were selected along the force profiles. Here, only those force peaks \( j \) were included whose height \( F_{\text{max},j} \) exceeded a certain threshold. This threshold was defined as

\[
F_{\text{max},j} \geq F_{\text{max},1} - k_{\text{AFM}} (\Delta z_{\text{spring},j} - \Delta z_{\text{spring},1} (t)),
\]

where \( k_{\text{AFM}} \) is the effective spring constant of the AFM cantilever and the attached linkers, and \( \Delta z_{\text{spring}} \) is the distance between the two springs attached to the C and N terminus, respectively, and \( j-1 \) denotes the force peak preceding peak \( j \). Here, \( k_{\text{AFM}} \) was estimated as 10 pN nm⁻¹, which is an upper limit. This selection procedure mimics the effect of the soft AFM cantilever, which, in contrast to the force probe simulations, is insensitive to minor force peaks that immediately follow a larger peak. Second, the number of unfolded residues \( N_{\text{unfinj}} \) and the length of the folded core \( \Delta L_{\text{fold},j} \) were determined for the conformations corresponding to each of these peaks. The contour length \( L_{j} \) was then calculated as \( L_{j} = N_{\text{unfinj}} \times 0.365 \text{ nm} + \Delta L_{\text{fold},j} \). Third, a Gaussian function was assigned to each of the peaks, weighted by its height \( F_{\text{max},j} ^{2} \):

\[
G_{j}(L_{i}) = F_{\text{max},j}^{2} \exp \left( \frac{(L_{i} - L_{j})^{2}}{2 \sigma^{2}} \right),
\]

with a width \( \sigma = 1 \text{ nm} \). The final contour length plot is obtained from the sum of all these Gaussian functions. As an exception, this procedure was not applied to the force peak marked with a plus sign in Fig. 3B Top. Structural analysis shows that peak arises from transient rupture and reformation of the interactions between Lys-36/Met-34 and ATP, similar to the subsequent ATP peak 2*.

Following the same argumentation as outlined above, this choice yields a barrier position for peak 2 of 35.5 nm, in good agreement with the 32.3 nm obtained from the AFM data. We note that, in contrast to peak 2*, where the anchor point can be easily assigned to the Met-34/Lys-36 motive, such a detailed assignment is more challenging for peak 2. Here, only a range of \( \beta R1 \) residues between Glu-5 and Asp-12 can be specified, thus leading to an uncertainty in the position of peak 2 of ~1.5 nm. Fig. S7 compares the barrier positions obtained from the AFM experiments and the two independent MD simulations of the complete TK in the absence of ATP (Fig. 3B Bottom). The MD simulations yield positions of peaks 2, 3, 4, and 5 at 35.5, 63.3, 80.1, and 86.8 nm, respectively. The corresponding peak positions obtained from the AFM experiments are 32.3, 61.3, 68.6, and 86.6 nm, respectively. For the force peaks 2, 3, and 5, the positions obtained from the simulations agree well with those obtained from the experiments, taking into account that the former are based on only two independent simulations. For peak 4, the difference between the simulations and the experiments is somewhat larger. We speculate that this difference results from the underlying free energy landscape that, at such partially unfolded conformations, is shallow and exhibits many barriers of comparable height along various unfolding directions, such that the pathways followed in the individual trajectories are different. Such heterogeneity of unfolding pathways is indeed seen from the color-coded unfolding events in Fig. 3B Bottom. A much larger number of trajectories would be required to thoroughly characterize this region of the energy landscape, which, however, is not the focus of this work.


Fig. S1. (Left) Wild type (WT) and K36A mutant of the titin kinase A168-M2 construct after two-step purification; 2.5 μg of both WT and KA mutant titin kinase were loaded. (Right) Titin kinase domain kin3 after three-step purification; 1 μg was loaded. Molecular mass marker (in kDa) from top: 94, 64, 43, 30, 20, and 14.

TK WT
MW marker
TK KA
TK kin3
MW marker
Fig. S2.  (a) Schematics of the experimental setup. An IR laser is coupled through a fiber into the AFM head where it is focused onto the cantilever with a microoptic. The reflected beam is detected by a computer adjustable four-quadrant photodiode. The AFM head is moved up and down by a Z-piezo drive, and the sample is moved after each trace by a x–y piezo stage. To avoid evaporation of the buffer, we used a sealed measurement chamber. (b) Schematic overview of the AFM experiment showing the initial attachment of the protein to surface and cantilever (1), straightening of the protein to its folded length of \(-25 \text{ nm}\) (2), unfolding of the kinase at low force (3), and sequential unfolding of the independently folding Ig and Fn3 domains at high forces (4–7). A rupture peak is observed when the completely unfolded peptide chain is pulled off the cantilever or surface (8).
Fig. S3. Selection of traces and identification of the TK unfolding pattern in the absence of ATP. “A” is the superposition of traces showing the unfolding of one to five spacer domains and the complete unfolding pattern of the titin kinase domain. Traces are shifted with respect to each other whereas the rupture of the first Ig-domain served as the barrier of reference. In this way the different offsets in the initial part of the force extension traces, caused by rupture of spacer domain fragments and unspecific interactions, are taken into account. For clarity the points are plotted semitransparent. Once we identified the unfolding pathway of the titin kinase by means of traces showing more than three spacer domains (e.g., B), we took also traces into account exhibiting the complete unfolding pattern of the titin kinase but less spacer domains (C–E).
Fig. S4. Histogram of experimental Ig/Fn domain contour length increments. The values are obtained by transforming each trace with the QM-WLC model (persistence length $P = 0.8 \text{ nm}$) into a barrier position histogram [Puchner EM, Franzen G, Gautel M, Gaub HE (2008) *Biophys J* 95:426–434] and by fitting the corresponding peaks with Gaussians. The mean value amounts to 30.45 nm, and the standard deviation values 1.68 nm.
Fig. S5. Superposition of traces from different TK protein constructs. The traces in black show the unfolding of the *E. coli*-expressed protein construct consisting of five spacer domains whereas the other one contains only three spacer domains (blue). The maximum number of even spacings at the end of the traces (ΔL = 30 nm) reflects the number of spacer domains in both cases.
Fig. S6. Unfolding pattern of the titin kinase at 37°C. The black points show the superposition of traces recorded at room temperature, whereas the red traces were recorded at 37°C. Although the unfolding forces are smaller by ~20 pN, the unfolding pattern is the same at 37°C.
Fig. S7. Barrier positions from AFM experiments and MD simulations in the absence of ATP. The latter were obtained from the simulations of the complete TK (321 residues) shown in Fig. 3B Bottom. The positions of the force peaks obtained from the AFM experiments are indicated by the dashed lines.
Movie S1. Structural changes during peak $2^*$: Unfolding of titin kinase (ribbon representation) upon mechanical stress, with ATP (ball-and-stick representation) bound to the active site. Interactions with ATP (dashed lines) cause an additional force peak ($2^*$ in Fig. 2) in both the AFM experiment and the molecular dynamics simulations, which is not seen in the ATP-free state. Particularly strong interactions contributing to this peak are seen with Met-34 and Lys-36, which are shown in ball-and-stick representation. The bond with Met-34 with the purine base breaks before that of Lys-36 with the $\alpha$-$\beta$ phosphate groups.
Table S1. Conversion of residues in N2B titin (NP_003310.3) to the numbering in the titin kinase crystal structure 1TKI

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<th>Residue in 1TKI</th>
<th>Residue in N2B titin</th>
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<tr>
<td>Met-34</td>
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Table S2. Expected experimental contour length increments of Ig/Fn-domain unfolding

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<th>Domain</th>
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<td>29.95</td>
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The theoretical contour length increments were calculated by multiplying the number of folded amino acids with 0.365 nm and subtracting the diameter of the folded domains. The mean value amounts to 30.86 nm.
Table S3. Schematic structure of ATP (above), defining the atom numbers used in the tables, and atomic partial charges for ATP (below)

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