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61/364,640 15 July 2010 (15.07.2010) US(71) Applicant (for all designated States except US): **THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK** [US/US]; 412 Low Memorial Library, 535 West 116th Street, New York, NY 10027 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **FERNANDEZ, Julio, M.** [US/US]; 1003 Fairchild Center, 1212 Amsterdam Avenue, New York, NY 10027 (US). **PEREZ-JIMENEZ, Raul** [ES/US]; 1003 Fairchild Center, 1212 Amsterdam Avenue, New York, NY 10027 (US). **KO-SURI, Pallav** [SE/US]; 331 W. 43rd St., Apt.5c, New York, NY 10036 (US).(74) Agents: **LOVE, Jane** et al.; Wilmer Cutler Pickering Hale And Dorr LLP, 399 Park Avenue, New York, NY 10022 (US).

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(54) Title: FORCE-CLAMP SPECTROMETER AND METHODS OF USE

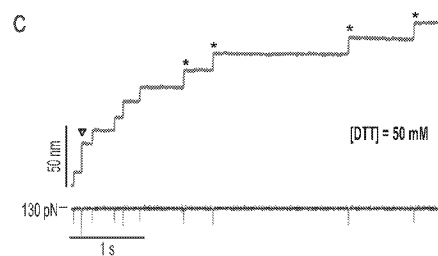
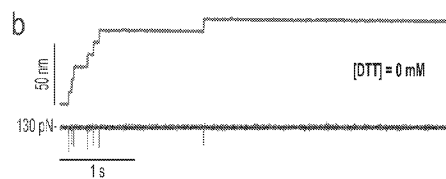
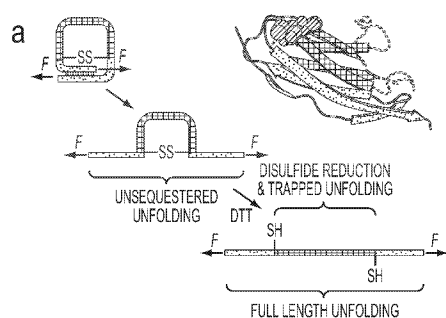


FIG. 5

(57) Abstract: The disclosed subject matter relates to a force-clamp spectrometer that enables operation in constant force mode and allows for automated data acquisition and analysis, using feedback electronics and software. The disclosed subject matter also relates to methods of using the force-clamp spectrometer for the measurement of the dynamics of chemical reactions. The methods may include, but are not limited to, the measurement of the dynamics of substrate folding and unfolding, as well as bond cleavage and bond formation.



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FORCE-CLAMP SPECTROMETER AND METHODS OF USE

[0001] This application claims priority to U.S. Provisional Application No. 61/364,208, filed on July 14, 2010, and U.S. Provisional Application No. 61/364640 filed on July 15, 2010, each of which is incorporated herein by reference in its entirety.

[0002] This invention was made with government support under HL66030 and HL61228 awarded by NIH. The government has certain rights in the invention.

[0003] All patents, patent applications, published patent applications, granted patents and publications cited herein are hereby incorporated by reference in their entirety. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described herein.

[0004] To conform to the requirements for International Patent Applications, many of the figures presented herein are in black and white. The original color versions can be viewed in Alegre-Cebollada et al., "Single-molecule Force Spectroscopy Approach to Enzyme Catalysis," *Journal of Biological Chemistry*, Vol 285, pp. 18961-18966 (2010); Perez-Jimenez et al., "Diversity of Chemical Mechanisms in Thioredoxin Catalysis Revealed by Single-Molecule Force Spectroscopy", *Nat. Struct. Mol. Biol.*, Vol 16(8), pp. 890-896 (2009); Wiita et al., "Probing the Chemistry of Thioredoxin Catalysis With Force", *Nature* 450, 124-127 (2007); and Wiita et al., "Force-Dependent Chemical Kinetics of Disulfide Bond Reduction Observed With Single-Molecule Techniques. *PNAS* 103:19, 7222-7227 (2006). For the purposes of the U.S., the contents of Alegre-Cebollada et al., "Single-molecule Force Spectroscopy Approach to Enzyme Catalysis," *Journal of Biological Chemistry*, Vol 285, pp. 18961-18966 (2010); Perez-Jimenez et al., "Diversity of Chemical Mechanisms in Thioredoxin Catalysis Revealed by Single-Molecule Force Spectroscopy," *Nat. Struct. Mol. Biol.* Vol 16(8), pp. 890-896 (2009); Wiita et al., "Probing the Chemistry of Thioredoxin Catalysis With Force," *Nature* 450, 124-127 (2007); and Wiita et al. Force-Dependent Chemical Kinetics of Disulfide Bond Reduction Observed With Single-Molecule Techniques. *PNAS* 103:19, 7222-7227 (2006)," are herein incorporated by reference.

BACKGROUND

[0005] This disclosure relates to force-clamp spectrometers and force-clamp spectroscopy techniques.

[0006] The intersection of force and chemistry has been studied for over a century, yet not much is known about this phenomenon compared with more common methods of chemical catalysis. There are a number of reasons for this discrepancy, but one of the most important factors remains that it is quite difficult to directly measure the effect of force on a bulk reaction. This difficulty arises because an applied force is not a scalar property of a system; it is associated with a vector. As a result, it is often not possible to directly probe the effect of force on a particular reaction because of heterogeneous application of force and a distribution of reaction orientations (Beyer, M. K. & Clausen-Schaumann, H. (2005) *Chem. Rev.* 105, 2921–2948). To fully quantify the effect of an applied force on a chemical reaction, it is necessary to generate an experimental system where the reaction of interest is consistently oriented with respect to the applied force.

[0007] The direct manipulation of single molecules allows for the application of force in a vector aligned with the reaction coordinate (Evans, E. & Ritchie, K. (1997) *Biophys. J.* 72, 1541–1555), avoiding the heterogeneity of bulk studies. Earlier works using single molecule techniques have described the rupture forces necessary to cleave single covalent bonds, including Si–C bonds in polysaccharide attachment (Grandbois, M., Beyer, M., Rief, M., Clausen-Schaumann, H. & Gaub, H. E. (1999) *Science* 283, 1727–1730), Au–Au bonds in nanowires (Marszalek, P. E., Greenleaf, W. J., Li, H., Oberhauser, A. F. & Fernandez, J. M. (2000) *Proc. Natl. Acad. Sci. USA* 97, 6282–6286; Rubio-Bollinger, G., Bahn, S. R., Agrait, N., Jacobsen, K. W. & Vieira, S. (2001) *Phys. Rev. Lett.* 87, 026101), and Ni²⁺–NTA attachments (Conti, M., Falini, G. & Samori, B. (2000) *Angew. Chem. Int. Ed.* 39, 215–218).

[0008] However, these studies have not been able to describe the effect of force on the dynamics and kinetics of these reactions, nor have they examined more complex chemical reactions beyond simple bond rupture.

[0009] Accordingly, there is a need in the art for devices and methods to describe the effect of force on the dynamics and kinetics of these reactions, and to examine more complex chemical reactions beyond simple bond rupture.

SUMMARY

[0010] The disclosed subject matter relates to a force-clamp spectrometer that enables operation in constant force mode and allows for automated data acquisition and analysis, using feedback electronics and software. The force-clamp spectrometer may be used in methods for the measurement of the dynamics of chemical reactions, including, but not

limited to, the dynamics of the measurement of substrate folding and unfolding, as well as bond cleavage and bond formation.

[0011] In some embodiments, the force-clamp spectrometer is used to manipulate substrates, including, but not limited to, single proteins, to study substrate folding and unfolding. For example, the method may directly measure the dynamics of folding and unfolding events in real-time. In one embodiment, the dynamics of folding and unfolding are identified through a fingerprint signal, thus removing the risk of false positives and the need for control experiments. In another embodiment, the dynamics are measured as a function of a force applied. The force may be applied to a bond, allowing for force spectroscopy reactions involving the bond. In other embodiments, the force-clamp spectrometer is used for the measurement of the dynamics of the cleavage of a bond. For example, the method may directly resolve bond cleavage events in real-time. In one embodiment, the dynamics are identified through a fingerprint signal, removing the risk of false positives and the need for control experiments. In another embodiment, the dynamics are measured as a function of a force applied. The force may be applied to a bond, allowing for force spectroscopy reactions involving the bond. In yet other embodiments, the force-clamp spectrometer is used for the measurement of the formation of a bond. For example, the method may measure the dynamics of bond formation in real-time. In one embodiment, the dynamics are identified through a fingerprint signal, removing the risk of false positives and the need for control experiments. In another embodiment, the dynamics are measured as a function of a force applied. The force may be applied to a bond, allowing for force spectroscopy reactions involving the bond.

[0012] In some embodiments, a single molecule is suspended between the tip of the cantilever and the coverslip. The extension of this molecule is controlled through a piezo, while the force generated is measured through the bending of the cantilever. The bending angle of the cantilever is constantly monitored by reflecting a laser beam off its backside, and detecting the reflected beam on a split photodetector. The force on the cantilever is thus directly proportional to the difference signal from the photodetector, i.e. the difference in voltage between its two sensors.

[0013] The cantilever chip is mounted in the fluid cell, which includes an inclined cantilever mount adjacent to a transparent surface where the laser beam can pass through. The laser can be focused on the cantilever by monitoring the beam spot through the camera. When the alignment is done, the camera and objective are swiveled out of the way. The

coverslip is mounted on the piezo, (liquid) sample is added to the coverslip, and the piezo can then be swiveled into place, facing the cantilever.

[0014] During force-clamp operation, the feedback system adjusts the extension of a suspended molecule until a set-point force is reached. This is achieved through a negative feedback circuit that takes the difference between the measured force and the setpoint force as error signal, processes it, and then feeds this signal back to the piezo.

[0015] In one aspect, the instant disclosure relates to a force-clamp spectrometer. The force-clamp spectrometer may include, for example, a cantilever chip; a piezo-electric positioner; a laser and focusing optics; a split photodetector; a data acquisition card; a computer; feed-back electronics; a cantilever holder or a fluid cell, at least one movable stage; a sample disc or a sample coverslip; a CCD camera; a microscope objective; a prism; at least one mount; an optical bread board; a vibration insulation table; and control and analysis software.

[0016] In some embodiments, a force-clamp spectrometer is provided that includes a cantilever, a force applicator mounted on a hinged stage; a coverslip positioned over the force applicator, wherein the coverslip is configured to suspend a single molecule between the tip of the cantilever and the coverslip; and a detector to measure bending of the cantilever. In one embodiment, the force applicator is a piezo-electric positioner. In another embodiment, the detector comprises a laser and optics. In yet another embodiment, the detector comprises a split photodetector.

[0017] In some embodiments, the cantilever chip is, for example, a MLCT chip from Veeco; the piezo-electric positioner is a PicoCube™ positioner from Physic Instrumente; the laser & focusing optics is 51nanoFCM, from Schafer + Kirchhoff; the split photodetector is a QP50 photodetector from Pacific Silicon Sensor; the data acquisition card is USB-6289, from National Instruments. In other embodiments, the cantilever holder or the fluid cell is a MMTMEC holder/cell from Veeco and the movable stages are Agilis™ mounts from Newport®.

[0018] The feedback settings, force protocol and data acquisition are all controlled from the computer, for example, through a custom made software package developed in IGOR (Wavemetrics). The software allows for automated operation for several days without manual intervention. Also included in the software are analysis features developed for several specific assays, including the study of bond cleavage, bond formation, and folding and unfolding.

[0019] In another aspect, the instant disclosure relates to a method for the measurement of the dynamics of chemical reactions. Dynamics may include, but are not limited to, kinetic rates and shifts in equilibrium. In one embodiment, the kinetic rates are measured. In another embodiment, shifts in equilibrium are measured.

[0020] In another embodiment, the instant disclosure relates to a method of measuring the cleavage of a bond. In some embodiments, the bond is a covalent bond. In other embodiments, the bond is a non-covalent bond. The method may include, for example, providing a sample and analyzing the sample using the force-clamp spectrometer of the instant disclosure. In one embodiment, the sample comprises a substrate. In another embodiment, the sample comprises one or more enzymes. In another embodiment, the sample comprises a substrate and an enzyme. In some embodiments, the enzyme is an oxidoreductase enzyme. In other embodiments, the enzyme is a thioredoxin. In still other embodiments, the enzyme is a protein disulfide isomerase. In another embodiment, the enzyme is protease, esterase, phosphodiesterase, or glycosidase. In another embodiment, the enzyme is a hydrolase. In some embodiments, the sample comprises a compound that reacts with a bond. The compound may be, but is not limited to, a reducing agent, an oxidizing agent, a nanoparticle, or a small molecule. In another embodiment, the sample comprises an ion. In some embodiments, the sample comprises an inhibitor. In other embodiments, the sample comprises an activator. Inhibitors and activators may be, but are not limited to, small molecules. In one embodiment, the inhibitor is an enzyme inhibitor. In another embodiment, the activator is an enzyme activator.

[0021] In another embodiment, the instant disclosure relates to a method of measuring the formation of a bond. In some embodiments, the bond is a covalent bond. In other embodiments, the bond is a non-covalent bond. The method may include, for example, providing a sample and analyzing the sample using the force-clamp spectrometer of the instant disclosure. In one embodiment, the sample comprises a substrate. In another embodiment, the sample comprises one or more enzymes. In another embodiment, the sample comprises a substrate and an enzyme. In some embodiments, the enzyme is an oxidoreductase enzyme. In other embodiments, the enzyme is a thioredoxin. In still other embodiments, the enzyme is a protein disulfide isomerase. In another embodiment, the enzyme is protease, esterase, phosphodiesterase, or glycosidase. In another embodiment, the enzyme is a hydrolase. In some embodiments, the sample comprises a compound that reacts with a bond. The compound may be, but is not limited to, a reducing agent, an oxidizing

agent, a nanoparticle, or a small molecule. In another embodiment, the sample comprises an ion. In some embodiments, the sample comprises an inhibitor. In other embodiments, the sample comprises an activator. Inhibitors and activators may be, but are not limited to, small molecules. In one embodiment, the inhibitor is an enzyme inhibitor. In another embodiment, the activator is an enzyme activator.

[0022] In another embodiment, the instant disclosure relates to a method of measuring the folding and/or the unfolding of a substrate. The method may include, for example, providing a sample and analyzing the sample using the force-clamp spectrometer of the instant disclosure. In one embodiment, the sample comprises a substrate. In another embodiment, the sample comprises one or more enzymes. In another embodiment, the sample comprises a substrate and an enzyme. In some embodiments, the enzyme is an oxidoreductase enzyme. In other embodiments, the enzyme is a thioredoxin. In still other embodiments, the enzyme is a protein disulfide isomerase. In some embodiments, the sample comprises a compound that reacts with a bond. The compound may be, but is not limited to, a reducing agent, an oxidizing agent, a nanoparticle, or a small molecule. In another embodiment, the sample comprises an ion. In some embodiments, the sample comprises an inhibitor. In other embodiments, the sample comprises an activator. Inhibitors and activators may be, but are not limited to, small molecules. In one embodiment, the inhibitor is an enzyme inhibitor. In another embodiment, the activator is an enzyme activator.

[0023] In another aspect, the instant disclosure relates to a substrate used for measuring the dynamics of chemical reactions. In one embodiment, the substrate is used for measuring the cleavage of a bond. In another embodiment, the substrate is used for measuring the formation of a bond. In yet another embodiment, the substrate is used for measuring folding and/or unfolding. In one embodiment, the substrate is a polymer. In one embodiment, the substrate is a protein or a polypeptide. In another embodiment, the substrate is a nucleic acid. In another embodiment, the substrate is a polysaccharide. In another embodiment, the substrate is an engineered polymer.

[0024] In one embodiment, the commercial opportunity for the disclosed subject matter lies in the precision of measurement of the reactivity of a reagent (such as an enzyme or a chemical compound) towards a specific bond. Coupled with force spectroscopy, the reaction kinetics measurements can reveal details about the reagent, for example, the direct detection of bond cleavage and/or bond formation events, as well as folding and/or unfolding events, as opposed to the measurement of coupled reactions that might introduce bias in the data.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] The following figures are provided for the purpose of illustration only and are not intended to be limiting.

[0026] **FIG. 1** is a view of an illustrative force clamp spectrometer according to some embodiments of the disclosed subject matter.

[0027] **FIG. 2** is a series of views showing an illustrative flip platform of an illustrative force clamp spectrometer according to some embodiments of the disclosed subject matter. FIG. 2A shows the illustrative flip platform in the measuring position. FIG. 2B shows the illustrative flip platform in the sample mounting position. FIG. 2C shows the illustrative flip platform in the laser focusing position.

[0028] **FIG. 3** is a top view of an illustrative flip piezo mount in the up position showing the sample coverslide, the piezo electric actuator and the piezo motor according to some embodiments of the disclosed subject matter.

[0029] **FIG. 4** is a detail front view of an illustrative force sensor according to some embodiments of the disclosed subject matter.

[0030] **FIG. 5A** is a schematic showing the unfolding of I27G32C–A75C. FIG. 5B shows the stepwise elongation (red trace) of an (I27G32C–A75C)₈ polypeptide pulled at a constant force of 130 pN (black trace) in the absence of DTT. FIG. 5C shows the stepwise elongation (red trace) of an (I27G32C–A75C)₈ polypeptide pulled at a constant force of 130 pN (black trace) in the presence of 50 mM DTT.

[0031] **FIG. 6A** shows a typical double-pulse force-clamp experiment pulling the (I27G32C–A75C)₈ protein first at 130 pN for 1 s and then stepping to a force of 200 pN for 7 s (black trace). FIG. 6B shows the same experiment in the presence of 12.5 mM DTT.

[0032] **FIG. 7** shows ensemble measurements of the kinetics of thiol/disulfide exchange. FIG. 7A shows three recordings are shown of single (I27G32C–A75C)₈ polypeptides that were extended with the same double-pulse protocol shown in Fig. 6B. FIG. 7B upper shows a four-trace average (red trace) of the double-pulse experiments shown in FIG. 7A and FIG. 6B. FIG. 7B lower shows the average force traces.

[0033] **FIG. 8** shows graphs of thiol/disulfide exchange events measured by using the double-pulse protocol as a function of force and DTT concentration.

[0034] **FIG. 9A** is a plot of the rate of the thiol/disulfide exchange. FIG. 9B is a plot of the energy landscape of the thiol/disulfide exchange reaction under force. FIG. 9C is an

illustration of the thiol/disulfide exchange reaction between a DTT molecule and a disulfide bond under a stretching force

[0035] **FIG. 10A** is a schematic illustrating molecular stretching. **FIG. 10B** shows the results of stretching a single (I27SS)8 molecule is stretched in the absence of Trx. **FIG. 10C** shows the results of stretching a single (I27SS)8 molecule is stretched in the presence of 8 μ M Trx.

[0036] **FIG. 11A** shows results of an experiment where multiple single-molecule recordings of the test pulse only ($n=10-30$) were averaged to monitor the kinetics of disulphide bond reduction under force F . A single exponential is fitted to each averaged trace (smooth line), and the rate constant of reduction $r=1/\tau$. **FIG. 11B** shows r as a function of force at $[\text{Trx}]=8 \mu\text{M}$. **FIG. 11C** shows r as a function of $[\text{Trx}]$ at various forces. Error bars in 11B and 11C represent the s.e.m. obtained from bootstrapping. Solid lines in **FIGS. 11B** and 11C are fits using the kinetic model shown in **FIG. 11D**.

[0037] **FIG. 12** is a plot of r as a function of force at $[\text{Trx(P34H)}] = 8 \mu\text{M}$.

[0038] **FIG. 13A** is a diagram of TRX (peptide-binding groove in dark green) bound to an NF- κ B peptide. The inset (yellow spheres are sulphur atoms A, B and C) shows the relative position of the disulphide bond between TRX Cys 32 (sulphur atom A) and the NF- κ B cysteine (sulphur atom B). **FIG. 13B** is a cartoon representation of the reduction by Trx of a disulphide bond in a stretched polypeptide. **FIG. 13C** shows force-dependent reduction by human TRX compared to E. coli Trx.

[0039] **FIG. 14** shows the phylogeny of Trx homologs from representative species of the three domains of life.

[0040] **FIG. 15** shows results from single-molecule force-clamp detection of disulfide bond-reduction events catalyzed by Trx enzymes. **FIG. 15A** is a graphic representation of the force clamp experiment. **FIG. 15B** is a trace showing the unfolding and consequent disulfide reductions of a (I27G32C-A75C)8 polyprotein. **FIG. 15C** and **15D** are plots showing the probability of reduction ($\text{Pred}(t)$) obtained by summing and normalizing traces of disulfide bond reductions at different forces (second pulse) for pea Trxm (10 mM) (**FIG. 15C**) and for poplar Trxh1 (10 mM) (**FIG. 15D**).

[0041] **FIG. 16** shows graphs depicting the force-dependency of the rate of disulfide reduction by Trx enzymes from different species.

[0042] **FIG. 17** shows three chemical mechanisms of disulfide reduction detected by force-clamp spectroscopy.

[0043] **FIG. 18** depicts structural analysis and molecular dynamics simulations of the binding groove in Trx enzymes.

[0044] **FIG. 19** is a schematic diagram of an illustrative single-molecule force spectroscopy assay for the detection of single reduction events by the enzyme thioredoxin.

[0045] **FIG. 20** presents data and a schematic diagram illustrating the use of an illustrative single-molecule force spectroscopy method as a probe to study chemical reactions and enzymatic catalysis.

[0046] **FIG. 21.** A single-molecule approach to the study of oxidative folding. FIG. 21A-21B, Protein disulfide isomerase (PDI) catalyzes disulfide formation in a polypeptide (blue) undergoing ER translocation. FIG. 21C-21D, With the use of an atomic force microscope, we could reproduce the *in vivo* cysteine separation (a) and initiate folding from this state. Disulfides and folded structures were subsequently identified through their mechanical fingerprints.

[0047] **FIG. 22.** PDI-catalyzed oxidative folding of Ig domains. FIG. 22A, Mechanical unfolding and refolding of an individual Ig domain in the presence of reduced PDI a. Applied force enables stochastic unfolding that is hindered by the presence of an intramolecular disulfide, yielding a stepwise extension of 11 nm and exposing the disulfide. This bond can now be cleaved by reduced PDI a, yielding an additional 14 nm extension step and establishing an intermediate complex. Switching off the force triggers collapse and folding. FIG. 22B, Representative trace using a polyprotein substrate consisting of sequential Ig domains. A [denature – Δt – probe] protocol was used, as described in the text. Arrowheads indicate disulfide cleavage events (14 nm steps). In this trace, six Ig domains were completely unfolded and reduced. Four of these subsequently underwent complete oxidative folding. Other traces revealed refolding without disulfide formation (25 nm step, inset). FIG. 22C, Step size histograms confirm that PDI a catalyzes oxidative folding in some domains and reduce other domains.

[0048] **FIG. 23.** Replacement of a single atom in TRX enables PDI-like catalysis of oxidative folding. FIG. 23A, The Ig polyprotein was unfolded and refolded in the presence of reduced human TRX. Arrowheads indicate disulfide cleavage events (14 nm steps). In this trace, seven domains were completely denatured. Four of these subsequently refolded, albeit without disulfide formation. FIG. 23B, Step size histograms confirm that TRX did not catalyze oxidative folding, as seen from the absence of 14 nm steps in the *probe* pulse. FIG 23C, Representative trace showing that TRX C35S can cleave disulfides and also catalyze

oxidative folding. In the *probe* pulse, 14 nm steps were occasionally seen without preceding 11 nm steps, indicating that disulfide formation had taken place whereas folding of these domains had not completed. FIG 23D, Step size histograms show that disulfides were formed in all refolded domains.

[0049] **FIG. 24.** Quantitative comparison of enzyme catalyzed oxidative folding. FIG. 24A, Substrate refolding after $\Delta t = 5$ s. All three enzymes allowed refolding to take place. FIG. 24B, Percent of refolded domains displaying intramolecular disulfides. FIG. 24C, Percent of substrate domains having successfully completed oxidative folding. PDI and TRX C35S displayed no significant difference in the catalysis of oxidative folding, whereas TRX showed no detectable activity. FIG. 24D, Substrate refolding kinetics, as percentage of initial number of folded substrate domains. FIG. 24E, Disulfide formation kinetics, as percentage of initial number of disulfides in substrate. Solid lines show exponential fits to the data. $N = 84$ traces (TRX), $N = 344$ traces (TRX C35S) FIG. 24F, In TRX, rapid nucleophilic attack by Cys35 Sy leaves the substrate reduced before folding can take place. FIG. 24G, Substitution of a single atom (S $\rightarrow$ O) eliminates the escape pathway of TRX, and the enzyme lingers on the extended substrate. Switching off the applied force (or continued translocation *in vivo*) triggers collapse and enables folding, while retention of the intermediate enzyme complex enables catalyzed oxidation of the substrate disulfide. Error bars indicate the s.e.m. as determined from bootstrap analysis.

[0050] **FIG. 25.** Conceptual summary. FIG. 25A, Thiol-disulfide oxidoreductases in the thioredoxin superfamily catalyze disulfide exchange by forming a mixed disulfide intermediate complex with the substrate. FIG. 25B, TRX catalyzes only reduction *in vivo*. FIG. 25C, PDI exhibits two reaction paths. The results show that the pathway preference results from a kinetic competition between the escape pathway and substrate folding.

[0051] **FIG. 26.** The Ig polypeptide unfolded in the presence of only 500 μ M DTT displayed 11 nm partial unfolding steps but did not show any 14 nm reduction steps.

[0052] **FIG. 27.** Experiments on the Ig polypeptide in the presence of 500 μ M DTT showed only unfolding and refolding of the unsequestered part of the substrate. FIG. 27A, Representative trace. FIG. 27B, Step size histograms.

[0053] **FIG. 28.** Oxidative folding with PDI **a** shows 25 nm steps in the *probe* pulse, evidence of incomplete oxidative folding.

[0054] **FIG. 29.** Functional assay to measure effect of inhibitor molecule on enzymatic activity. Using a force-clamp spectrometer, the enzymatic activity of 200 μ M PDI was

measured by detecting the cleavage of a disulfide in a substrate I27 protein. Accumulation of hundreds of such events yielded a kinetic rate given as events per second. By adding the inhibitor to the reaction volume, the measured rate decreased in a concentration-dependent manner.

[0055] FIG. 30 is a view of an illustrative force clamp spectrometer according to some embodiments of the disclosed subject matter

DETAILED DESCRIPTION

[0056] In one aspect, the instant disclosure relates to a device that comprises a flip force spectrometer, a USB electronic controller box, and a computer for data acquisition and analysis.

[0057] In a typical experiment, a single molecule is suspended between the tip of the cantilever and the coverslip. The extension of this molecule is controlled through the piezo, while the force generated is measured through the bending of the cantilever. The bending angle of the cantilever is constantly monitored by reflecting a laser beam off its backside, and detecting the reflected beam on a split photodetector. The force on the cantilever is thus directly proportional to the difference signal from the photodetector, i.e. the difference in voltage between its two sensors.

[0058] The cantilever chip is mounted in the fluid cell, which in its simplest version consists of an inclined cantilever mount adjacent to a transparent surface where the laser beam can pass through. The laser can easily be focused on the cantilever by monitoring the beam spot through the camera. When the alignment is done, the camera and objective are swiveled out of the way. The coverslip is mounted on the piezo, (liquid) sample is added to the coverslip, and the piezo can then be swiveled into place, facing the cantilever.

[0059] During force-clamp operation, the feedback system adjusts the extension of a suspended molecule until a set-point force is reached. This is achieved through a negative feedback circuit that takes the difference between the measured force and the setpoint force as error signal, processes it, and then feeds this signal back to the piezo.

[0060] The feedback settings, force protocol and data acquisition can be all controlled from the computer, for example, through a custom made software package developed in IGOR (Wavemetrics). The software allows for automated operation for several days without manual intervention. Also included in the software are analysis features developed for several

specific assays, including the study of bond cleavage and formation and protein folding and unfolding.

[0061] This device is uniquely designed for doing force-clamp spectroscopy of single proteins. As shown in FIG. 1, the device includes a PID controller for the force-clamp function together with a high voltage power supply for piezo actuator displacement (gray box). The device includes a USB controller for the piezo motors (smaller elongated controller with buttons) and a USB data acquisition and control unit (white box with cable). The device is mounted on a small perforated optical table and placed on top of a vibration isolation table (black box underneath the spectrometer). The entire instrument may be connected to a computer via, for example, a single USB cable.

[0062] The combined effect of these components is the ability, with this device, to mechanically manipulate single proteins to study protein unfolding/folding and chemical reactions. The instrument is easy to use. An operator without any experience, for example, can learn to use it effectively in a few hours of training. High quality single protein data can be rapidly obtained, on demand.

[0063] As shown in FIG. 2 and FIG. 3, the device includes flip-force spectrometer components. The flip design minimizes the number of moving parts of the spectrometer, thereby greatly reducing drift and difficulty of operation. In some embodiments, the flip design includes a flip platform onto which a remotely operated piezo-motor controls the positioning of the main piezoelectric actuator that actually pulls the molecules under feedback. In its measuring position (FIG. 2A), the flip platform is down and presents the sample to a cantilever mounted on the force sensor. In the set up configuration (FIG. 2B and FIG. 3), the piezo mount is flipped up allowing access to the cantilever holder for cantilever placement, and allowing the protein sample to be placed on the surface of the piezo. Once a cantilever is mounted in the force sensor, the video microscope is flipped-in to observe the cantilever at high magnification (FIG. 2C), allowing the operator to precisely focus the laser beam on the cantilever. Once this is done, the video microscope is flipped back, and the piezoelectric actuator with the new sample is flipped down into its measuring position (FIG. 2A). This arrangement and sequence of events is a feature of this device.

[0064] The force sensor may be a modification of AFM instrumentation altered to suit the requirements of a single protein force spectrometer, which include but are not limited to reduce drift, improve ease of operation, and minimize the number of moving parts. As shown in FIG. 4, the device includes a fixed cantilever holder cell mounted with the cantilever

facing up to enable the flip design and facilitate cantilever removal and laser focusing. As shown in FIG. 3, the laser is focused on the cantilever using a tilt platform while observing the operation with the video microscope. As shown in FIG. 4, the reflected beam is steered towards the split photodiode using a prism mounted on magnets to eliminate drift. As shown in FIG. 4, the split photodiode is placed on top of an Agilis piezo motor to precisely position it remotely and automatically by the controlling computer. The device may include, for example, upwards placement of the cantilever, a magnetic mount for the deflecting prism, and an Agilis mounted photodiode which permits a fully hands-off remote operation.

[0065] The instrument as it stands is fully automatic and may operate for hours or days without intervention of the operator.

[0066] In another aspect, the instant disclosure relates to the use of the force-clamp spectrometer in a method for the measurement of the dynamics of chemical reactions. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium.

[0067] In some embodiments, the force-clamp spectrometer is used in a method for the measurement of the folding of a substrate. In other embodiments, the force-clamp spectrometer is used in a method for the measurement of the unfolding of a substrate. In some embodiments, the dynamics of folding and/or unfolding are measured in real-time. In other embodiments, the dynamics of folding and/or unfolding are identified through a fingerprint signal, thus removing the risk of false positives and the need for control experiments. A fingerprint may include knowledge of the number of bonds within the substrate and/or the location of these bonds. The fingerprint can thereby be verified as the number of steps and/or the amplitude of these steps when monitoring the substrate extension during the reaction, for example. In other embodiments, the dynamics are measured as a function of a force applied. This force may be applied to a bond, allowing for force spectroscopy of the reactions involving the bond. In some embodiments, the folding or unfolding of a substrate is catalyzed by an enzyme.

[0068] In other embodiments, the force-clamp spectrometer is used in a method for the measurement of the cleavage of a bond in a substrate. In some embodiments, the dynamics of bond cleavage are measured in real-time. In other embodiments, the dynamics of bond cleavage are identified through a fingerprint signal, thus removing the risk of false positives and the need for control experiments. A fingerprint may include knowledge of the number of bonds within the substrate and/or the location of these bonds. The fingerprint can thereby be verified as the number of steps and/or the amplitude of these steps when monitoring the

substrate extension during the reaction, for example. In other embodiments, the dynamics of bond cleavage are measured as a function of a force applied. In some embodiments, the bond is cleaved by a compound that reacts with the bond. In other embodiments, bond cleavage is catalyzed by an enzyme.

[0069] In yet other embodiments, the force-clamp spectrometer is used in a method for the measurement of the formation of a bond in a substrate. In some embodiments, the dynamics of bond formation are measured in real-time. In other embodiment, the dynamics of bond formation are identified through a fingerprint signal, thus removing the risk of false positives and the need for control experiments. A fingerprint may include knowledge of the number of bonds within the substrate and/or the location of these bonds. The fingerprint can thereby be verified as the number of steps and/or the amplitude of these steps when monitoring the substrate extension during the reaction, for example. In other embodiments, the dynamics of bond formation are measured as a function of a force. In some embodiments, bond formation is catalyzed by an enzyme.

[0070] In one aspect, the instant disclosure relates to a method of measuring the dynamics of bond cleavage in a substrate by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate and; c) detecting the absence of a bond(s), wherein the absence is indicative of bond cleavage. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates. The absence of a bond may be detected, for example, by the total length of the substrate, and/or the length increase upon cleavage of a bond. In one embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0071] In another aspect, the instant disclosure relates to a method of measuring the dynamics of bond formation in a substrate by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate; and; c) detecting the presence of bond(s), wherein the presence is indicative of bond formation. In one embodiment, the method further comprises measuring the dynamics of substrate folding, wherein the presence of a second bond is indicative of substrate folding. The presence of a bond may be detected, for example, by the total length of the substrate,

and/or the length increase upon formation of a bond. In one embodiment, the dynamics of substrate folding and bond formation are measured independently. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates. In another embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0072] In one aspect, the instant disclosure relates to a method of measuring substrate folding by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate; and; c) detecting the presence of bond(s), wherein the presence is indicative of substrate folding. The presence of a bond may be detected, for example, by the total length of the substrate, and/or the length increase upon formation of a bond. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates. In one embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0073] In another aspect, the instant disclosure relates to a method of measuring substrate unfolding by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate; and; c) detecting the absence of bond(s), wherein the absence is indicative of substrate unfolding. The absence of a bond may be detected, for example, by the total length of the substrate, and/or the length increase upon cleavage of a bond. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates. In one embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0074] In one aspect, the instant disclosure relates to a method of measuring the dynamics of folding and bond formation in a substrate by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate; and; c) detecting the presence of a first bond(s) and a second bond(s), wherein the presence of a first bond(s) is indicative of substrate folding and the presence of a second bond(s) is indicative of bond formation. In one embodiment, the dynamics of substrate

folding and bond formation are measured independently. The presence of a bond may be detected, for example, by the total length of the substrate, and/or the length increase upon formation of a bond. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the first bond is a non-covalent bond. In another embodiment, the first bond is a covalent bond. In one embodiment, the second bond is a non-covalent bond. In another embodiment, the second bond is a covalent bond. In another embodiment, the first bond is a non-covalent bond and the second bond is a covalent bond. In another embodiment, the first bond is a covalent bond and the second bond is a non-covalent bond. In one embodiment, the dynamics include kinetic rates. In one embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0075] In another aspect, the instant disclosure relates to a method of measuring the dynamics of unfolding and bond cleavage of a substrate by single-molecule force spectroscopy, the method comprising a) placing a sample comprising a substrate in the force-clamp spectrometer described herein; b) applying a force to the sample, wherein the force extends the substrate; and c) detecting the absence of a first bond(s) and a second bond(s), wherein the absence of a first bond is indicative of substrate unfolding and the absence of a second bond is indicative of bond cleavage. The absence of a bond may be detected, for example, by the total length of the substrate, and/or the length increase upon cleavage of a bond. In one embodiment, the first bond is a non-covalent bond. In another embodiment, the first bond is a covalent bond. In one embodiment, the second bond is a non-covalent bond. In another embodiment, the second bond is a covalent bond. In another embodiment, the first bond is a non-covalent bond and the second bond is a covalent bond. In another embodiment, the first bond is a covalent bond and the second bond is a non-covalent bond. In one embodiment, the dynamics of substrate unfolding and bond cleavage are measured independently. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates. In another embodiment, steps b) and c) of the method described are repeated over time. In another embodiment, the method further comprises measuring the dynamics of one or more additional substrates.

[0076] In some embodiments, the sample comprises a compound that reacts with one or more bond(s). The compound may be, but is not limited to, a reducing agent (for example, dithiothreitol or DTT), an oxidizing agent, a nanoparticle, or a small molecule. In another embodiment, the sample may comprise an ion.

[0077] In some embodiments, the sample comprises an inhibitor. In other embodiments, the sample comprises an activator. Inhibitors and activators may be, but are not limited to, small molecules. In one embodiment, the inhibitor is an enzyme inhibitor. In another embodiment, the activator is an enzyme activator.

[0078] In one embodiment, the sample comprises one or more enzyme(s). In one embodiment, the enzyme is an oxidoreductase enzyme. In another embodiment, the enzyme is an oxidase enzyme. In one embodiment, the enzyme is thioredoxin. In another embodiment, the oxidase enzyme is protein disulfide isomerase. In another embodiment, the enzyme is a protease, esterase, phosphodiesterase, or glycosidase. In another embodiment, the enzyme comprises one or more mutations. Mutations may include, but are not limited to, substitutions, deletions and/or point mutations.

[0079] In another aspect, the instant disclosure relates to a substrate used for measuring the dynamics of chemical reactions. In one embodiment, the substrate is a polymer. In another embodiment, the substrate has a folded structure. In one embodiment, the substrate is a protein or a polypeptide. In one embodiment, the substrate is a natural protein or natural polypeptide. In one embodiment, the substrate is cadherin, selectin, IgCAM, fibronectin, fibrillin or titin. In another embodiment, the substrate is an engineered polymer. In one embodiment, the polymer contains domain repeats. In one embodiment, the substrate is a nucleic acid. A nucleic acid may include, but is not limited to, a deoxyribonucleic acid, a ribonucleic acid or an oligonucleotide. In another embodiment, the substrate is a polysaccharide. In another embodiment, the substrate contains domain repeats. In one embodiment, the substrate comprises one or more bonds. In one embodiment, the bond is a single bond. In another embodiment, the bond is a double bond. In one embodiment, the bond is a covalent bond. In another embodiment, the bond is a non-covalent bond. In another embodiment, the bond is a disulfide bond. In another embodiment, the substrate comprises one or more mutations. Mutations may include, but are not limited to, substitutions, deletions and/or point mutations. Such mutations can be made using any suitable mutagenesis method known in the art.

[0080] In one embodiment, the substrate is used for measuring the dynamics of folding. In another embodiment, the substrate is used for measuring the dynamics of unfolding. In one embodiment, the substrate is used for measuring the dynamics of cleavage of a bond. In another embodiment, the substrate is used for measuring the dynamics of formation of a

bond. Dynamics may include, but are not limited to, kinetic rates and/or shifts in equilibrium. In one embodiment, the dynamics include kinetic rates.

[0081] Although several substrates are described herein, one of skill in the art will recognize that other substrates can also be used in the methods described herein. Substrates can be also generated using the methods described herein, including, but not limited to therapeutic proteins and proteins susceptible to industrial use.

[0082] Substrates produced according to the methods described herein can be from any source or origin and can include a substrate found in prokaryotes, viruses, and eukaryotes, including fungi, plants, yeasts, insects, and animals, including mammals (e.g. humans). Substrates that can be produced according to the methods described herein include, but are not limited to any polypeptide sequences, known or hypothetical or unknown, which can be identified using common sequence repositories. Example of such sequence repositories include, but are not limited to GenBank EMBL, DDBJ and the NCBI. Other repositories can easily be identified by searching on the internet. Substrates that can be produced using the methods described herein also include polypeptides have at least about 60%, 70%, 75%, 80%, 90%, 95%, or at least about 99% or more identity to any known or available polypeptide (e.g., a therapeutic polypeptide, a diagnostic polypeptide, an industrial enzyme, or portion thereof, and the like). Substrates that can be produced using the methods described herein also include nucleic acids that have at least about 60%, 70%, 75%, 80%, 90%, 95%, or at least about 99% or more identity to any known or available nucleic acid.

[0083] Protein and polypeptide substrates that can be produced according to the methods described herein also include polypeptides comprising one or more non-natural amino acids. As used herein, a non-natural amino acid can be, but is not limited to, an amino acid comprising a moiety where a chemical moiety is attached, such as an aldehyde- or keto-derivatized amino acid, or a non-natural amino acid that includes a chemical moiety. A non-natural amino acid can also be an amino acid comprising a moiety where a saccharide moiety can be attached, or an amino acid that includes a saccharide moiety.

[0084] Protein and polypeptide substrates can also comprise peptide derivatives (for example, that contain one or more non-naturally occurring amino acids). In specific embodiments, the library members contain one or more non-natural or non-classical amino acids or cyclic peptides. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, -amino isobutyric acid, 4-aminobutyric acid, Abu, 2-amino butyric acid; -Abu, -Ahx, 6-amino hexanoic acid; Aib, 2-amino isobutyric acid; 3-

amino propionic acid; omithine; norleucine; norvaline, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, .beta.-alanine, designer amino acids such as .beta.-methyl amino acids, C-methyl amino acids, N-methyl amino acids, fluoro-amino acids and amino acid analogs in general. Furthermore, the amino acid can be D (dextrorotary) or L (levorotary).

[0085] A substrate may comprise substrates that are well known to those of skill in the art and have been described in detail in the scientific literature. Several common modifications, such as glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance Creighton, *Protein Structure and Molecular Properties*, 2nd ed., W. H. Freeman and Company (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, in Johnson (ed.), *Posttranslational Covalent Modification of Proteins*, pgs. 1-12, Academic Press (1983); Seifter et al., *Meth. Enzymol.* 182: 626-646 (1990) and Rattan et al., *Ann. N. Y Acad. Sci.* 663: 48-62 (1992).

[0086] One can prepare a protein or polypeptide substrate that has post-translational modifications. Examples of types of post-translational modifications include, but are not limited to: (Z)-dehydrobutyrine; 1-chondroitin sulfate-L-aspartic acid ester; 1'-glycosyl-L-tryptophan; 1'-phospho-L-histidine; 1-thioglycine; 2'-(S-L-cysteinyl)-L-histidine; 2'-[3-carboxamido (trimethylammonio)propyl]-L-histidine; 2'-alpha-mannosyl-L-tryptophan; 2-methyl-L-glutamine; 2-oxobutanoic acid; 2-pyrrolidone carboxylic acid; 3'-(1'-L-histidyl)-L-tyrosine; 3'-(8alpha-FAD)-L-histidine; 3'-(S-L-cysteinyl)-L-tyrosine; 3', 3'', 5'-triiodo-L-tyronine; 3'-4'-phospho-L-tyrosine; 3-hydroxy-L-proline; 3'-methyl-L-histidine; 3-methyl-L-lanthionine; 3'-phospho-L-histidine; 4'-(L-tryptophan)-L-tryptophyl quinone; 42 N-cysteinyl-glycosylphosphatidylinositoethanolamine; 43-(T-L-histidyl)-L-tyrosine; 4-hydroxy-L-arginine; 4-hydroxy-L-lysine; 4-hydroxy-L-proline; 5'-(N6-L-lysine)-L-topaquinone; 5-hydroxy-L-lysine; 5-methyl-L-arginine; alpha-1-microglobulin-Ig alpha complex chromophore; bis-L-cysteinyl bis-L-histidino diiron disulfide; bis-L-cysteinyl-L-N3'-histidino-L-serinyl tetrairon' tetrasulfide; chondroitin sulfate D-glucuronyl-D-galactosyl-D-galactosyl-D-xylosyl-L-serine; D-alanine; D-allo-isoleucine; D-asparagine; dehydroalanine; dehydrotyrosine; dermatan 4-sulfate D-glucuronyl-D-galactosyl-D-galactosyl-D-xylosyl-L-serine; D-glucuronyl-N-glycine; dipyrrolylmethanemethyl-L-cysteine; D-leucine; D-methionine; D-phenylalanine; D-serine; D-tryptophan; glycine amide; glycine oxazolecarboxylic acid; glycine thiazolecarboxylic acid; heme P450-bis-L-cysteine-L-

tyrosine; heme-bis-L-cysteine; hemediol-L-aspartyl ester-L-glutamyl ester; hemediol-L-aspartyl ester-L-glutamyl ester-L-methionine sulfonium; heme-L-cysteine; heme-L-histidine; heparan sulfate D-glucuronyl-D-galactosyl-D-galactosyl-D-xylosyl-L-serine; heme P450-bis-L-cysteine-L-lysine; hexakis-L-cysteinyl hexairon hexasulfide; keratan sulfate D-glucuronyl-D-galactosyl-D-galactosyl-D-xylosyl-L-threonine; L oxoalanine- lactic acid; L phenyllactic acid; 1'-(8alpha-FAD)-L-histidine; L-2'.4',5'-topaquinone; L-3',4'-dihydroxyphenylalanine; L-3'.4'.5'-trihydroxyphenylalanine; L-4'-bromophenylalanine; L-6'-bromotryptophan; L-alanine amide; L-alanyl imidazolinone glycine; L-allysine; L-arginine amide; L-asparagine amide; L-aspartic 4-phosphoric anhydride; L-aspartic acid 1-amide; L-beta-methylthioaspartic acid; L-bromohistidine; L-citrulline; L-cysteine amide; L-cysteine glutathione disulfide; L-cysteine methyl disulfide; L-cysteine methyl ester; L-cysteine oxazolecarboxylic acid; L-cysteine oxazolinecarboxylic acid; L-cysteine persulfide; L-cysteine sulfenic acid; L-cysteine sulfinic acid; L-cysteine thiazolecarboxylic acid; L-cysteinyl homocitryl molybdenum-heptairon-nonasulfide; L-cysteinyl imidazolinone glycine; L-cysteinyl molybdopterin; L-cysteinyl molybdopterin guanine dinucleotide; L-cystine; L-erythro-beta-hydroxyasparagine; L-erythro-beta-hydroxyaspartic acid; L-gamma-carboxyglutarnic acid; L-glutamic acid 1-amide; L-glutamic acid 5-methyl ester; L-glutamine amide; L-glutamyl 5-glycerolphosphorylethanolarnine; L-histidine amide; L-isoglutamyl-polyglutamic acid; L-isoglutamyl-polyglycine; L-isoleucine amide; L-lanthionine; L-leucine amide; L-lysine amide; L-lysine thiazolecarboxylic acid; L-lysinoalanine; L-methionine amide; L-methionine sulfone; L-phenylalanine thiazolecarboxylic acid; L-phenylalanine amide; L-proline amide; L-selenocysteine; L-selenocysteinyl molybdopterin guanine dinucleotide; L-serine amide; L-serine thiazolecarboxylic acid; L-seryl imidazolinone glycine; L-T-bromophenylalanine; L-T-bromophenylalanine; L-threonine amide; L-thyroxine; L-tryptophan amide; L-tryptophyl quinone; L-tyrosine amide; L-valine amide; meso-lanthionine; N-(L-glutamyl)-L-tyrosine; N-(L-isoaspartyl)-glycine; N-(L-isoaspartyl)-L-cysteine; N,N,N-trimethyl-L-alanine; N,N-dimethyl-L-proline; N2-acetyl-L-lysine; N2-succinyl-L-tryptophan; N4-(ADP-ribosyl)-L-asparagine; N4-glycosyl-L-asparagine; N4-hydroxymethyl-L-asparagine; N4-methyl-L-asparagine; N5-methyl-L-glutamine; N6-1-carboxyethyl-L-lysine; N6-(4-amino hydroxybutyl)-L-lysine; N6-(L-isoglutamyl)-L-lysine; N6-(phospho-5'-adenosine)-L-lysine; N6-(phospho-5'-guanosine)-L-lysine; N6,N6,N6-trimethyl-L-lysine; N6,N6-dimethyl-L-lysine; N6-acetyl-L-lysine; N6-biotinyl-L-lysine; N6-carboxy-L-lysine; N6-formyl-L-lysine; N6-glycyl-L-lysine; N6-lipoyl-L-lysine; N6-methyl-L-lysine; N6-methyl-N6-poly(N-methyl-

propylamine)-L-lysine; N6-mureinyl-L-lysine; N6-myristoyl-L-lysine; N6-palmitoyl-L-lysine; N6-pyridoxal phosphate-L-lysine; N6-pyruvic acid 2-iminyl-L-lysine; N6-retinal-L-lysine; N-acetylglycine; N-acetyl-L-glutamine; N-acetyl-L-alanine; N-acetyl-L-aspartic acid; N-acetyl-L-cysteine; N-acetyl-L-glutamic acid; N-acetyl-L-isoleucine; N-acetyl-L-methionine; N-acetyl-L-proline; N-acetyl-L-serine; N-acetyl-L-threonine; N-acetyl-L-tyrosine; N-acetyl-L-valine; N-alanyl-glycosylphosphatidylinositoethanolamine; N-asparaginyl-glycosylphosphatidylinositoethanolamine; N-aspartyl-glycosylphosphatidylinositoethanolamine; N-formylglycine; N-formyl-L-methionine; N-glycyl-glycosylphosphatidylinositoethanolamine; N-L-glutamyl-poly-L-glutamic acid; N-methylglycine; N-methyl-L-alanine; N-methyl-L-methionine; N-methyl-L-phenylalanine; N-myristoyl-glycine; N-palmitoyl-L-cysteine; N-pyruvic acid 2-iminyl-L-cysteine; N-pyruvic acid 2-iminyl-L-valine; N-seryl-glycosylphosphatidylinositoethanolamine; N-seryl-glycosylphosphatidylinositoethanolamine; O-(ADP-ribosyl)-L-serine; O-(phospho-5'-adenosine)-L-threonine; O-(phospho-5'-DNA)-L-serine; O-(phospho-5'-DNA)-L-threonine; O-(phospho-5'rRNA)-L-serine; O-(phosphoribosyl dephospho-coenzyme A)-L-serine; O-(sn-1-glycerophosphoryl)-L-serine; O4'-(8alpha-FAD)-L-tyrosine; O4'-(phospho-5'-adenosine)-L-tyrosine; O4'-(phospho-5'-DNA)-L-tyrosine; O4'-(phospho-5'-RNA)-L-tyrosine; O4'-(phospho-5'-uridine)-L-tyrosine; O4-glycosyl-L-hydroxyproline; O4'-glycosyl-L-tyrosine; O4'-sulfo-L-tyrosine; O5-glycosyl-L-hydroxylysine; O-glycosyl-L-serine; O-glycosyl-L-threonine; omega-N-(ADP-ribosyl)-L-arginine; omega-N-omega-N'-dimethyl-L-arginine; omega-N-methyl-L-arginine; omega-N-omega-N-dimethyl-L-arginine; omega-N-phospho-L-arginine; O-octanoyl-L-serine; O-palmitoyl-L-serine; O-palmitoyl-L-threonine; O-phospho-L-serine; O-phospho-L-threonine; O-phosphopantetheine-L-serine; phycoerythrobilin-bis-L-cysteine; phycourobilin-bis-L-cysteine; pyrroloquinoline quinone; pyruvic acid; S-hydroxycinnamyl-L-cysteine; S-(2-aminovinyl)methyl-D-cysteine; S-(2-aminovinyl)-D-cysteine; S-(6-FW-L-cysteine; S-(8alpha-FAD)-L-cysteine; S-(ADP-ribosyl)-L-cysteine; S-(L-isoglutamyl)-L-cysteine; S-12-hydroxyfamesyl-L-cysteine; S-acetyl-L-cysteine; S-diacylglycerol-L-cysteine; S-diphytanylglycerol diether-L-cysteine; S-famesyl-L-cysteine; S-geranylgeranyl-L-cysteine; S-glycosyl-L-cysteine; S-glycyl-L-cysteine; S-methyl-L-cysteine; S-nitrosyl-L-cysteine; S-palmitoyl-L-cysteine; S-phospho-L-cysteine; S-phycobiliviolin-L-cysteine; S-phycocyanobilin-L-cysteine; S-phycoerythrobilin-L-cysteine; S-phytochromobilin-L-cysteine; S-selenyl-L-cysteine; S-sulfo-L-cysteine; tetrakis-L-cysteinyl diiron disulfide; tetrakis-L-cysteinyl iron; tetrakis-L-cysteinyl tetrairon tetrasulfide; trans-2,3-

cis 4-dihydroxy-L-proline; tris-L-cysteinyl triiron tetrasulfide; tris-L-cysteinyl triiron trisulfide; tris-L-cysteinyl-L-aspartato tetrairon tetrasulfide; tris-L-cysteinyl-L-cysteine persulfido-bis-L-glutamato-L-histidino tetrairon disulfide trioxide; tris-L-cysteinyl-L-N3'-histidino tetrairon tetrasulfide; tris-L-cysteinyl-L-NM'-histidino tetrairon tetrasulfide; and tris-L-cysteinyl-L-serinyl tetrairon tetrasulfide.

[0087] Additional examples of post translational modifications can be found in web sites such as the Delta Mass database based on Krishna, R. G. and F. Wold (1998).

Posttranslational Modifications. Proteins--Analysis and Design. R. H. Angeletti. San Diego, Academic Press. 1: 121-206.; Methods in Enzymology, 193, J. A. McClosky (ed) (1990), pages 647-660; Methods in Protein Sequence Analysis edited by Kazutomo Imahori and Fumio Sakiyama, Plenum Press, (1993) "Post-translational modifications of proteins" R. G. Krishna and F. Wold pages 167-172; "GlycoSuiteDB: a new curated relational database of glycoprotein glycan structures and their biological sources" Cooper et al. Nucleic Acids Res. 29; 332-335 (2001) "O-GLYCBASE version 4.0: a revised database of O-glycosylated proteins" Gupta et al. Nucleic Acids Research, 27: 370-372 (1999); and "PhosphoBase, a database of phosphorylation sites: release 2.0.", Kreegipuu et al. Nucleic Acids Res 27(1):237-239 (1999) see also, WO 02/211 39A2, the disclosure of which is incorporated herein by reference in its entirety.

[0088] Exemplary substrates that can be produced according to the methods described herein include but are not limited to, cytokines, inflammatory molecules, growth factors, their receptors, and oncogene products or portions thereof. Examples of cytokines, inflammatory molecules, growth factors, their receptors, and oncogene products include, but are not limited to e.g., alpha-1 antitrypsin, Angiostatin, Antihemolytic factor, antibodies (including an antibody or a functional fragment or derivative thereof selected from: Fab, Fab', F(ab)2, Fd, Fv, ScFv, diabody, tribody, tetrabody, dimer, trimer or minibody), angiogenic molecules, angiostatic molecules, Apolipopolypeptide, Apopolypeptide, Asparaginase, Adenosine deaminase, Atrial natriuretic factor, Atrial natriuretic polypeptide, Atrial peptides, Angiotensin family members, Bone Morphogenic Polypeptide (BMP-1, BMP-2, BMP-3, BMP-4, BMP-5, BMP-6, BMP-7, BMP-8a, BMP-8b, BMP-10, BMP-15, etc.); C--X--C chemokines (e.g., T39765, NAP-2, ENA-78, Gro-a, Gro-b, Gro-c, IP-10, GCP-2, NAP-4, SDF-1, PF4, MIG), Calcitonin, CC chemokines (e.g., Monocyte chemoattractant polypeptide-1, Monocyte chemoattractant polypeptide-2, Monocyte chemoattractant polypeptide-3, Monocyte inflammatory polypeptide-1 alpha, Monocyte inflammatory polypeptide-1 beta,

RANTES, 1309, R83915, R91733, HCC1, T58847, D31065, T64262), CD40 ligand, C-kit Ligand, Ciliary Neurotrophic Factor, Collagen, Colony stimulating factor (CSF), Complement factor 5a, Complement inhibitor, Complement receptor 1, cytokines, (e.g., epithelial Neutrophil Activating Peptide-78, GRO alpha/MGSA, GRO beta, GRO gamma, MIP-1 alpha, MIP-1 delta, MCP-1), deoxyribonucleic acids, Epidermal Growth Factor (EGF), Erythropoietin ("EPO", representing a preferred target for modification by the incorporation of one or more non-natural amino acid), Exfoliating toxins A and B, Factor IX, Factor VII, Factor VIII, Factor X, Fibroblast Growth Factor (FGF), Fibrinogen, Fibronectin, G-CSF, GM-CSF, Glucocerebrosidase, Gonadotropin, growth factors, Hedgehog polypeptides (e.g., Sonic, Indian, Desert), Hemoglobin, Hepatocyte Growth Factor (HGF), Hepatitis viruses, Hirudin, Human serum albumin, Hyalurin-CD44, Insulin, Insulin-like Growth Factor (IGF-I, IGF-II), interferons (e.g., interferon-alpha, interferon-beta, interferon-gamma, interferon-epsilon, interferon-zeta, interferon-eta, interferon-kappa, interferon-lambda, interferon-T, interferon-zeta, interferon-omega), glucagon-like peptide (GLP-1), GLP-2, GLP receptors, glucagon, other agonists of the GLP-1R, natriuretic peptides (ANP, BNP, and CNP), Fuzeon and other inhibitors of HIV fusion, Hirudin and related anticoagulant peptides, Prokineticins and related agonists including analogs of black mamba snake venom, TRAIL, RANK ligand and its antagonists, calcitonin, amylin and other glucoregulatory peptide hormones, and Fc fragments, exendins (including exendin-4), exendin receptors, interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, etc.), I-CAM-1/LFA-1, Keratinocyte Growth Factor (KGF), Lactoferrin, leukemia inhibitory factor, Luciferase, Neurturin, Neutrophil inhibitory factor (NIF), oncostatin M, Osteogenic polypeptide, Parathyroid hormone, PD-ECSF, PDGF, peptide hormones (e.g., Human Growth Hormone), Oncogene products (Mos, Rel, Ras, Raf, Met, etc.), Pleiotropin, Polypeptide A, Polypeptide G, Pyrogenic exotoxins A, B, and C, Relaxin, Renin, ribonucleic acids, SCF/c-kit, Signal transcriptional activators and suppressors (p53, Tat, Fos, Myc, Jun, Myb, etc.), Soluble complement receptor 1, Soluble I-CAM 1, Soluble interleukin receptors (IL-1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15), soluble adhesion molecules, Soluble TNF receptor, Somatomedin, Somatostatin, Somatotropin, Streptokinase, Superantigens, i.e., Staphylococcal enterotoxins (SEA, SEB, SEC1, SEC2, SEC3, SED, SEE), Steroid hormone receptors (such as those for estrogen, progesterone, testosterone, aldosterone, LDL receptor ligand and corticosterone), Superoxide dismutase (SOD), Toll-like receptors (such as Flagellin), Toxic shock syndrome toxin (TSST-1), Thymosin a 1, Tissue

plasminogen activator, transforming growth factor (TGF- alpha, TGF- beta), Tumor necrosis factor beta (TNF beta), Tumor necrosis factor receptor (TNFR), Tumor necrosis factor-alpha (TNF alpha), transcriptional modulators (for example, genes and transcriptional modular polypeptides that regulate cell growth, differentiation and/or cell regulation), Vascular Endothelial Growth Factor (VEGF), virus-like particle, VLA-4/VCAM-1, Urokinase, signal transduction molecules, estrogen, progesterone, testosterone, aldosterone, LDL, corticosterone.

[0089] Other substrates that can be produced according to the methods described herein include, but are not limited to, agriculturally related polypeptides such as insect resistance polypeptides (e.g., Cry polypeptides), starch and lipid production enzymes, plant and insect toxins, toxin-resistance polypeptides, Mycotoxin detoxification polypeptides, plant growth enzymes (e.g., Ribulose 1,5-Bisphosphate Carboxylase/Oxygenase), lipoxygenase, and Phosphoenolpyruvate carboxylase.

[0090] Other substrates that can be produced according to the methods described herein include, but are not limited to, antibodies, immunoglobulin domains of antibodies and their fragments. Examples of antibodies include, but are not limited to antibodies, antibody fragments, antibody derivatives, Fab fragments, Fab' fragments, F(ab)₂ fragments, Fd fragments, Fv fragments, single-chain Fv fragments (scFv), diabodies, tribodies, tetrabodies, dimers, trimers, and minibodies.

[0091] In another embodiment, the disclosed subject matter is directed to a composition comprising a substrate for use in the methods described herein and produced according to the methods described herein, and an additional component selected from the group consisting of pharmaceutically acceptable diluents, carriers, excipients and adjuvants.

[0092] Substrates that can be produced according to the methods described herein can also further comprise a chemical moiety selected from the group consisting of: cytotoxins, pharmaceutical drugs, dyes or fluorescent labels, a nucleophilic or electrophilic group, a ketone or aldehyde, azide or alkyne compounds, photocaged groups, tags, a peptide, a polypeptide, a polypeptide, an oligosaccharide, polyethylene glycol with any molecular weight and in any geometry, polyvinyl alcohol, metals, metal complexes, polyamines, imidizoles, carbohydrates, lipids, biopolymers, particles, solid supports, a polymer, a targeting agent, an affinity group, any agent to which a complementary reactive chemical group can be attached, biophysical or biochemical probes, isotopically-labeled probes, spin-label amino acids, fluorophores, aryl iodides and bromides.

[0093] In some embodiments, the disclosed subject matter involves mutating nucleotide sequences of substrates to add/create or remove/disrupt sequences. Such mutations can be made using any suitable mutagenesis method known in the art, including, but not limited to, site-directed mutagenesis, oligonucleotide-directed mutagenesis, positive antibiotic selection methods, unique restriction site elimination (USE), deoxyuridine incorporation, phosphorothioate incorporation, and PCR-based mutagenesis methods. Details of such methods can be found in, for example, Lewis et al. (1990) Nucl. Acids Res. 18, p3439; Bohnsack et al. (1996) Meth. Mol. Biol. 57, p1; Vavra et al. (1996) Promega Notes 58, 30; Altered Sites II in vitro Mutagenesis Systems Technical Manual #TM001, Promega Corporation; Deng et al. (1992) Anal. Biochem. 200, p81; Kunkel et al. (1985) Proc. Natl. Acad. Sci. USA 82, p488; Kunke et al. (1987) Meth. Enzymol. 154, p367; Taylor et al. (1985) Nucl. Acids Res. 13, p8764; Nakamaye et al. (1986) Nucl. Acids Res. 14, p9679; Higuchi et al. (1988) Nucl. Acids Res. 16, p7351; Shimada et al. (1996) Meth. Mol. Biol. 57, p157; Ho et al. (1989) Gene 77, p51; Horton et al. (1989) Gene 77, p61; and Sarkar et al. (1990) BioTechniques 8, p404. Numerous kits for performing site-directed mutagenesis are commercially available, such as the QuikChange II Site-Directed Mutagenesis Kit and the Altered Sites II in vitro mutagenesis system. Such commercially available kits may also be used to optimize sequences. Other techniques that can be used to generate modified nucleic acid sequences are well known to those of skill in the art. See for example Sambrook *et al.* (2001) Molecular Cloning: A Laboratory Manual, 3rd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

[0094] In another aspect, the instant disclosure relates to an enzyme used in the methods described herein. Examples of enzymes include, but are not limited to amidases, amino acid racemases, acylases, dehalogenases, dioxygenases, diarylpropane peroxidases, epimerases, epoxide hydrolases, esterases, isomerases, kinases, glucose isomerases, glycosidases, glycosyl transferases, haloperoxidases, monooxygenases (e.g., p450s), lipases, lignin peroxidases, nitrile hydratases, nitrilases, proteases, phosphatases, subtilisins, transaminase, and nucleases. In one embodiment, the enzyme is an oxidoreductase enzyme. In another embodiment, the enzyme is thioredoxin. In one embodiment, the enzyme is an oxidase enzyme. In another embodiment, the enzyme is protein disulfide isomerase. In another embodiment, the enzyme is a protease, esterase, phosphodiesterase, or glycosidase. In another embodiment, the enzyme is a hydrolase. In one embodiment, the enzyme comprises one or more mutations. Mutations may include, but are not limited to, substitutions, deletions

and/or point mutations. Such mutations can be made using any suitable mutagenesis method known in the art.

[0095] Although there are different ways to carry out the methods described herein, in one embodiment, the method is carried out as follows: a force-clamp spectrometer is used to stretch a specifically engineered polymer substrate containing a bond of interest, in an environment containing a bond-cleaving or a bond-forming reagent of interest. The force-clamp spectrometer applies a constant force to the substrate while measuring its extension. The force applied is high enough to break non-covalent bonds and elongate the substrate, but not high enough to break covalent bonds. The substrate is a polymer crosslinked with linker molecules containing the bond of interest. Each time one of the bonds of interest breaks, a concomitant substrate extension is detected. This stepwise extension corresponds to elongation of the substrate region that was previously sequestered behind the crosslink. The extension step has a precise length and thus constitutes a fingerprint for the bond cleavage reaction.

[0096] Although different types of substrates can be used, in one embodiment, a substrate is constructed by chemically ligating a polymer scaffold molecule with a customized linker molecule. In one embodiment, a protein polymer consisting of repeats of the I27 domain from human cardiac titin, expressed in *E. coli* and purified using affinity and size exclusion chromatography, is used as a scaffold. This protein is designed to contain in each domain two exposed cysteine residues, which are then crosslinked using a commercially available linker derivitized at both ends with maleimide. In another embodiment, the linker is designed to contain any bond not present in the scaffold molecule.

Disulfide bond reduction studied by single-molecule force spectroscopy

[0097] Single-molecule force spectroscopy by Atomic Force Microscope (AFM) may be used to study the force dependency of the reduction of disulfide bonds both by chemicals and different Trxs. Although different types of substrates can be used in this approach, in one embodiment, the substrate is a polyprotein composed of several copies of an immunoglobulin domain from human cardiac titin (I27) is held between the tip of an AFM cantilever and a gold surface on top of a piezoelectric positioner (Fig. 19A). Polyproteins composed of eight I27 modules are usually employed. Each one of the I27 modules includes an engineered disulfide bond between residues 32 and 75, (I27G32C-A75C)₈ (Fig. 19B). In the force-clamp mode of the AFM, it is possible to set the force exerted to the polyprotein up to several hundreds of pico Newtons. In this mode of operation, the deflection of the cantilever is held

constant by an electronic feedback system that controls the extension of the polyprotein via the piezoelectric positioner. Response times around 5 ms can be easily achieved with the current instrumentation.

[0098] The behavior of both I27 and I27G32C-A75C upon application of mechanical loads has been thoroughly examined by AFM. The unfolding rate of I27G32C-A75C around 200 pN is high (30 s⁻¹). Thus, a double pulse protocol is employed to detect the reduction of disulfide bonds. A first pulse of force (160 - 190 pN during 0.3 - 1.0 s) unfolds the domains of the polyprotein. In these experiments, the disulfide bonds act as force transducers; therefore, the I27G32C-A75C modules extend only up to the disulfide bonds (Figs. 19C and 19D), as forces higher than 1 nN are required to cleave covalent bonds. The individual unfolding events can be detected as step increases of ~10.8 nm in the length of the polyprotein, which give rise to a well-defined staircase in a length vs. time plot (Fig. 20A). Each step in size is accompanied by a sudden decrease in the force, which is rapidly compensated by the feedback (Fig. 20A). Therefore, this series of unfolding steps serves as a well-defined fingerprint that distinguishes the polyprotein of interest from any other spurious interactions. In addition, the unfolding events promote a steric switch which now allows the disulfide bonds that were buried in the protein to be reduced by reducing agents contained in the solution (Fig. 19D). After the unfolding of the domains, a second pulse of force is applied for up to minutes to monitor single disulfide reductions. When a disulfide bond is reduced, the region of the protein whose unfolding was hampered by the disulfide bond now extends. These extensions are recorded as a second series of steps of ~13.2 nm per disulfide bond reduction (Fig. 20A). As in the unfolding steps, a sudden decrease in the force accompanies the reduction events (Fig. 20A). Generally, 15-50 traces as that shown in Fig. 20A are accumulated per force. In the more straightforward analysis, the traces are averaged and fitted with a single exponential with time constant τ (Fig. 20B). From this fit, it is possible to obtain the reduction rate at a given force ($r = 1/\tau$). It has been observed that, as a consequence of the detachment kinetics of the polyprotein from the surface or the cantilever, the results can be biased to faster rates. In order to avoid this artifact, only traces with long detachment times should be included in the analysis. As an alternative to exponential fits, a dwell time analysis technique has been recently implemented for the study of single-molecule mechanochemical reactions. This procedure overcomes the limitations of exponential fits when multiple reaction pathways occur simultaneously; however, a large pool of events (over one thousand) needs to be collected.

Chemical reactions under force at the single-molecule level

[0099] In order to better understand the effects of force on a reaction catalyzed by an enzyme, first consider the case of more simple uncatalyzed chemical reactions was considered. It has been shown that the rate of reduction of the disulfide bonds in (I27G32CA75C)⁸ by small reducing agents such as DTT or LCys is exponentially dependent on the applied force (Fig. 20C). The exponential dependency is given by a Bell-like relationship: $r(F) = A \exp((F \cdot \Delta x - E_a)/k_B \cdot T)$. In this expression, A is the attempt frequency, Δx is the distance to the transition state of the reaction, E_a is the activation energy barrier for the reaction, k_B is the Boltzmann's constant, and T is the absolute temperature. Fitting the experimental results for the reduction by DTT to the equation above yields $\Delta x = 0.34 \text{ \AA}$. Interestingly, theoretical calculations have suggested that the length of the disulfide bond at the transition state of a simple S_N2 thiol/disulfide exchange reaction increases by approximately $0.37 \text{ \AA}$. Then, these results indicate that the changes in distance between the sulfur atoms at the transition state are responsible for the force dependency of the reaction. Indeed, when different reducing agents are used, the measured distances to the transition states of the corresponding reactions are in agreement with the physicochemical characteristics of the reactants. For thiol-initiated disulfide bond reductions, such as DTT, β -mercaptoethanol, or glutathione, $\Delta x = 0.31 \pm 0.05 \text{ \AA}$. When phosphine-based reducing agents (TCEP and THP) are employed, Δx is significantly higher, $0.44 \pm 0.03 \text{ \AA}$. Such an increase in the distance to the transition state for the latter compounds is again in agreement with quantum chemical calculations, which show that the distance between sulfur atoms in the transition states of phosphine-based reactions is longer than in thiol-initiated reductions. In addition, it has been demonstrated for phosphine-initiated reductions that Δx decreases when glycerol is incorporated into the aqueous solution. This result provides a direct test of theoretical calculations of the role of solvent molecules in the transition state of a bimolecular S_N2 reaction. In summary, the force-clamp experiments using small reducing agents show that the mechanical force imposes a 1-D reaction coordinate for the reaction (Fig. 20D). In this context, Δx reports on the progression along that reaction coordinate with sub-Ångström resolution, providing valuable information about the geometry of the transition state. In addition, the information gained about the transition state from force-clamp determinations is probably independent of its lifetime. The rationale behind this is that no matter how short- or long-lived a transition state is, it will always be subjected to force. Thus, in theory, the force-

spectroscopy methodology applied to chemical reactions might be able to inspect the geometric properties of transition states independently of their life times.

Force as a new probe of enzyme catalysis

[0100] From the experiments using small reducing agents, it is clear that chemical reactions resulting in changes in bond distance will be force dependent and that single-molecule force spectroscopy is able to provide sub-Ångström information about the transition state of the reaction. The same approach has also been employed to investigate the mechanism of disulfide bond reduction by members of the Trx family of enzymes. Trxs show a highly conserved active site (CXXC) that catalyzes the reduction of target disulfide bonds involved in a multitude of cellular processes. Several methods based on bulk spectrophotometry have been widely used to determine the activity of Trxs. These methods are based on the oxidation of NADPH in the presence of thioredoxin reductase (TrxR) or ribonucleotide reductase; the increase in turbidity of insulin solutions concomitant to the reduction of the disulfide bonds in that peptide; or the use of Ellman's reagent (DTNB), which generates colored products upon reduction by thiol groups. The change in the intrinsic fluorescence of Trx has also been used to measure rates of enzyme oxidation and reduction. While highly effective in monitoring the overall activity of Trx enzymes, these methods do not probe the chemical mechanisms underlying their catalysis. The main reason is that many factors influence the measurements, such as the kinetics of reduction of Trx by TrxR, or the kinetics of insulin aggregation after disulfide bond reduction. In addition, they have the limitations inherent to bulk assays, as they only provide average measurements of activity. In the case of the single-molecule force spectroscopy assays, the enzyme is kept in the reduced form due to the presence of TrxR and NADPH (the so-called Trx system). Therefore, the amount of oxidized Trx is negligible and the measured activity only reflects the reduction of the disulfide bond in (I27G32C-A75C)₈ at a given Trx concentration.

[0101] In contrast to DTT and other small reducing agents, human Trx-mediated disulfide reduction is strongly inhibited by force, with $\Delta x = -0.79$ Å (Fig. 20C). A molecular interpretation of this result has been obtained from the crystal structure of human Trx in complex with a substrate peptide (PDB code 1MDI). A peptide-binding groove is identified on the surface of the protein close to the catalytic cysteine. It is known that the reduction of a disulfide bond proceeds via an S_N2 mechanism, in which the three participating sulfur atoms form a $\sim 180^\circ$ angle. Given the fact that the disulfide bond in 1MDI forms an angle of $\sim 70^\circ$

with respect to the axis of the groove, it is evident that the target disulfide bond must rotate with respect to the pulling axis to acquire the correct geometry for reaction (Fig. 20E). Taking into account the orientation of the disulfide bond with respect to the pulling force, it can be estimated that a 0.77 Å shortening of the substrate polypeptide is needed in order to align the participating sulfur atoms, in extraordinary agreement with the experimental Δx (-0.79 Å). This interpretation is supported by molecular dynamics simulations and a theoretical model that treats the substrate backbone as a freely jointed chain. Therefore, it appears that, differently to what is observed for the reductions by small reducing agents, the change in bond distance at the transition state is not the main determinant of the force dependency for enzyme-catalyzed reactions. On the contrary, the dynamics of enzyme and substrate during catalysis are the main contributors to the measured Δx .

[0102] When Trx from *E. coli* was assayed, a similar force-dependency up to 200 pN was observed. However, this enzyme shows a second chemical pathway that becomes apparent only at higher forces. The two pathways seem to be independent of each other, since the mutants P34H and G47S selectively inhibit only the first pathway. The second pathway of *E. coli* Trx is force accelerated with $\Delta x = 0.22$ Å (Fig. 20C). This catalytic mode might be explained by the reduction happening without the peptide binding the groove; in this regard, the second pathway would be similar to the reduction by agents such as DTT or L-Cys (Fig. 20C).

[0103] In summary, the application of single molecule force spectroscopy to the study of catalysis by Trxs, in combination with molecular dynamics simulations, provides detailed information about the dynamics of enzyme and substrate during catalysis. This information has been used to detect residue co-evolution in enzymatic activity, which would have gone unnoticed to standard bulk assays.

Single-molecule force spectroscopy assays for other enzymatic activities

[0104] The results obtained with Trx suggest that it will be highly informative to apply the single molecule force spectroscopy methodology to other enzymes. In principle, the single-molecule assay for the reduction of disulfide bonds by thioredoxin might be adapted to any other enzyme that catalyzes the cleavage of covalent bonds. This would allow a deeper understanding of different mechanisms of catalysis. Proteases, esterases, phosphodiesterases, glycosidases, and glycosyltransferases are enzymes with the ability to cleave covalent bonds. From what has been learned from the single-molecule assay for disulfide bond reduction, it is clear that any new experimental setup aimed to study single-molecule bond cleavage under

force should fulfill the following requirements: i) the substrate should be incorporated into a macromolecule providing an unambiguous fingerprint after mechanical unfolding; ii) the rate of substrate cleavage by the enzyme should be low when put together in solution, so that no significant cleavage occur in the timescale needed to conduct AFM experiments; iii) the unfolding of the macromolecule should promote a steric switch in the substrate, rendering it sensitive to cleavage; iv) cleavage should be translated into a new increment in length of the macromolecule.

[0105] Single-molecule force spectroscopy may be used to probe the catalytic mechanisms of enzymes. In the examples below, this approach has been used to study the reduction of disulfide bonds by thioredoxin. From the force dependency of the reaction rate, new light has been shed on the dynamics of enzyme and substrate during catalysis. In particular, the Δx parameter, which is derived from exponential fits to the measured force dependency, reports on the spatial rearrangements of the participating atoms at the transition state of the reaction. These rearrangements can be dissected at the sub-Ångström scale, in a manner unachievable by any other current experimental technique.

[0106] The following examples illustrate the disclosed subject matter, and are set forth to aid in the understanding of the invention, and should not be construed to limit in any way the scope of the invention as defined in the claims which follow thereafter.

EXAMPLES

EXAMPLE 1

[0107] To test the hypothesis that mechanical force can directly influence the kinetics of a chemical reaction, thiol/disulfide exchange was studied, the reduction of disulfide bonds in a protein. The disulfide bond itself is a covalent bond formed between the thiol groups of two vicinal cysteine residues. In the first step of thiol_disulfide exchange, a new disulfide bond is formed between a thiolate anion of the reducing molecule (in this case DTT) and one cysteine on a protein, whereas the sulfur of the other cysteine reverts to the free thiolate state. This reaction has been extensively studied and is known to be important in the function and folding processes of proteins. This reaction is also of particular interest because it is known that many proteins that are exposed to mechanical stress in vivo contain disulfide bonds. Thus, the effect of force on this reaction could be of significance in biological systems.

[0108] Disulfide bonds have been studied in previous atomic force microscopy (AFM) experiments where a protein molecule is stretched at a constant velocity whereas the applied force varies (force-extension AFM). Most of these experiments could identify the presence or absence of a disulfide bond but could not determine when the disulfide reduction reaction occurred. Engineered disulfide bonds were used to precisely correlate disulfide reduction events with increases in protein contour length, developing a molecular fingerprint for identifying individual chemical reactions. In the present work, this fingerprint is used to investigate the kinetics of thiol/disulfide exchange as a function of pulling force using force-clamp AFM. This method provides the only direct means by which to observe the exponential chemical kinetics of thiol/disulfide exchange under a calibrated pulling force. This technique has been used to study the unfolding kinetics as well as refolding of single protein molecules as a function of force, offering insight into the link between protein dynamics and force. By using force-clamp AFM, it was demonstrated that thiol/disulfide exchange, a bimolecular chemical reaction, is catalyzed by mechanical force. The force-dependency of the reaction rate is determined by the structure of the transition state, a result that may be generalized to other chemical reactions. These findings demonstrate that force-clamp AFM is a powerful tool with which to study chemistry at the single molecule level.

Results and Discussion

[0109] In the studies herein, the 27th immunoglobulin-like domain of cardiac titin (I27), an 89-residue, β -sandwich protein with well characterized mechanical properties is used. Through cysteine mutagenesis, a disulfide bond in the I27 domain between the 32nd and 75th residues, which are closely positioned in space as determined by the NMR structure of wild-type I27 (Protein Data Bank ID code 1TIT) is engineered. An eight-repeat polyprotein of this modified domain was constructed and expressed, (I27G32C–A75C)₈, and used single-molecule force-clamp spectroscopy to manipulate and stretch single polyproteins. Under force-clamp conditions, stretching a polyprotein results in a well defined series of step increases in length, marking the unfolding and extension of the individual modules in the chain. Previous work has demonstrated that there is a close correlation between the size of the observed steps and the number of amino acids released by each unfolding event. Upon stretching a single (I27G32C–A75C)₈ polyprotein in an oxidizing environment (FIG. 5B), a series of steps of approximately 10.6 nm were observed, which are significantly shorter than those expected for native I27 unfolding (23.6 nm). This shortening indicates the formation of

the engineered disulfide bond within the protein module. The unfolding of 46 “unsequestered” residues (1–31 and 76–89) has a predicted step size of 10.4 nm at 130 pN, very similar to the observed value. At this stage of unfolding, the disulfide bond in each module is directly exposed to the applied stretching force (Fig. 5A), forming a covalent barrier “trapping” residues 33–74 and preventing complete module unfolding. If the bond were to be ruptured by force alone, a second step corresponding to the extension of the trapped polypeptide is expected to be observed. Yet any such steps under these oxidizing conditions (Fig. 5B) were not observed. This outcome was predicted by previous experimental and theoretical studies, where forces <1 nN cannot break a covalent bond. After unsequestered unfolding, the disulfide bond is exposed to the solvent, and thiol-disulfide exchange can occur if DTT is present in solution. In Fig. 1C, a single (I27G32C–A75C)₈ molecule in the presence of 50 mM DTT was pulled. During the first second, a series of steps of approximately 10.8 nm as unsequestered unfolding occurs in individual domains is observed. The one step of 24.0 nm denotes a domain with its disulfide reduced before mechanical unfolding, giving a full-length step approximately equal to that for wild-type I27. Such full-length unfolding was rare, however; previous studies have indicated that the disulfide bond in I27G32C–A75C is particularly solvent-inaccessible in the folded protein. After this first series of steps relating to protein unfolding, which occur over approximately 1 s, a second series of steps of approximately 13.8 nm over approximately 4 s was then observed. The predicted step size for trapped residue extension as determined from force-extension experiments is 13.5 nm at 130 pN. In addition, these steps were observed only after unsequestered protein unfolding and only in the presence of DTT. Hence, it is concluded that at a pulling force of 130 pN, the 13.8-nm steps monitor the thiol/disulfide exchange reaction as single disulfide bonds are reduced in each protein module, allowing for the extension of the trapped residues.

[0110] To study the kinetics of disulfide bond reduction as a function of the pulling force, a double pulse protocol in force-clamp was designed. The first pulse to 130 pN allows the monitoring of the unfolding of the unsequestered region of the I27G32C–A75C modules in the polyprotein, exposing the disulfide bonds to the solution. With the second pulse, the rate of reduction of the exposed disulfides at various pulling forces was tracked. Fig. 6 demonstrates the use of the double-pulse protocol in the absence (Fig. 6A) and in the presence of DTT (Fig. 6B). In both cases, the first pulse elicits a rapid series of steps of approximately 10.6 nm marking the unfolding and extension of the 46 unsequestered

residues. The second pulse in Fig. 6 increases the stretching force up to 200 pN. Upon application of the second pulse, an elastic extension of the polyprotein by approximately 10 nm was observed. In the presence of DTT (12.5 mM), this elastic extension is followed by a series of five additional approximately 14.2-nm steps that mark single thiol/disulfide exchange reactions (expected steps of 14.2 nm at 200 pN, whereas no further steps were observed in the absence of DTT (Fig. 6A).

[0111] To measure the rate of reduction at 200 pN and at a DTT concentration of 12.5 mM, the pulse pattern shown in Fig. 6B was repeated many times, obtaining an ensemble of single-molecule recordings. Fig. 7A shows three additional recordings demonstrating the stochastic nature of both the unsequestered unfolding and of the thiol/disulfide exchange events. The red trace in Fig. 3B was obtained by simple averaging of these four recordings. The green trace in Fig. 7B was obtained following similar procedures with the second force pulse to 300 pN. By comparison, the blue trace in Fig. 7B corresponds to the averaging of four traces obtained with the second force pulse set to 200 pN and in the absence of DTT. It is apparent from Fig. 7B that protein unfolding during the first pulse to 130 pN is independent of DTT, following a similar exponential time course in all cases at this force. However, thiol/disulfide exchange during the second pulse appears both DTT- and force-dependent. The double-pulse protocol as shown here effectively separates protein unfolding from the disulfide-bond reduction events. Hence, in the subsequent analysis the unsequestered unfolding observed during the first pulse and only analyze thiol/disulfide exchange events in the second pulse was ignored.

[0112] Multiple double-pulse experiments were conducted, all with an identical first force pulse to 130 pN lasting 1 s. Fig. 8A shows multiple (>25) trace averages of only the second pulse at four different forces (100, 200, 300, and 400 pN) and at a constant DTT concentration of 12.5 mM. Only traces that included a clear unsequestered unfolding fingerprint in the first pulse and that contained only disulfide reduction events in the second pulse were included in this analysis. By fitting a single exponential to the traces shown in Fig. 8A, the observed rate of thiol/disulfide exchange as $r = 1/\tau_r$, where τ_r is defined as the time constant measured from the exponential fits. Fig. 8B, shows that r is exponentially dependent on the applied force (12.5 mM DTT) ranging from 0.211 s⁻¹ (100 pN) up to 2.20 s⁻¹ (400 pN). Fig. 8C shows the second-pulse averages (>20) of experiments conducted at 200 pN, at different concentrations of DTT (0, 1.25, 12.5, 31, 50, 83, and 125 mM). Fig. 8D, shows that

r has a first-order dependence on the concentration of DTT, demonstrating that the thiol/disulfide exchange reaction in this system is bimolecular.

[0113] Given these observations, an empirical relationship $r = k(F)[\text{DTT}]$ is derived, where $k(F)$ depends exponentially on the applied force and is given by a Bell-like (25) relationship: $k(F) = A \exp((F\Delta x_r - E_a)/k_B T)$. In this equation, A is a constant with units of $\text{M}^{-1}\cdot\text{s}^{-1}$, Δx_r is the distance to the transition state for the reaction, and E_a is the activation energy barrier for the thiol/disulfide exchange at zero force. Fitting this equation to the data presented in Fig. 4B, a value of $\Delta x_r = 0.34 \text{ \AA}$ and a value of $k(0) = 6.54 \text{ M}^{-1}\cdot\text{s}^{-1}$ are obtained, which is similar to the second-order rate constant for DTT reduction of disulfide bonds in insulin at neutral pH [$k = 5 \text{ M}^{-1}\cdot\text{s}^{-1}$]. Other studies have found that under certain solution conditions, thiol_disulfide exchange can occur with a second-order rate constant as high as $132,000 \text{ M}^{-1}\cdot\text{s}^{-1}$ but also can be up to 6 orders of magnitude slower, in accord with other observations. From the linear fit in Fig. 8D, an applied force is seen that alters the second-order rate constant in the system; $k(200 \text{ pN}) = 27.6 \text{ M}^{-1}\cdot\text{s}^{-1}$, a 4-fold increase from zero force. Assuming that A ranges from 10^5 to $10^{12} \text{ M}^{-1}\cdot\text{s}^{-1}$, E_a is estimated to be in the range of 30–65 kJ/mol. The upper values in this range overlap with the calculated energy barriers for a number of thiol_disulfide exchange reactions in solution [60–66 kJ/mol], and each 100 pN of force lowers the energy barrier by approximately 2 kJ/mol.

[0114] Although the empirical Bell-like model is a useful first approximation to examine the data, it may not hold over all combinations of DTT and force. It also cannot completely describe the effect of a force on the thiol/disulfide exchange reaction. The force constant for an S–S bond, found by vibrational spectrum in the gas phase, is 4.96 N/cm. As a result, an applied force of 400 pN will stretch this bond by only $0.008 \text{ \AA}$, which is a negligible effect on the geometry of the S–S bond. However, as pointed out by Beyer, the reactivity of a stretched molecule is likely to depend on the pulling force despite only minor changes in bond geometry. Furthermore, a reorganization of the energy landscape of the bond is likely to occur during bond lengthening. These effects are not accounted for by this model. Hence, further theoretical developments on the effect of a mechanical force on the thiol/disulfide exchange reaction will be required to fully understand these experiments. These limitations notwithstanding, useful parameters can be extracted from this analysis. For example, the sensitivity of the rate of reduction to a pulling force is well represented by the measured value of Δx_r , which can be contrasted to that of unfolding the unsequestered region of the protein. By fitting a single exponential to an average of traces containing solely unsequestered

unfolding events of the type shown in Fig. 1B (no DTT), the rate of unfolding is measured, $\alpha u = 1/\tau_u$, at different pulling forces. Fig. 9A shows a semilogarithmic plot of both αu and r as a function of the pulling force. The dashed line corresponds to a fit of $\alpha u(F) = \alpha u(0) \exp(F\Delta x_u/kBT)$ (19), obtaining $\Delta x_u = 1.75 \text{ \AA}$ for the unsequestered unfolding. The solid line is a fit of the Bell-like model described earlier, where $\Delta x_r = 0.34 \text{ \AA}$ for the thiol/disulfide exchange reaction. Fig. 9A confirms the difference in force sensitivity between the unsequestered unfolding and the thiol/disulfide exchange reaction, which are two distinct processes occurring within the same protein.

[0115] From the measurements above a preliminary description of the energy landscape for the thiol/disulfide exchange chemical reaction was obtained under a stretching force (Fig. 9B). Recent theoretical calculations have proposed that the length of an S–S bond at the transition state of a simple SN2 thiol/disulfide exchange reaction in solution increases by 0.36 $\text{\AA}$ or 0.37 $\text{\AA}$. These values suggest that the value of Δx_r that has been measured experimentally corresponds to the lengthening of the S–S bond during an SN2 reaction with a DTT molecule (Fig. 9C). Furthermore, in these theoretical studies, varying the reaction mechanism could result in S–S lengthening at the transition state as small as 0.24 $\text{\AA}$ or as large as 0.78 $\text{\AA}$ (28). Different values of Δx_r would result in very different sensitivities of the reaction to a pulling force. Hence, it is proposed that by experimentally measuring the value of Δx_r , as demonstrated here for a single disulfide bond, different types of reaction mechanisms can be distinguished. Conversely, this work also suggests that other bimolecular reactions that result in bond lengthening may be force dependent.

[0116] Although force-dependent thiol/disulfide exchange in an engineered protein has been demonstrated, there are many native proteins that contain disulfide bonds and that are exposed to mechanical forces in vivo. Some examples include cellular adhesion proteins such as cadherins, selectins, and IgCAMs. Others are important in maintaining the extracellular matrix, such as fibronectin, or in tissue elasticity, such as fibrillin and titin. It has been shown that thiol/disulfide exchange in integrin $\alpha IIb\beta III$ as well as disulfide reduction in von Willebrand factor multimers is necessary for hemostasis and regulating clot formation under high shear forces generated by blood flow. Even the mechanical process of HIV virus fusion and entry into helper T cells has been shown to require disulfide bond reduction in both gp120 of HIV and the CD4 cell surface receptor.

[0117] Forces $>100 \text{ pN}$ are necessary to achieve a measurable increase in the rate of thiol/disulfide exchange. Such forces are thought to be toward the high end of the range

experienced in biology: single protein complexes may produce forces >100 pN, and single selectin–ligand bonds can withstand forces >200 pN. Although it is not yet known how often a single disulfide bond in vivo will be exposed to the force levels explored in this study, it does seem likely that the sensitivity of any particular thiol/disulfide exchange reaction to a pulling force will depend very specifically on the environment surrounding the bond as well as the type of chemical reaction involved. For example, Δx_r is likely to depend on a number of factors that also affect the rate of the thiol/disulfide exchange reaction, including the temperature, type of reducing agent, pH, electrostatics the reaction mechanism, and the torsional strain present in the protein structure. Any combination of these effects that cause Δx_r to be >1 Å would lead to a near 2-fold increase in reduction rate over just 20 pN of applied force, suggesting that force-catalyzed disulfide reduction may play an important role in vivo.

Materials and Methods

[0118] Protein Engineering and Purification. The QuikChange site-directed mutagenesis kit (Stratagene) was used to mutate residues Gly-32 and Ala-75 in the 27th Ig-like domain of human cardiac titin to Cys residues. Native Cys-47 and Cys-62, which do not form a disulfide bond, were mutated to alanines. An eight-domain N–C linked polypeptide of this I27G32C–A75C domain was constructed through rounds of successive cloning in modified pT7Blue vectors and then expressed the gene using vector pQE30 in *Escherichia coli* strain BL21(DE3) as described. Pelleted cells were lysed by sonication and the His-6-tagged soluble protein was purified first by immobilized metal ion affinity chromatography (IMAC) and then by gel filtration. The protein was stored at 4°C in 50 mM sodium phosphate/150 mM sodium chloride buffer (pH 7.2).

[0119] Single-Molecule Force–Clamp Spectroscopy. A custom-built atomic force microscope equipped with a PicoCube P363.3-CD piezoelectric translator (Physik Instruments, Karlsruhe, Germany) controlled by an analog proportional–integral–differential feedback system is described elsewhere (Schlierf et al., *PNAS USA*, 101:7299-7304). All data were obtained and analyzed by using custom software written for use in IGOR 5.0 (WaveMetrics, Lake Oswego, OR). There was ≈0.5 nm of peak-to-peak noise and a feedback response time of ≈5 ms in all experiments. The spring constant of silicon–nitride cantilevers (Veeco, Santa Barbara, CA) was calibrated; the average spring constant was ≈15 pN/nm. All experiments were conducted in PBS buffer with the indicated amount of DTT (Sigma).

Buffers were all controlled to pH 7.2. All experiments were conducted over ≈ 8 h at room temperature (298 K) in an atmosphere open to air. Small changes in active DTT concentration due to evaporation and air-oxidation of DTT did not appear to greatly affect the results, because traces compiled over the course of 1 day's experiment at the same force demonstrated similar single-exponential kinetics. Approximately 5 μL of protein sample (≈ 0.1 mg/mL) in phosphate buffer was added to ≈ 70 μL of DTT-containing buffer in each experiment. Single protein molecules were stretched by first pressing the cantilever on the gold-coated coverslide for 3 s at 350–500 pN, then retracting at a constant force. The success rate at picking up a single molecule was $\approx 1\%$ of trials. Gold-coated coverslides were used because they resulted in a better success rate than glass coverslides even in the absence of thio-gold bonds. In all force-dependent experiments (Fig. 8A), the molecule was stretched for 1 s at 130 pN and then 5–7 s at the second pulse force. In the concentration-dependent experiments (FIG. 8C), the molecule was pulled at 130 pN for 1 s (0–31 mM DTT) or 140–145 pN for 0.2–0.5 s (50–125 mM DTT; the shorter first pulse times are to reduce thiol/disulfide events during the first pulse), then the force increased to 200 pN for 5–7 s. The interaction between protein and cantilever/coverslide is nonspecific. Thus, in most cases fewer than eight domains were unfolded when a molecule was stretched.

[0120] Data Analysis. The fingerprint of a single $(\text{I27}_{\text{G32C-A75C}})_8$ was considered to be two well resolved steps of ≈ 10.5 nm during the first pulse. No traces that included unsequestered unfolding events during the second pulse were included in the analysis. Such mixed spectra were very rarely observed ($<1\%$) at forces of 200 pN or greater because of the very rapid kinetics of unsequestered unfolding at these forces. At a second pulse force of 100 pN, such mixed spectra were observed $\approx 15\%$ of the time; such traces were not included in the averaging analysis because the unsequestered unfolding steps would corrupt the time course of disulfide reduction. It is assumed that disulfide reduction in this protein is Markovian (i.e., each reduction event is independent of all others); thus, averaging traces with different numbers of reduction steps will result in invariant exponential kinetics. Error bars in FIG. 8B and FIG 8D and FIG 9A were obtained by partitioning the entire set of traces into random subsets (for example, if 20 total single-molecule traces were used, then two subsets of 10 traces each were constructed). These traces in these subsets then were averaged and fit with a single exponential. The rate of thiol_disulfide exchange (FIG. 8A and FIG 8B) or rate of unsequestered unfolding (FIG. 9A) was determined as described from the exponential fit. The standard deviation of the rate was then calculated with $n =$ the number of subsets (in the

case of the example, $n = 2$). This value then was used as the magnitude of the error bar shown in the figures.

EXAMPLE 2

[0121] One of the principal challenges of understanding enzyme catalysis, a central problem in biology, is resolving the dynamics of enzyme–substrate interactions with sub-ångström resolution—the length scale at which chemistry occurs. Although nuclear magnetic resonance (NMR) and X-ray crystallography determinations of protein structures can reach down to the sub- ångström level, they cannot yet provide dynamic information about enzyme catalysis at this length scale. This demonstrates the ability of single-molecule techniques in probing the dynamics of enzyme catalysis at the sub- ångström scale.

[0122] A polyprotein made of eight repeats of the I27 domain of human cardiac titin with engineered cysteines, (I27SS)8, was used as a substrate protein to monitor the Trx-catalysed reduction of individual disulphide bonds (SS) placed under a stretching force. In these experiments, an atomic force microscopy in force-clamp mode to extend single (I27SS)8 polyproteins was used (FIG. 10A, far left). The constant applied force caused individual domains to unfold, resulting in a stepwise increase in the length of the molecule after each unfolding event. This is illustrated in Fig. 10b, in which a single (I27SS)8 polyprotein was first mechanically unfolded at 165 pN for 400 ms. A series of 10.8-nm steps was rapidly observed (FIG. 10B, inset); each step corresponds to the partial unfolding of a single I27SS domain up to the disulphide bond (red, FIG. 10A). The disulphide bond is buried in the folded protein and is exposed to the bathing solution only after partial unfolding. The unfolding force pulse was followed by a test pulse (100pN in this case). No further steps were observed during the test pulse because the disulphide bond could not be broken by the applied force alone in the absence of Trx. After unfolding, the stretching force was applied directly to the disulphide bond and, if Trx is present in solution, the bond can be chemically reduced by the enzyme. Such a result is shown in FIG. 10C, with a similar experiment in the presence of 8 μ M Trx. Now, during the test pulse, seven steps of ~ 13.2 nm were observed as individual disulphide bonds were reduced by single Trx enzymes, allowing for the immediate extension of the residues previously trapped behind the disulphide bond (blue, FIG. 10A). The size of the increases in step length observed during these force-clamp experiments corresponds to the number of amino acids released, serving as a precise fingerprint to identify the reduction events.

[0123] An ensemble of single-molecule recordings to measure the kinetics of disulphide bond reduction by Trx was used. At each force and Trx concentration, 10–30 test-pulse recordings of the type shown in FIG. 10C were averaged. Averaged traces at various forces are shown in FIG. 11A. The averaged traces were fitted by a single exponential with a time constant τ (see Supplementary FIG. 11 and FIG. 12). The observed rate constant of disulphide reduction is defined as $r = 1/\tau$.

[0124] FIG. 11B shows a plot of r as a function of the applied (test pulse) force. The figure shows that the rate of reduction decreases fourfold between 25 and 250 pN, and then increases approximately threefold when the force is increased up to 600 pN, demonstrating a biphasic force dependency. This result is in contrast with the uniform acceleration of dithiothreitol (DTT) reduction rate with increasing force⁴, underlining a much more complex chemical reaction catalysed by Trx. Furthermore, the rate of reduction becomes saturated as the concentration of Trx is increased (FIG. 11C).

[0125] To explain the data, different kinetic models of force-dependent Trx catalysis were tested. It was found that the model that could best describe the data incorporates an intermediate state as well as two different force-dependent rate constants (Fig. 11D). Path I (red, Fig. 11D) is similar to a Michaelis–Menten mechanism, with a catalytic step inhibited by force. Path II (blue, Fig. 11D) is governed solely by the rate constant κ_{02} (where subscripts refer to steps in Fig. 11D), which is accelerated by force. The model can be globally fitted to the data of Fig. 11D and 11C (solid lines), obtaining values for the model parameters. The goodness of fit for this model was measured using statistical methods (see Methods and Supplementary Table 3; $\chi^2_{\nu} = 0.835$, and $\nu = 26$, six free parameters; $P(\chi^2_{\nu} = 0.705)$). An extrapolation to zero force predicts a second-order rate constant for Trx reduction of $2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. This is $\sim 30,000$ times faster than that found for I27SS disulphide reduction by DTT ($6.5 \text{ M}^{-1} \text{ s}^{-1}$). This result is consistent with bulk biochemical experiments, in which Trx has been found to reduce insulin disulphide bonds $\sim 20,000$ times faster than DTT ($1 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for Trx versus $5 \text{ M}^{-1} \text{ s}^{-1}$ for DTT at pH7).

[0126] The experimental data shown in Fig. 11B suggest that there are two separate pathways for disulphide bond reduction by Trx. Further support for this hypothesis was gained by probing the force-dependent reduction kinetics of an active site mutant, Trx(P34H) (Fig. 12). In the single-molecule experiments, the extrapolated zero-force rate of reduction for Trx(P34H) is less than one-half of that for the wild-type enzyme ($8.8 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ versus $2.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, showing a similar relationship to bulk biochemical experiments ($3 \times 10^3 \text{ M}^{-1}$

s-1 for Trx(P34H) versus $2 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for wild-type Trx at pH8 and 15 °C. In Trx(P34H), the rate of Trx binding to the substrate (κ_{01}) decreased significantly, whereas the other kinetic parameters remain mostly unchanged. By fitting this data with two alternate kinetic models, it was found that the Trx(P34H) mutant supports the view that Trx has two distinct forms of catalysis, without a common intermediate.

[0127] In the kinetic model shown in Fig. 11D, the catalytic rate constants are described by a straightforward Arrhenius term. For example, $\kappa_{12} = \beta_0 \exp(F\Delta x_{12}/\kappa_B T)$, where β_0 is the rate constant at zero force, κ_B is Boltzmann's constant, T is the temperature and Δx_{12} is the distance to the transition state along the length coordinate. Fits of the kinetic model (Fig. 11D) to the data of Fig. 11B and 11C gave values of $\Delta x_{12} = -0.79 \pm 0.09 \text{ \AA}$ for the catalytic step of path I and $\Delta x_{12} = 0.17 \pm 0.02 \text{ \AA}$ for the catalytic step of path II ($\pm$ s.e.m. obtained by downhill simplex procedure for model fitting). Similar parameters were also found for the Trx(P34H) mutant. Thus, the two catalytic pathways are very different: the transition state of reduction by way of path I requires a shortening of the substrate polypeptide by $\sim 0.8 \text{ \AA}$, whereas path II requires an elongation by $\sim 0.2 \text{ \AA}$.

[0128] The experiments show that sub-ångström-level distortions of the substrate disulphide bond take place dynamically during Trx catalysis. A glimpse of the transition state for Trx catalysis can be obtained from the NMR structure of human TRX (also known as TXN) a homologue of the E. coli enzyme, in a complex with a substrate peptide from the signalling protein NF- κ B (Fig. 13A, PDB accession number 1MDI). In this structure, as well as in the structure of human TRX bound to REF-1 (also known as APEX1), a peptide-binding groove is identified on the surface of TRX in the vicinity of the catalytic Cys 32. The sulphur atom in Cys 32 (sulphur atom A) of the active site of TRX forms a disulphide bond with the sulphur atom of the NF- κ B peptide (sulphur atom B).

[0129] The orientation of the disulphide bond within the Trx active site was used in an attempt to predict the structure of the catalytic transition state in the experiments. It is known that disulphide bond reduction proceeds by means of an $\text{S}_\text{N}2$ mechanism. This reaction is highly directional, proceeding via a transition state in which the three involved sulphur atoms form an $\sim 180^\circ$ angle. Thus, the relative positions of these sulphur atoms must be important for efficient Trx catalysis. It was found that the disulphide bond in 1MDI forms an angle of $\sim 70^\circ$ with respect to the axis of the peptide-binding groove. Assuming that this orientation applies to the $\text{S}_\text{N}2$ reaction that reduces the I27SS bond of the experiments, and that the

stretched polypeptide is bound to the groove, it is apparent that the target disulphide bond must rotate with respect to the pulling axis to acquire the correct SN2 geometry (Fig. 13B). Given that the disulphide bond in the stretched polypeptide is aligned within $\sim 20^\circ$ of the pulling force, a further rotation by an angle $\theta = 50^\circ$ would be required for catalysis (Fig. 13B), causing a contraction of the target polypeptide by $\sim 1.2 \text{ \AA}$, close to the measured value of $\Delta x_{12} \approx -0.8 \text{ \AA}$. However, molecular dynamics simulations have previously identified multiple conformations of the catalytic thiol in glutaredoxin, a member of the thioredoxin superfamily.

[0130] Similarly, molecular dynamics simulations of the 1MDI structure was performed to examine the conformational diversity of the NF- κ B to Cys 32 disulphide bond. The simulations show that the disulphide bond samples a range of conformations with $\theta = 50^\circ - 80^\circ$ in either the clockwise or the counterclockwise direction (shaded area in the inset of Fig. 13A). The results of these molecular dynamics simulations were combined with a theoretical model that treats the substrate backbone as a freely jointed chain. This model predicts the likelihood of the substrate disulphide achieving the correct geometry for the reaction transition state under a pulling force. It was found that, in the cases of NF- κ B, REF-1 and the apo TRX, an average bond rotation on the sub-ångström scale (resulting in Δx_{12} values of -0.77, -0.45 and -0.19 Å, respectively) must take place to allow SN2 chemistry in the TRX active site.

[0131] To probe this model of catalysis, which is based on the structure of human TRX complexes, the force-dependent mechanism of disulphide bond reduction by human TRX (Fig. 13C) was also tested. At low forces, it is clear that human TRX catalyses disulphide bond reduction in I27SS much more rapidly than Trx from *E. coli*. However, at high forces it appears that path II is quite diminished in the human TRX variant. Thus, the data for human TRX resemble a simple Michaelis–Menten model (dashed green line in Fig. 11B), indicating that the two thioredoxin variants differ in their catalytic mechanisms at high force. The three-state kinetic model also describes the human TRX data well with a fixed $\Delta x_{12} = -0.79 \text{ \AA}$ (Fig. 13C). Thus, it is clear that the mechanism that governs the force-dependence of path I is conserved between these homologues, and the results for human TRX at low forces can also be explained by the structural model.

[0132] The origin of the $\Delta x_{12} = -0.79 \text{ \AA}$ elongation at the transition state of catalysis for *E. coli* Trx, measured from the force-dependency of path II, is less clear. However, as demonstrated in the theoretical calculations of thiol/disulphide exchange, other reaction

geometries are possible, even if they are typically unfavourable energetically. Thus, Δx_{02} may correspond to the lengthening of the I27SS disulphide bond at a transition state other than the standard SN_2 form.

[0133] The results show that a mechanical force can alter the chemistry of the catalytic site in thioredoxin significantly. This is a novel concept in biology, that mechanical stresses applied to tissues may completely change the enzymatic chemistry from that observed in solution biochemistry. These effects may be particularly significant in tissues exposed to pathological force levels such as those that occur during mechanical injury. For example, it is well known that the increased mechanical stress during hypertension triggers an oxidative stress response in vascular endothelium and smooth muscle that is compensated by an increase in the activity of thioredoxin. In this context, it is predicted that the increased mechanical forces applied to target disulphide bonds would inhibit the activity of thioredoxin, diminishing the effectiveness of the antioxidant properties of the enzyme. The capability of single-molecule atomic force microscopy techniques directly to probe the dynamic sub-ångström molecular rearrangements during catalysis may prove to be an important tool in understanding the fundamental mechanisms underlying enzymatic chemistry.

Methods Summary

[0134] The buffer used in the experiments contained 10mM HEPES, 150mM NaCl, 1mMEDTA, 2mM NADPH, 50 nM thioredoxin reductase (from *E. coli* for Trx and from rat liver for TRX) and the indicated concentration of Trx or TRX, and was controlled to pH 7.2. Single (I27SS)8 protein molecules were stretched by first pressing the cantilever on the coverslide at a constant force of 800 pN for 3 s, then retracting to a constant force of 165 pN for 400 ms during the unfolding pulse. The indicated test-pulse force was applied for ~5 s. All data were obtained and analysed using custom software written for use in Igor 5.0 (Wavemetrics). The test-pulse portions of numerous ($n=10-30$) recordings that contained only disulphide reduction events and no unsequestered unfolding events were summed and normalized to obtain the experimental value r . The differential rate equations were solved using matrix analysis methods, and error analysis was performed using the nonparametric bootstrap method in combination with the downhill simplex method. All error bars shown represent standard error.

METHODS

[0135] Protein engineering, expression and purification. In brief, the QuikChange site-directed mutagenesis method (Stratagene) was used to introduce Gly 32 Cys and Ala 75 Cys mutations into the I27 module from human cardiac titin. Multiple rounds of successive cloning were used to create an amino-carboxy linked, eight-domain polypeptide gene, (I27G32C–A75C)₈. In this work, this construct is called (I27SS)₈. This gene was encoded in vector pQE30 and expressed in *E. coli* strain BL21(DE3). Pelleted cells were lysed by sonication, and the His 6-tagged protein was first purified using an immobilized Talon-Co²⁺ column (Clontech) and then further purified by gel filtration on a Superdex 200 column (GE Healthcare). The purified protein was verified by SDS–PAGE and stored at 4°C in a buffer of 10mM HEPES, 150mM NaCl, 1mM EDTA and 0.02% NaN₃ (w/v), pH 7.2.

[0136] Both wild-type Trx and Trx(P34H) were expressed and purified by the same method described previously²⁶. Briefly, the *E. coli* Trx gene encoded in plasmid pTK100, was expressed in *E. coli* strain JF521. Cell pellets were lysed using a French press and stirred with streptomycin sulphate (10% w/v) at 4°C for 16 h. The filtered supernatant was then loaded onto a 2-l Sephacryl S-100 High Resolution (GE Healthcare) gel filtration column. Trx fractions were pooled and applied to a 250-ml Fractogel EMD DEAE(M) (Merck) ion exchange column equilibrated in a buffer containing 1mM EDTA and 30mM TRIZMA, pH 8.3. The protein was eluted by a linear gradient between 0 and 0.5M NaCl. The proteins were pure, as measured by SDS–PAGE gel densitometry. The molecular weight of pure proteins was confirmed by mass spectrometry. Trx fractions were dialysed into a buffer of 10mM HEPES, 150mM NaCl and 1mM EDTA, pH7.2. Trx concentration was determined spectrophotometrically at 280nm using a molar absorption coefficient ϵ_{280} of 13,700 M⁻¹ cm⁻¹. The bulk activity of Trx and Trx(P34H) was confirmed by monitoring spectrophotometrically at 412 nm the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) as described (Perez-Jimenez et al., *Biophys. Chem.*, 115:105-107 (2005)).

[0137] TRX was purified as previously described. Briefly, the pACA/TRX plasmid was expressed in BL21(DE3) cells. Cell pellets were lysed using a French press and stirred with 7% w/v streptomycin sulphate. Protein was then precipitated by adding ammonium sulphate to 85% saturation. The crude extracts were applied to a DEAE 52 column equilibrated with 50mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.1mM DTT. Protein was eluted with an NaCl gradient, pooled and concentrated, and then applied to a Sephadex G-50 column equilibrated with 50 mM Tris-HCl, pH 7.5, 1 mM EDTA and 0.1 mM DTT. Fractions were pooled, concentrated and further purified using *E. coli* Trx antibody affinity chromatography. Protein

concentration was determined spectrophotometrically at 280 nm using an ϵ_{280} of 8,050 M⁻¹ cm⁻¹.

[0138] Single-molecule force-clamp spectroscopy. Typical resolution in extension was ~0.5 nm and typical analogue feedback lag in the force-clamp following unfolding was ~5 ms. The spring constant of silicon nitride cantilevers (Veeco), typically ~20 pN nm⁻¹, was calibrated as described previously. The buffer used for all experiments contained 10 mM HEPES, 150 mM NaCl, 1 mM EDTA and 2 mM NADPH, and was controlled to pH 7.2. Before beginning the experiment, thioredoxin reductase (Sigma; from *E. coli* for *E. coli* Trx experiments, or from rat liver for human TRX experiments) was added to the experimental buffer to a final concentration of 50 nM. Thioredoxin was then added to the experimental buffer to the indicated concentration. An excess of NADPH and a catalytic amount of thioredoxin reductase are both necessary to maintain ~98% of Trx in the active, reduced form during the experiment. In the Trx system, reducing equivalents are donated from NADPH to the FAD domain of thioredoxin reductase, and these electrons subsequently reduce a catalytic disulphide bond in thioredoxin reductase. Reduced *E. coli* thioredoxin reductase is very specific for reducing the disulphide bond in oxidized Trx1 and does not non-specifically reduce other disulphides. When Trx was not included in the solution, no disulphide reduction in I27SS was observed even in the presence of thioredoxin reductase and NADPH.

[0139] In the experiment, ~5 μ l (I27SS)₈ solution was added to a ~100 μ l droplet of Trx-containing experimental buffer deposited on a substrate coverslide. Single (I27SS)₈ protein molecules were stretched by first pressing the cantilever on the coverslide at a constant force of 800 pN for 3 s, then retracting to a constant force of 165 pN for 400 ms during the unfolding pulse. The indicated test-pulse force was applied for ~5 s. In these experiments, the precise point of attachment between the (I27SS)₈ molecule and the cantilever was not controlled; thus, varying numbers of disulphide reduction events may be observed in a given single-molecule recording.

[0140] Data analysis. All data were recorded and analysed using custom software written in Igor Pro 5.0 (Wavemetrics). Only recordings that exhibited disulphide reduction events of the expected step size in the test pulse were analyzed. (For a discussion of expected disulphide reduction step size as a function of force, see Wiita et al., *PNAS USA*, 103: 7222-7227(2006)). The test-pulse portions of numerous (n = 10–30) recordings that contained only disulphide reduction events and no unsequestered unfolding events were summated and normalized. These averaged traces were fitted with a single exponential to obtain the

observed rate constant of reduction, r . This type of summation procedure is standard in the ion channel literature and has been used in many contexts to obtain macroscopic kinetics from single-molecule recordings. It is assumed that disulphide reduction in (I27SS)8 is markovian (that is, that each reduction event is independent of all others); thus, averaging traces with different numbers of reduction steps will result in invariant exponential kinetics⁴. To estimate the error on the experimentally obtained rate constant, the nonparametric bootstrap method was carried out. At a given value of force and $[Trx]$, n staircases were randomly drawn with replacement from the original data set. These were summed and fitted to obtain a rate constant. This procedure was repeated 1,000 times for each data set, resulting in a distribution that provided the standard error of the mean for the reduction rate constant, shown as the error bars in FIG. 11B and 11B, FIG. 12 and FIG. 13B.

[0141] Kinetic model. In the kinetic model shown in FIG. 11D, three states are used to describe the experimental system. The rate equations for the concentrations of states 0, 1 and 2 as a function of time τ are:

$$d[0]/d\tau = -\kappa_{01}[0] - \kappa_{02}[0] + \kappa_{10}[1] \quad (1)$$

$$d[1]/d\tau = \kappa_{01}[0] - \kappa_{10}[1] - \kappa_{12}[1] \quad (2)$$

$$d[2]/d\tau = \kappa_{02}[0] + \kappa_{12}[1] \quad (3)$$

[0142] Where each rate constant is defined by the following parameters (α_0 , β_0 , γ_0 and δ_0 are coefficients used to calculate each rate constant as a function of force and $[Trx]$):

$$\kappa_{01} = \alpha_0[Trx] \quad (4)$$

$$\kappa_{12} = \beta_0 \exp(F\Delta x_{12}/k_B T) \quad (5)$$

$$\kappa_{02} = \gamma_0[Trx] \exp(F\Delta x_{02}/k_B T) \quad (6)$$

$$\kappa_{10} = \delta_0 \quad (7)$$

[0143] As shown in FIG. 11D, rate constants κ_{01} and κ_{02} are linearly dependent on the concentration of Trx and have units of $\mu\text{M}^{-1} \text{s}^{-1}$. κ_{10} is a constant with units of s^{-1} . κ_{12} is in units of s^{-1} and is modelled to be exponentially dependent on the applied force, following the Bell equation. κ_{02} also demonstrates an exponential dependence on the applied force. There are no reverse rate constants for the $0 \rightarrow 2$ and $1 \rightarrow 2$ transitions (that is, $\kappa_{20} = 0$ and $\kappa_{21} = 0$). Immediately after disulphide bond reduction in an I27SS module by Trx, the two thiol groups in I27SS are pulled more than 10 nm apart by the applied force. This prevents any reoxidation of the disulphide bond in I27SS, so the formation of state 2 is irreversible in the experiment. It is assumed that the concentration of Trx remains constant throughout the experiment because the rare oxidation of single enzymes will not significantly affect the overall solution concentration of active Trx. The [Trx] term is input as a constant from the experimental conditions. The I27SS concentration is not a factor in the rate equations because only single molecules are monitored at any given time and all results are unaffected by the bulk concentration of I27SS.

[0144] To describe the obtained experimental data, this kinetic model using matrix analysis was solved. By determining the eigenvalues and eigenvectors of the kinetic matrix (see equation (8)) it is possible to calculate the probability of a single I27SS module being in a given state as a function of time.

$$A = \begin{pmatrix} -(\kappa_{01} + \kappa_{02}) & \kappa_{10} & 0 \\ \kappa_{01} & -(\kappa_{10} + \kappa_{12}) & 0 \\ \kappa_{02} & \kappa_{12} & 0 \end{pmatrix} \quad (8)$$

[0145] If values are inputted for the parameters α_0 , β_0 , γ_0 , δ_0 , Δx_{12} and Δx_{02} as well as the experimental [Trx], the matrix for a discrete set of forces in the range of 0–600 pN can be solved. The output of the analysis shows the probability of a single disulphide bond existing in state 0, state 1 or state 2 as a function of time. It is noted that the model is solved with the initial condition of $P(0)=1$ at time =0. State 2, where disulphide reduction by Trx has occurred, is the only state that can be directly monitored using the experimental technique.

Thus, the calculated probability of being in state 2 as a function of time directly corresponds to the observed single-molecule recordings shown in FIG. 11A. By fitting these calculated probabilities with a single exponential, the observed rate constant of reduction ($r=1/\tau$ from the exponential fit to the model plot) can be obtained in the same manner that r was determined for the experimental data.

[0146] To find the optimal kinetic parameters to describe the experimental data, the kinetic model for several, widely ranging values for each parameter (typically over three orders of magnitude) was solved first. Then the model r values were compared to those obtained experimentally (see FIGS. 2C, D for wild-type Trx, and FIG. 3B for Trx(P34H)) and calculated the goodness of the

[0147] fit χ^2 , where $\chi^2 = \sum_{i=1}^N \left(\frac{r_i - f(x_i)}{\sigma} \right)^2$; here, N is the number of data points, r_i is the experimentally observed rate, $f(x_i)$ is the calculated rate from the kinetic model, and σ is the magnitude of the error of the observed rate. The combination of parameter values with the lowest χ^2 then served as the starting point for the downhill simplex method to optimize further the global fit of the model to the data.

[0148] Errors for each parameter were again obtained with the bootstrap method in combination with the downhill simplex method. At each given value of force and $[\text{Trx}]$, a value for the rate constant from the distribution obtained with the bootstrap method (see the 'Data Analysis' section above) was picked at random. By using different values for the rate at each force, extracted from the bootstrap analysis, the experimental error in each rate constant is accounted for when performing fits to the model. The downhill simplex method was then applied to these rate constants, giving the best fitting values for each parameter for that particular combination of rates. The downhill simplex simultaneously varied all six fitting parameters to globally fit the 32 data points for wild-type Trx (14 at 8 μM Trx; 18 at other concentrations). This procedure was repeated 200 times, resulting in distributions, and thereby standard errors, for each model parameter.

[0149] To determine the goodness of fit of the various kinetic models, the above methods were first used to globally fit each model to the force-dependent and concentration-dependent data for wild-type Trx. An overall χ^2 value was measured for the best fit to each model (best-fitting parameters shown in Table 1).

Table 1. Parameters obtained from global fits to alternate kinetic models (see Supplementary Figs. 5 and 6). Error bars are given by the standard error for each parameter obtained from the downhill simplex fitting procedure (see "Kinetic Model" in Methods).

WT Trx	m_0 (μM $\times 10^3$)	β_0 (5° or $\mu\text{M}^{-1} \times 10^3$)	γ_0 ($^\circ$)	δ_0 ($^\circ$)	$\Delta x_{1,0}$ ($\text{\AA}$)	$\Delta x_{2,0}$ ($\text{\AA}$)	$\Delta x_{3,0}$ ($\text{\AA}$)	$\Delta x_{4,0}$ ($\text{\AA}$)
Model 1	0.24 ± 0.07	0.060 ± 0.008	-	-	-	-	-0.9 ± 0.2	-
Model 2	0.25 ± 0.04	0.030 ± 0.007	-	-	-	-	-0.6 ± 0.1	0.15 ± 0.03
Michaelis-Menten	0.22 ± 0.03	25 ± 4	-	3.5 ± 0.9	-0.87 ± 0.07	-	-	-
Model 3	0.24 ± 0.03	28 ± 4	2.7 ± 0.3	4.1 ± 0.6	-1.2 ± 0.2	-	-	-
Model 4	0.24 ± 0.03	33 ± 4	0.4 ± 0.1	4.4 ± 0.3	-0.8 ± 0.1	0.20 ± 0.03	-	-
Trx P34H								
Model 4	0.14 ± 0.02	30 ± 2	0.37 ± 0.08	3.3 ± 0.6	-0.9 ± 0.2	0.36 ± 0.03	-	-

A reduced chi-squared value, $\chi^2_{\nu} = \chi^2/\nu$, where ν is the number of degrees of freedom in the fit ($\nu = N - c$, where N is the number of data points and c is the number of free fitting parameters) was then obtained. To determine the statistical goodness of fit, $P(\chi^2_{\nu})$ was calculated, the likelihood of obtaining the observed χ^2_{ν} if the experimental data are truly represented by the proposed kinetic model. This method has been used previously to determine the goodness of fits of various kinetic models to single-molecule data. $P(\chi^2_{\nu})$ was calculated using the web-based program available at <http://www.fourmilab.ch/rpkp/experiments/analysis/chiCalc.html>. Parameters relating to the analysis of various kinetic models are shown in Table 2.

Table 2. Statistical Analysis of kinetic model fitting (see Supplementary Figs. 5 and 6). For WT Trx, number of data points $N = 32$; for Trx P34H, $N = 10$; for hTrx, $N = 10$ and $\Delta x_{1,0} = 0.079$ (fixed)

WT Trx	Reduced chi-squared (χ^2_{ν})	Degrees of Freedom (ν)	Free parameters	$P(\chi^2_{\nu})$
Final Model	0.835	26	6	0.795
Model 1	1.96	29	3	0.0015
Model 2	1.39	28	4	0.110
Michaelis-Menten	5.06	28	4	$<1 \cdot 10^{-20}$
Model 3	1.21	27	5	0.211
Model 4	0.973	26	6	0.503
Trx P34H				
Final Model	0.580	4	6	0.677
Model 4	3.09	4	6	0.015
hTrx				
Final Model	0.750	5	5	0.586

[0150] Force-probe molecular dynamics and structural modeling. Simulations were carried out with the Gromacs 3.3.1 simulation suite (<http://www.gromacs.org>). The simulations were started from the NMR structure of human TRX in an intermediate complex with a disulphide bond to a substrate, the NF- κ B peptide (PDB accession number 1MDI). Protonation states of the standard amino acids were adopted from the solution structure.

[0151] The OPLS (optimized potentials in liquid simulations) force field was applied. The protein was solvated in a 7.3 x 7.3 x 7.4 nm³ box of TIP4P water molecules. Twenty-two sodium and 18 chloride ions were added to the simulation system to compensate for the overall positive charge of the protein and to mimic physiological conditions. This yielded a total system size of 49,220 atoms. Simulations were carried out with periodic boundary conditions. Application of the Lincs and Settle methods allowed for an integration time step of 2 fs. Electrostatic and Lennard-Jones interactions were calculated within a cut-off of 1 nm, and the neighbour list was updated every ten steps. For the long-range electrostatic interactions, the Particle-Mesh-Ewald (PME) method⁴¹ with a grid spacing of 0.12 nm was used. An N, p, T ensemble, where N is the number of atoms, p is the pressure and T is the temperature, was simulated, with separate coupling of the protein, solvent and ions to a 300K heat bath ($\tau = 0.1$). The system was isotropically coupled to a 1 bar pressure bath ($\tau = 1.0$). Initially, the system was energy-minimized (steepest descent, 1,000 steps), before equilibrating the solvent for 700 ps with positional restraints on protein heavy atoms. Then, the whole system was equilibrated (300 K).

[0152] To model an approximate transition state geometry for the SN2 reaction in the active site of Trx, in a subsequent simulation the Trx-NF- κ B disulphide bond was elongated from 2.05 Å to 2.60 Å (the length of the extended bond found for the transition state in an SN2 reaction) within 160 ps using the free-energy perturbation code in Gromacs and starting from the equilibrated system. Next, the third sulphur atom taking part in the SN2 reaction was placed along the resulting vector of the extended disulphide bond between Trx and the NF- κ B peptide in a distance of 2.40 Å, as found for the SN2 transition state. The cysteine residue to which the third sulphur atom is bound was placed into the location defined by the sulphur atom, and was oriented such that it did not clash with Trx or peptide residues. Using 20 different starting structures of equilibrated Trx for the modelling of the reduction transition state resulted in somewhat different active-site geometries. The angle between the peptide-binding groove and the axis of the sulphur atoms varies and exists in the range

between 50° and 130°. The average conformation of the disulphide bond was observed to fall into two populations. The resulting structures were plotted with Pymol.

[0153] In another set of simulations, titin I27 with residues 32 and 75 mutated to cysteines was unfolded to monitor the disulphide bond orientation in the unfolded state with respect to the pulling direction. The OPLS force field was applied for I27. The wild-type protein (PDB accession number 1TIT) was solvated in TIP4P water in a 6.8 X 5.7 x 5.0 nm³ box. Sixteen sodium and ten chloride atoms were added to neutralize the protein charges and to give physiological ion strength. The resulting system size was 23,524 atoms. I27 was minimized, the solvent initially equilibrated with restraints on the protein heavy atoms (500 ps), and then the entire system subsequently equilibrated for a further 8 ns. The simulation software and parameters as described above were applied. Residues 32 and 75 of the equilibrated structure were mutated to cysteine residues using the program WHATIF. The mutant I27SS was re-solvated in a larger box (19.2 x 5.5 x 5.0 nm³), allowing sufficient space to completely unfold the protein, yielding a system size of 112,156 atoms. The system was minimized, resulting in a shortening of the S–S bond to the value typical for an S–S bond (2.05 Å). The solvent was equilibrated with restraints on the protein heavy atoms (2 ns), followed by the equilibration of side chains with restraints on the protein backbone atoms (2 ns), and finally by the equilibration of the whole system (11 ns). No distortion of the structure adjacent to the point mutations was observed. Force-probe molecular dynamics simulations of the equilibrated I27SS mutant were performed. The C α -atoms of the terminal residues were subjected to harmonic pulling potentials with a spring constant of 500 kJ mol⁻¹ nm⁻², and were moved away from each other with a constant velocity of 0.4 nm ns⁻¹. As expected, the unfolded structure, obtained after ~14 ns of the force-probe molecular dynamics simulation time, showed alignment of the disulphide bond within ~20° of the pulling direction, with a projection of the S–S bond length on the pulling axis of ~1.9 Å.

[0154] For comparison of the active-site geometry, additional standard equilibrium molecular dynamics simulations have been performed for the reduced state of Trx in the absence of a peptide, and for the other available Trx intermediate, the Trx–Ref-1 complex. For the simulation of the reduced state, the NF- κ B peptide in the 1MDI structure was deleted. The apo structure with an unprotonated Cys 32 was solvated in water. After addition of ions to yield physiological ion strength, the system comprised 33,606 atoms. The Trx–Ref-1 complex (PDB accession number 1CQH) was solvated in water with physiological ion strength, resulting in a system size of 38,760 atoms.

EXAMPLE 3

Selection of Trxs from different species

[0155] To investigate the various catalytic mechanisms developed by Trx, a set of Trxs belonging to a representative group of species from different kingdoms were selected: Animalia, Eubacteria, Protista and Plantae (covering two domains of life: bacteria and eukaryotes). Trx is widely distributed in all living organisms from bacteria to mammals. In addition, the existence of a second paralogous Trx gene (Txn2) seems to be common in animals, protists and Gram-negative bacteria. In protists and animals, Trx1 is located in the cytoplasm, whereas Trx2 is present in mitochondria. Notably, mitochondrial Trx2 from mammals has been shown to have higher similarity with *E. coli* Trx1 than with cytosolic Trx1 from mammals. In the case of plants, a rich variety of Trx genes can be found encoding more than 20 different types of Trxs that are classified into six isoforms: Trxf, h, m, x, y and o. The f, m, x and y forms are plastidic Trxs, h forms are mainly cytosolic and o forms are found in mitochondria. In this study, both human cytosolic and mitochondrial Trxs from animals were included, poplar Trxh1 (featuring a CPPC active site instead of the canonical CGPC), poplar Trxh3 and pea chloroplastic Trxm from plants, *E. coli* Trx1 and Trx2 from bacteria and, finally, *Plasmodium falciparum* (malaria parasite) Trx1 from protists.

[0156] A sequence alignment of the Trxs of interest shows that the residues around the active site are highly conserved. The construction of a phylogenetic tree (Fig. 14), incorporating additional Trxs from the three domains of life, classifies *E. coli* Trx1 and Trx2, human TRX2 and pea Trxm as 'bacterial-type' Trxs (upper branches in Fig. 14) and human TRX1, poplar Trxh1 and Trxh3 and *P. falciparum* Trx1 as 'eukaryotic-like' Trxs (lower branches in Fig. 14). The construction of a larger tree incorporating more than 200 Trx sequences corroborates the suggestion that the sequences used are widely distributed and that they are representative for the entire Trx family.

Force-dependent chemical kinetics of disulfide reduction

[0157] An atomic force microscope was used in its force-clamp mode to study the chemistry of disulfide reduction by Trx. Briefly, a polyprotein composed of eight domains of the 27th module of human cardiac titin was chosen for a substrate, in which each module contains an engineered disulfide bond between the 32nd and 75th positions (I27G32C-A75C)⁸. The first pulse of force (175 pN, 0.3 s) applied to the polyprotein allows the rapid

unfolding of the I27G32C-A75C modules up to the disulfide bond. The individual unfolding events can be registered as steps of B10.5 nm per module. After the first pulse, the disulfide bonds become exposed to the solvent, where the Trx molecules are present in the reduced form owing to the presence of Trx reductase and NADPH (Trx system)⁶. A second pulse of force is applied to monitor single disulfide reductions by Trx enzymes, recorded as a second series of steps of B13.5 nm per domain (Fig. 15A, 15B). Several traces per force (15–50) have been accumulated, which have been averaged and fit with a single exponential with a time constant τ (Fig. 15C, 15D). Thus, the reduction rate at a given force ($r = 1/\tau$) is obtained.

[0158] The single-molecule assay was applied to obtain the force-dependency of the rate of reduction by the selected Trxs (Fig. 16). From these data three different types of force-dependencies can be readily distinguished. First, all tested Trx enzymes showed a negative force-dependency in the range 30–200 pN. Second, all Trx enzymes from bacterial origin show that, after reaching a minimum rate at around 200 pN, the rate of reduction increases exponentially at greater forces. Third, at forces greater than 200 pN, enzymes from eukaryotic origin show a rate of reduction that becomes force-independent. Therefore, the previous observations in *E. coli* and human TRXs can now be generalized to bacterial-origin and eukaryotic-origin Trxs.

[0159] As previously proposed, the reduction mechanism observed when the substrate is stretched at low forces (30–200 pN) is similar to a Michaelis-Menten (MM-SN2) reaction in which the formation of an enzyme-substrate complex is determinant. Upon binding, the substrate disulfide bond needs to rotate to achieve the correct geometry necessary for an SN2 reaction to occur, that is, the three involved sulfur atoms form an $\sim 180^\circ$ angle. This rotation causes the shortening of the substrate polypeptide along the stretching force axis, as determined by the negative value of Δx_{12} in the kinetic model (Table 3, Fig. 17). This mechanism is rapidly inhibited as the force increases, generating the negative dependence of the reduction rate with the pulling force in all Trx enzymes (Fig. 16). Here it is demonstrated that, whereas the absolute rate of reduction varies from enzyme to enzyme, the general characteristics of this mechanism of reduction are apparent in all of them.

Table 3 Kinetic parameters for Trx enzymes from different species

	α_0 ($\mu\text{M}^{-1} \text{s}^{-1}$)	β_0 (s^{-1})	γ_0 ($\mu\text{M}^{-1} \text{s}^{-1}$)	k_{12} (s^{-1})	Δx_{12} ($\text{\AA}$)	Δx_{22} ($\text{\AA}$)	k_0 (s^{-1})
<i>E. coli</i> Trx1	0.25 ± 0.02	24 ± 2	0.012 ± 0.002	4.7 ± 0.5	-0.75 ± 0.05	0.18 ± 0.01	0.38 ± 0.02
<i>E. coli</i> Trx2	0.18 ± 0.04	23 ± 3	0.009 ± 0.002	4.5 ± 0.5	-1.41 ± 0.03	0.19 ± 0.01	0.37 ± 0.03
Human TRX2	0.55 ± 0.15	25 ± 2	0.019 ± 0.004	3.8 ± 0.6	-0.94 ± 0.05	0.19 ± 0.02	0.35 ± 0.04
Popl Trxm	0.28 ± 0.03	22 ± 2	0.012 ± 0.002	5.0 ± 0.6	-0.93 ± 0.08	0.17 ± 0.02	0.39 ± 0.04
<i>S. turgidum</i> Trx1	0.43 ± 0.05	25 ± 2	..	4.8 ± 0.5	-1.01 ± 0.05	..	0.39 ± 0.02
Human TRX1	0.52 ± 0.05	33 ± 2	..	3.3 ± 0.9	-0.71 ± 0.05	..	0.35 ± 0.02
Poplar Trxh3	0.12 ± 0.03	30 ± 3	..	4.4 ± 0.4	-1.16 ± 0.08	..	0.17 ± 0.02
Poplar Trxh1	0.22 ± 0.02	28 ± 3	..	5.5 ± 0.6	-0.68 ± 0.07	..	0.17 ± 0.03

[0160] According to the parameters obtained from the fit of the data to a simple MM-SN2-type kinetic model (Table 3), it was found that an extrapolation to zero force predicts rate constants ranging from $1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for poplar Trxh3 to $6.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ for human TRX2. These values are markedly similar to those obtained previously using insulin disulfides as substrates and *E. coli* Trx8. The value of Δx_{12} remained below $1 \text{ \AA}$, except for *E. coli* Trx2 and poplar Trxh3, which gave values of more than $1 \text{ \AA}$ (Table 3). These high values of Δx_{12} represent a higher rotation angle of the substrate disulfide bond for the SN2 reaction. This mechanism is unique to Trx enzymes, and it seems to be the result of evolutionary pressure toward developing an efficient mechanism of disulfide reduction that is not possible with simple chemical reagents.

[0161] When forces of more than 200 pN were applied to the substrate, the MM-SN2 mechanism was blocked, and a second force-dependent mechanism of reduction became dominant. This was true for all types of Trx enzymes that were tested. In enzymes of bacterial origin, this high-force mechanism (Fig. 16A) seems to be analogous to that of simple chemical compounds such as cysteine, glutathione or DTT, which reduce disulfide bonds through a force-dependent SN2 thiol-disulfide exchange reaction with bond elongation at the transition state. This reaction was incorporated into the kinetic model (k_{02}), obtaining a value for the elongation of the disulfide bond at the transition state of $\sim 1.8 \text{ \AA}$ (Δx_{02} in Table 3), a value that is similar to those obtained when using cysteine as a nucleophile ($\sim 0.2 \text{ \AA}$). Absence of the SN2-like mechanism seems to be common to all eukaryotic-origin Trx, where the rate of the reaction became force-independent at greater forces (Fig. 16B). This force-independent mechanism was measured as a constant rate of reduction that ranged from 0.2 s^{-1} to 0.4 s^{-1} (Fig. 16B, inset). It is speculated that in enzymes of bacterial origin the minimum value of the reduction rate is also limited by this force-independent ‘floor’ in the rate of reduction, which varies in the range $0.2\text{--}0.4 \text{ s}^{-1}$ (Fig. 16A, inset). This mechanism was

incorporated into the kinetic model in the form of a constant parameter, l , which contributes equally to the reduction rate throughout the entire range of forces (Table 3).

[0162] A possibility that may explain this force-independent chemical mechanism is a SET reaction via tunneling, a process that has been observed in enzymes containing metallic complexes. In addition, it has been suggested that SET reactions are highly favored when steric hindrance occurs. To test whether an electron transfer mode of reduction would be force-independent, the kinetics of disulfide reduction under force by a metal was investigated. Some metals participate in oxidation-reduction processes in proteins via electron-transfer reactions that are governed by the reduction potential. The reduction of disulfide bonds by zinc nanoparticles (diameter <50 nm) was experimentally studied. In sharp contrast to all other reducing agents that were studied, the rate of reduction of disulfide bonds by zinc was force-independent (Fig. 17A). Owing to the experimental difficulty of working at low forces with zinc nanoparticles, only experiments done using forces of more than 200 pN were included. The results support the idea that the force-independent mechanism is a barrier-free electron-tunneling reaction that does not require the precise orientation for the S_N2 reaction.

[0163] Another piece of evidence in support of the SET mechanism can be obtained from the analysis of the concentration-dependencies of the MM- S_N2 and SET reduction mechanisms. Reduction kinetics by human TRX1 were measured at different forces and concentrations (50–600 pN and 2–15 μ M of TRX1) and found that the low-force MM- S_N2 mechanism (50–200 pN) is clearly dependent on the concentration of the enzyme, whereas the high-force SET mechanism (>300 pN) is essentially concentration-independent. As expected for a first-order MM- S_N2 mechanism, where substrate binding to the groove is determinant, the rate of reduction showed linear concentration-dependence when working below saturating concentrations of TRX1 enzyme (<15 μ M). However, given that the TRX1 and NADPH system was in equilibrium owing to the presence of TrxR, the redox potential of TRX1 would be expected to remain constant (from the Nernst equation). Therefore, the potential difference between TRX1 and substrate, and thus the rate of electron transfer, would also be constant in this TRX1 concentration range. Hence, the SET mechanism should be essentially independent of the enzyme concentration.

[0164] The results suggest that there are three distinct mechanisms of reduction that operate simultaneously in a Trx enzyme. These mechanisms are identified by their force-dependency, as shown in Figure 17. The most complex mechanism is characterized by a negative force-dependency and is unique to enzymatic catalysis by Trx (Fig. 17A, 17B). This

enzymatic mechanism of reduction is characterized by a MM-SN2 reaction between the substrate polypeptide and the binding groove of the enzyme, followed by a rotation of the substrate disulfide bond to gain position for the SN2 reduction mechanism (Fig. 17A, 17B). A much simpler mechanism is that of a regular SN2 reaction, characterized by a rate of reduction that increases exponentially with the applied force. This mechanism is well represented by nucleophiles such as L-cysteine (Fig. 17A), glutathione and DTT. By this mechanism, the substrate disulfide bond and the catalytic cysteine of the enzyme orient themselves with the pulling force, without needing a rotation of the substrate disulfide bond (Fig. 17C). It was suspected that this mechanism would be possible only if the Trx enzyme had a shallow binding groove that allowed many other orientations of the substrate-enzyme complex. Finally, the third mechanism is the force-independent, barrier-free electron-tunneling transfer mechanism, revealed by the action of metallic zinc (Fig. 17D). It is inevitable that, if the disulfide bond gets close enough to the thiolate anion of the catalytic cysteine, the electron tunneling will occur, albeit at a low rate.

[0165] Thus, comparing the data in Figures 16 and 11, it is clear that the main difference between enzymes of bacterial and eukaryotic origin is the elimination of the high-force, simple SN2-like mechanism of reduction. This drastic change in the catalytic chemistry may be caused by changes in the structure of the enzyme as it evolved. The most salient feature in the structure of Trx enzymes is the binding groove into which the target polypeptide first binds, to be subsequently reduced by the exposed thiol of the catalytic cysteine (Fig. 18A).

Structural analysis and molecular dynamics simulations

[0166] To study the role of the structure in the chemical behavior of Trxs, the structure of the binding groove of a set of bacterial-origin and eukaryotic-origin Trxs were analyzed. Three eukaryotic-origin enzymes—human TRX1, *Arabidopsis thaliana* Trxh1 and spinach Trxf—and three bacterial-origin enzymes—human mitochondrial TRX2, *E. coli* Trx1 and *Chlamydomonas reinhardtii* Trxm were studied. From the X-ray or NMR structures, structural axes were defined that allowed the calculation of the depth and width of the binding groove in the region surrounding the catalytic cysteine (Fig. 18A). It was found that eukaryotic Trx enzymes have binding grooves that are several angstroms deeper than those of bacterial origin (Fig. 18B). By contrast, the width of the binding groove remained the same.

[0167] The consequences of a deepening binding groove using molecular dynamics simulations to probe the mobility of a bound polypeptide were explored. For these

simulations, a set of enzyme structures obtained with mixed disulfide intermediates between the catalytic cysteine and a cysteine in the bound substrate were considered. Such structures capture the general disposition of the substrate in the catalytic site of the Trx enzyme. Three eukaryotic complexes—human TRX1 with the substrate REF-1, human TRX1 with NF- κ B and barley Trxh2 with protein BASI—and two bacterial complexes—*E. coli* Trx1 with Trx reductase and *B. subtilis* Trx with ArsC complex were used. To compare these structures, 13 residues of the substrates, with the binding cysteine always set as the seventh residue were taken into account. For the molecular dynamics simulations, the substrate-enzyme disulfide bond was removed to allow substrate mobility. The shallow binding groove of bacterial Trxs allows the substrate to be mobile (Fig. 18C). By contrast, the deeper groove found in Trx enzymes of eukaryotic origin tends to freeze the substrate into a much smaller range of conformations. Similarly, the measured distribution of the distances between the reacting sulfur atoms is smaller and more narrowly distributed in the deeper binding groove of Trx enzymes of eukaryotic origin than in those with the shallower grooves found in enzymes of bacterial origin (Fig. 18D).

[0168] As an additional test, molecular dynamics simulations were carried out in which the substrate was removed from the Protein Data Bank structures for two Trx complexes: one from eukaryotic origin, barley Trxh2 with protein BASI (PDB 2IWT), and the other from bacterial origin, *B. subtilis* Trx with ArsC complex (PDB 2IPA). No appreciable difference in the dynamics of the groove between the bacterial and the eukaryotic Trxs was found. In fact, the averaged value of the r.m.s. deviation difference is 0.035 ± 0.028 for PDB 2IWT and 0.023 ± 0.031 for PDB 2IPA (the error indicates s.d.). These results support the idea that the large differences in the mobility of the substrate that is being reported (Fig. 18C, 18D) are due to the different binding constraints of the groove.

[0169] Finally, the B-factor distribution of the substrate from the PDB structures were compared. The B-factors of protein crystal structures reflect the fluctuation of atoms around their average positions and provide information about protein dynamics. In particular, the B-factors of eukaryotic barley Trxh2 bound to protein BASI (PDB 2IWT) were compared with that of *E. coli* Trx bound to Trx reductase (PDB 1F6M), both from X-ray crystallographic experiments. Consistent with the simulation results (Fig. 18C), the substrate in 1F6M (bacterial-origin Trx) has larger B-factors than does eukaryotic Trx 2IWT.

[0170] These structural observations suggest that a major feature in the evolution of Trx enzymes has been an increase in the depth of the binding groove, increasing the efficiency of the MM-SN2 mechanism and eliminating the simple SN2 mechanism of catalysis.

Discussion

[0171] Over the past 4 billion years, the chemistry of living organisms has changed continuously in response to changes in atmospheric conditions and biological phenomena. For example, the large increase in the level of atmospheric oxygen that occurred about ~2.5 billion years ago is thought to have triggered a chemical expansion that had a large impact on the chemistry of enzymatic reactions, especially those involving redox transformations. However, understanding how enzymes have adapted their chemical mechanisms to evolutionary pressures remains a challenge in molecular biology.

[0172] Here it is shown that single-molecule force-clamp spectroscopy can be a valuable tool to examine the evolution of Trx catalysis by studying the chemistry of eight Trx enzymes from four different kingdoms. It is shown that three different chemical mechanisms for disulfide reduction can be distinguished in Trx enzymes by their sensitivity to a mechanical force applied to their substrate. Common to all Trx enzymes is a highly efficient Michaelis-Menten-type mechanism of disulfide bond reduction, characterized by a negative force-dependency (Fig. 17A, 17B). Also common to all enzymes is a low-rate, force-independent mechanism of reduction that, owing to its similarity to metallic zinc, may be due to a barrier-free electron-tunneling mechanism (Fig. 17A, 17D). Finally, enzymes of bacterial origin show an additional mechanism of reduction, comparable to that of a simple SN2 reaction and showing a force-dependency similar to that of glutathione or cysteine (Fig. 17A, 17C). This simple SN2 mechanism seems to have been eliminated from Trx enzymes of eukaryotic origin, suggesting that the mechanism of disulfide bond reduction by Trx enzymes was altered at an early stage of eukaryotic evolution.

[0173] The physical characteristics of the binding groove are identified as important factors in the evolution of Trx catalysis. The appearance of the hydrophobic binding groove allowed Trxs to bind the substrate in a specific fashion, generating a stabilizing interaction that allows the enzyme to regulate the geometry and orientation of the substrate disulfide bond in the catalytic site of the enzyme. This binding mechanism results in the Michaelis-Menten-type kinetics of reduction observed in all Trx. It is noteworthy that, as the binding groove deepens in enzymes of eukaryotic origin, the SN2-like mechanism of the evolution of Trx catalysis. The appearance of the hydrophobic binding groove allowed Trxs to bind the

substrate in a specific fashion, generating a stabilizing interaction that allows the enzyme to regulate the geometry and orientation of the substrate disulfide bond in the catalytic site of the enzyme. This binding mechanism results in the Michaelis-Menten-type kinetics of reduction observed in all Trx. It is noteworthy that, as the binding groove deepens in enzymes of eukaryotic origin, the SN2-like mechanism of reduction disappears. These observations are in agreement with the view that the SN2-like mechanism of reduction observed in bacterial Trx enzymes results from less specific enzyme-substrate interactions (Fig. 17C). The emergence of eukaryotes gave rise to vastly more complex biological systems, resulting in a myriad of new functions and targets. It is tempting to speculate that the deepening of the binding groove in eukaryotic Trx (Fig. 18) may have been an important structural adaptation that improved the specificity of substrate-enzyme interactions.

[0174] However, evolutionary optimization of Trx activity is clearly a much more complex multiparameter function involving other structural features and cofactors. Most importantly, Trxs work together with TrxRs, which convert oxidized Trx to its active dithiol form. There are major differences in the structure and mechanism of TrxR across the evolutionary tree, and it is reasonable to consider that the evolution of the chemical mechanisms found in Trx has been tightly associated with the evolution of TrxR. In the experiments, generic bacterial and eukaryotic TrxR have been used to keep the Trx enzymes in the reduced state. It is anticipated that this assay can be expanded by contrasting the effect of different TrxR enzymes in the observed chemistry of Trx.

[0175] From a biological point of view, an interesting hypothesis is that the simple SN2-like mechanism present in bacterial Trxs might be related to their ability to live in extreme environments, where elevated mechanical forces might result as a consequence of the high pressures or extreme salinity that cause cells to swell or shrink. Under such conditions the enzymatic Michaelis-Menten-type mechanism of reduction would become inoperative. In support of this view, Trx has been shown to promote high-pressure resistance in *E. coli*.

[0176] This work generally demonstrates the usefulness of combining single-molecule force spectroscopy together with molecular dynamics simulations in probing enzymatic chemistry. Substantial differences in the chemical mechanisms of extant Trx enzymes are observed. It would be interesting to track the evolution of these chemical mechanisms using resurrected ancient Trx enzymes. Owing to an extensive sequence database and the development of sophisticated maximum-likelihood algorithms for the reconstruction of ancient DNA sequences, reconstructing the evolution of chemical mechanisms in this class of

important enzymes now seems entirely plausible. It is anticipated that the enzymatic studies carried out on Trx at the single-molecule level can serve as a starting point to investigate the chemistry of other enzymes, such as C-S lyases or proteases, for which the catalyzed rupture of covalent bonds is the fundamental process.

Methods

[0177] Protein expression and purification. Preparation of (I27G32S-A75C)₈ polyprotein has been described extensively. The expression and purification of the different Trxs used have also been described: *P. falciparum*, *Drosophila melanogaster* Trx1, poplar Trxh1 and Trxh3, pea Trxm60, *E. coli* Trx1, *E. coli* Trx2 and human TRX2.

[0178] Sequence analysis. Sequence alignment was carried out using ClustalW and modified by hand. Tree topology and branch lengths of the tree were estimated using Mr. Bayes (v. 3.5) (<http://mr bayes.csit.fsu.edu/>), with rate variation modeled according to a gamma distribution. The following GI numbers were accessed from GenBank. Bacteria: *E. coli* Trx1 (67005950), *Salmonella* Trx1 (16767191), *E. coli* Trx2 (16130507), *Salmonella* Trx2 (16765969), human TRX2 mitochondria (21361403), bovine Trx2 mitochondria (108935910), *Rickettsia* Trx (15603883), *Nostoc* Trx (17227548), *Prochlorococcus* Trx (126696505), spinach Trxm chloroplast (2507458), pea Trxm chloroplast (1351239), *Thermus* Trx (46199687), *Deinococcus* Trx (15805968), Archaea *Aeropyrum* Trx (118431868), *Hyperthermus* Trx (124027987), *Sulfolobus* Trx (15897303). Eukaryote: *P. falciparum* Trx (75024181), poplar Trxh1 (19851972), poplar Trxh3 (2398305), pea Trx (27466894), *Dictyostelium* Trx (165988451), bovine Trx (27806783), human TRX1 (135773).

[0179] Single-molecule force-clamp experiments. Silicon nitride cantilever (Veeco) was used with a typical spring constant of 20 pN nm⁻¹, calibrated using the equipartition theorem. The force-clamp mode provides an extension resolution of ~0.5 nm and a piezoelectric actuator feedback of ~5 ms. The buffer used in all the experiments was 10 mM HEPES, 150 mM NaCl, 1mM EDTA, 2mM NADPH at pH 7.2. Before the beginning of the experiment, TrxR, bacterial or eukaryotic depending on the case, was added to a final concentration of 50 nM. The different Trxs were added to the desired concentration. The reaction mixture and the substrate were added and allowed to absorb onto a freshly evaporated gold coverslip before the experiments. The force-clamp experiment consisted of a double-pulse force protocol. The first pulse was set at 175 pN during 0.3–0.4 s. The second

pulse can be set at different forces and was held long enough to capture all the possible reduction events. The experiments using metallic zinc in 100 mM citrate buffer at pH 6 were carried out. After adding zinc nanoparticles (Sigma) to a concentration of 10 mM, the solution was sonicated to allow resuspension. The pH of the buffer was verified during the time of the experiment, and no appreciable changes were observed. In addition, to verify the behavior of the substrate in citrate buffer, several control experiments in the absence of zinc nanoparticles were carried out, and detected no reduction events. Data using custom-written software in IGOR Pro 6.03 (Wavemetrics) was collected and analyzed. The collected traces (15–50 per force) containing the reduction events were summated and averaged. The resulting averaged traces were fit with a single exponential from which the rate constant was obtained.

[0180] As will be apparent to one of ordinary skill in the art from a reading of this disclosure, the present disclosed subject matter can be embodied in forms other than those specifically disclosed above. The particular embodiments described above are, therefore, to be considered as illustrative and not restrictive. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific embodiments described herein. The scope of the disclosed subject matter is as set forth in the appended claims and equivalents thereof, rather than being limited to the examples contained in the foregoing description.

EXAMPLE 4

[0181] Disulfide bonds are formed as essential posttranslational modifications in a third of human proteins (Hatahet, F. *et al. Antioxid Redox Signal* 11, 2807-2850 (2009)). Formation of disulfides also plays a critical role in numerous pathologies including bacterial infection (Heras, B. *et al. Nat Rev Microbiol* 7, 215-225 (2009)), virus replication (Land, A. & Braakman, I. *Biochimie* 83, 783-790 (2001)) and protein misfolding disease (Uehara, T. *et al. Nature* 441, 513-517 (2006)); Culotta, V. C., Yang, M. & O'Halloran, T. V. *Biochim Biophys Acta* 1763, 747-758 (2006)). In the cytosol of eukaryotic cells, thioredoxin (TRX) maintains cysteines reduced (Holmgren, A. Thioredoxin. *Annu Rev Biochem* 54, 237-271 (1985)). Disulfides are formed in the endoplasmic reticulum where the thioredoxin-like protein disulfide isomerase (PDI) catalyzes co-translocational oxidative folding (Hatahet, F. *et al. Antioxid Redox Signal* 11, 2807-2850 (2009); Mamathambika, B. S. & Bardwell, J. C. Disulfide-linked protein folding pathways. *Annu Rev Cell Dev Biol* 24, 211-235 (2008); Di Jeso, B. *et al. Mol Cell Biol* 25, 9793-9805 (2005)). However, the precise involvement of PDI

during protein folding has remained elusive. A kinetic model for PDI activity during catalyzed oxidative folding is presented, including a method enabling, for the first time, independent kinetic measurements of folding and disulfide formation in a single protein substrate. The data indicate that catalyzed disulfide formation is rate limited by structural folding. Enzyme-substrate intermediate complexes do not impede folding and are necessary for disulfide formation. It is proposed that the spontaneous rate of enzyme release modulates oxidative catalysis during co-translocational folding. Replacement of a single atom in TRX was shown to inhibit spontaneous release and enable efficient catalysis of disulfide formation. These findings show that contrary to the prevailing view, oxidative folding is best described as a non-equilibrium process governed by enzyme kinetics.

[0182] In both pro- and eukaryotes, the Sec translocase machinery mediates transport of unfolded polypeptides from the cytosol to compartments where disulfide formation takes place (Sevier, C. S. & Kaiser, C. A. Formation and transfer of disulphide bonds in living cells. *Nat Rev Mol Cell Biol* **3**, 836-847 (2002); Wickner, W. & Schekman, R. Protein translocation across biological membranes. *Science* **310**, 1452-1456 (2005); Bechtluft, P. *et al.* Direct observation of chaperone-induced changes in a protein folding pathway. *Science* **318**, 1458-1461 (2007)). Oxidase enzymes in these compartments engage with exposed cysteines in translocating substrates by forming covalently linked complexes (FIG. 21A). Identification of such complexes (Di Jeso, B. *et al.* *Mol Cell Biol* **25**, 9793-9805 (2005); Kadokura, H., Tian, H., Zander, T., Bardwell, J. C. & Beckwith, J. *Science* **303**, 534-537 (2004); Kadokura, H. & Beckwith, J. *Cell* **138**, 1164-1173 (2009)) has established their role as obligatory intermediates during oxidative folding. An intermediate complex can be resolved upon emergence of a second substrate cysteine from the translocase channel, whereby the enzyme is released and an intramolecular disulfide is formed in the substrate (FIG. 21B). Oxidative folding *in vivo* is thus governed by the kinetic interplay between disulfide oxidation and polypeptide collapse from an extended state. However, conventional experimental approaches are unable to measure these processes independently.

[0183] A force-clamp atomic force microscope was used to extend individual polypeptides to an initial unfolded state wherein the two substrate cysteines were spatially separated and an intermediate complex was formed (FIG. 21C), in analogy to ongoing translocation. Allowing the polypeptide to collapse, the progress of oxidative folding could be directly probed (FIG. 21D). This technique allows to separately monitor the two concurrent processes of folding and disulfide formation within the same molecule.

[0184] PDI consists of two catalytically active **a** domains and two redox-inactive **b** domains (Hatahet, F. *et al. Antioxid Redox Signal* 11, 2807-2850 (2009)). Each a domain exhibits the same oxidase activity in isolation as in full-length PDI (Darby, N. J., Kemmink, J. & Creighton, T. E. *Biochemistry* 35, 10517-10528 (1996)). The interaction of PDI a with immunoglobulin (Ig) proteins undergoing oxidative folding was studied. The model substrate consisted of sequential repeats of the 27th Ig domain from human cardiac titin, each with a buried disulfide between residues 32 and 75 (Wiita, A. P. *et al. Nature* 450, 124-127 (2007)). A calibrated stretching force was applied to a single substrate protein while measuring its extension (Alegre-Cebollada, J., Perez-Jimenez, R., Kosuri, P. & Fernandez, J. M. Single-molecule Force Spectroscopy Approach to Enzyme Catalysis. *Journal of Biological Chemistry* 285, 18961-18966 (2010)). During mechanical denaturation of the substrate, folded structures and disulfide bonds were identified as obstructions in the unfolding pattern (Wiita, A. P., Ainavarapu, S. R., Huang, H. H. & Fernandez, J. M. *Proc Natl Acad Sci U S A* 103, 7222-7227 (2006)). Reduced PDI **a** was used to cleave substrate disulfides, thereby attaining mixed disulfide complexes of the sort identified *in vivo* (Di Jeso, B. *et al. Mol Cell Biol* 25, 9793-9805 (2005)) (FIG. 22A). Folding by switching off the force was initiated, and after a time delay the substrate was probed by again applying force, resulting in a [*denature* – Δt – *probe*] protocol.

[0185] A representative trace is displayed in FIG 22B. During the *denature* pulse, stepwise extensions of 11 and 14 nm were seen. As previously shown (Wiita, A. P., Ainavarapu, S. R., Huang, H. H. & Fernandez, J. M. *Proc Natl Acad Sci U S A* 103, 7222-7227 (2006)), an 11 nm step corresponds to an unfolding event wherein a single Ig domain extends up to the disulfide bond. This exposes the disulfide and enables enzymatic cleavage. Upon cleavage of the disulfide bond, an additional 14 nm of the polypeptide chain is released and a mixed disulfide complex is generated. After a few seconds, the force was switched off and the substrate was allowed to refold ($\Delta t = 5$ s). Folding and disulfide formation was then probed by again applying a stretching force (*probe* pulse). The reappearance of 11 and 14 nm steps in the *probe* pulse indicate that oxidative folding had taken place. In other traces, the *probe* pulse revealed 25 nm steps (FIG. 28 & FIG 22B, inset), identified as the sum of unfolding (11 nm) and reduction (14 nm) steps. A 25 nm step thus indicates the presence of a folded domain in which the disulfide bond is not formed, suggesting that in these cases, PDI had failed to induce disulfide formation and instead acted as a reductase. Step size histograms

generated from multiple experiments underline this finding (FIG. 22C), revealing an apparent dichotomy in the activity of PDI.

[0186] This dichotomy may be explained through the similarity of PDI to TRX, a cytosolic reductase. Both enzymes feature the thioredoxin fold and a CXXC active site motif (Sevier, C. S. & Kaiser, C. A. *Nat Rev Mol Cell Biol* 3, 836-847 (2002); Martin, J. L. *Structure* 3, 245-250 (1995)). The reaction path of both PDI and TRX involves formation of a mixed disulfide intermediate between the substrate and the N-terminal active site cysteine of the enzyme (FIG. 25A). The symmetry is then broken as the enzymes generally resolve this intermediate in different ways (Lundstrom, J., Krause, G. & Holmgren, A. *J Biol Chem* 267, 9047-9052 (1992); Xiao, R., Lundstrom-Ljung, J., Holmgren, A. & Gilbert, H. F. *J Biol Chem* 280, 21099-21106 (2005)). During substrate reduction, TRX resolves the intermediate through the “escape” pathway, whereby nucleophilic attack by the C-terminal cysteine in the enzyme active site yields a reduced substrate (Holmgren, A. *Annu Rev Biochem* 54, 237-271 (1985)) (FIG. 25B). During oxidative folding, PDI resolves the intermediate through attack by a substrate cysteine, leaving the substrate oxidized (Hatahet, F. *et al. Antioxid Redox Signal* 11, 2807-2850 (2009); Mamathambika, B. S. & Bardwell, J. C. *Annu Rev Cell Dev Biol* 24, 211-235 (2008)) (FIG. 25C). Experiments with PDI suggest flexibility in the pathway by which PDI resolves the intermediate. Similar observations have been made, including activity reversal for both PDI and TRX (Stewart, E. J., Aslund, F. & Beckwith, J. *EMBO J* 17, 5543-5550 (1998); Lundstrom, J. & Holmgren, A. *J Biol Chem* 265, 9114-9120 (1990)). These findings raise the questions of how the apparent flexibility affects oxidative folding, and what determines the pathway preference.

[0187] The effects of human TRX on the oxidative folding of the Ig substrate were studied. The experimental procedure outlined in FIG. 22 was repeated using reduced TRX instead of PDI to cleave the substrate disulfides. The trace in FIG. 23A reveals that despite successful refolding of the substrate, none of the disulfides were reformed. Histograms in FIG. 23B further strengthen the conclusion that TRX is a poor catalyst of oxidative folding. The reaction mechanism of TRX has been well characterized (Holmgren, A. *Annu Rev Biochem* 54, 237-271 (1985)), allowing the conclusion that a mixed disulfide intermediate was formed upon each 14 nm step in the *denature* pulse. However, the successful folding of the substrate without formation of intramolecular disulfides suggests the TRX enzymes had dissociated through the escape pathway prior to substrate refolding. A simple explanation for the different propensities of PDI and TRX in catalyzing oxidative folding could thus be a

difference in the escape pathway kinetics for the two enzymes; TRX escapes from the intermediate rapidly, leaving behind an unreactive substrate thiol; PDI lingers as part of the intermediate complex for a longer time, allowing a second substrate cysteine to attack the mixed disulfide upon substrate collapse.

[0188] To test if this hypothesis was sufficient to explain the catalytic difference between PDI and TRX, the escape pathway in TRX was disabled and its influence on a folding protein was studied. Through site-directed mutagenesis the TRX C-terminal cysteine was replaced with a serine (TRX C35S), thereby switching the identity of only one atom, from sulfur to oxygen. The vastly higher pK_a of a hydroxyl renders it much less reactive than a thiol at physiological pH . TRX C35S is thus still able to initially cleave a substrate disulfide but cannot resolve a mixed disulfide intermediate through the escape pathway.

[0189] The collapse of Ig - TRX C35S intermediate complexes was studied, and a representative trace can be seen in FIG. 23C. The trace shows the reappearance of both 11 and 14 nm steps in the *probe* pulse, indicating that the substrate had undergone oxidative folding involving both structural folding and formation of the intramolecular disulfide. As seen from the absence of a 25 nm peak in the probe pulse histogram (FIG. 23D), every folded domain had undergone disulfide oxidation. The stark contrast between the histograms in FIG. 23B and 23D strongly suggests the role of the Sy atom in TRX Cys35 as an enzyme functionality switch.

[0190] A quantitative comparison of substrate folding at $\Delta t = 5s$ revealed no significant difference between PDI and TRX (FIG. 24A); however TRX C35S seemed to impede folding slightly. PDI had introduced disulfides in 60% of the refolded Ig domains (FIG. 24B). TRX had not introduced any disulfides, whereas TRX C35S had introduced a disulfide in every refolded domain. Overall, there was no significant difference in the number of domains that had undergone complete oxidative folding with PDI and with TRX C35S (FIG. 24C). It seems unlikely that the introduced hydroxyl in TRX C35S generated this gain of function. Rather, the results suggest TRX being intrinsically adept at catalyzing oxidative folding. This is not a natural activity of TRX, but can be explained if the requirements for catalysis of substrate disulfide cleavage and formation are identical. Cys35 acts as a release trigger, breaking the symmetry between oxidases and reductases. If the time constant of the escape pathway determines whether or not a disulfide is formed in a folding substrate, then FIG. 24B reflects this time constant for the different enzymes. TRX shows rapid escape whereas TRX C35S lingers indefinitely. PDI escape kinetics appear to fall somewhere between these two

extremes. Tuning the kinetics of the escape pathway can thus modulate the oxidase activity of a TRX-like enzyme, and can explain the ability of PDI to catalyze oxidative folding in a subset of folded domains. Mutational studies may hold the key to understanding which residues play a role in this tuning (Lundstrom, J., Krause, G. & Holmgren, A. *J Biol Chem* 267, 9047-9052 (1992); Karala, A. R., Lappi, A. K. & Ruddock, L. W. *J Mol Biol* 396, 883-892 (2010); Ren, G. *et al. J Biol Chem* 284, 10150-10159 (2009)).

[0191] The folding and disulfide formation kinetics for TRX and TRX C35S were compared by varying Δt in the pulse protocol (FIG. 24D, E). Folding appeared to be an exponential process with a rate of $k_{\text{fold}} = 0.2 \pm 0.1 \text{ s}^{-1}$ for both TRX and TRX C35S, consistent with earlier measurements (Ainavarapu, S. R. *et al. Biophys J* 92, 225-233 (2007)). Even though kinetics were not altered with the mutant, the total fraction of folded domains was slightly lower, possibly reflecting a subpopulation of complexes unable to fold. FIG. 24D reveals no substrate disulfides at any of the time points with TRX, whereas TRX C35S disulfide formation kinetics followed a single exponential with $k_{\text{ox}} = 0.32 \pm 0.04 \text{ s}^{-1}$, indicating a single rate limiting step in the process. The similarity of the rates of disulfide formation and unhindered folding suggests that structural rearrangement of the substrate is this rate limiting step during catalyzed disulfide formation.

[0192] The properties of thiol-disulfide oxidoreductases are often described in terms of their reduction potential E_R , a measure of how likely they are to oxidize or reduce substrate disulfides under equilibrium conditions (Sevier, C. S. & Kaiser, C. A. *Nat Rev Mol Cell Biol* 3, 836-847 (2002)). However, oxidative folding is not well characterized as an equilibrium process, as the reactivity of cysteines can be vastly different in the unfolded and folded state of a protein (Hatahet, F. *et al. Antioxid Redox Signal* 11, 2807-2850 (2009); Mamathambika, B. S. & Bardwell, J. C. *Annu Rev Cell Dev Biol* 24, 211-235 (2008)). *In vivo*, proteins undergo oxidative folding during ongoing translocation. In these cases, disulfide formation is determined not by equilibrium energetics but by the presence of a mixed disulfide complex upon folding. In the case of TRX, escape is fast and the complex is likely to dissociate prior to or during folding (FIG. 24F), failing to oxidize the substrate. If this escape is slowed down or prevented altogether, as in the case of TRX C35S, the complex is stable upon folding and allows catalysis of native disulfide formation (FIG. 24G). The reactivity of the C-terminal cysteine in the enzyme active site can thus function as a switch, enabling or disabling catalysis of oxidative folding depending on its reactivity. In support of this conclusion, recent *in vivo* experiments have suggested excessively slow kinetics of the escape pathway in the

prokaryotic disulfide oxidase DsbA (Kadokura, H., Tian, H., Zander, T., Bardwell, J. C. & Beckwith, J. *Science* 303, 534-537 (2004); Kadokura, H. & Beckwith, J. *Cell* 138, 1164-1173 (2009)).

Methods

[0193] The polyprotein substrate, TRX and TRX C35S were expressed and purified as described elsewhere (Wiita, A. P. *et al. Nature* 450, 124-127 (2007); Ren, X., Bjornstedt, M., Shen, B., Ericson, M. L. & Holmgren, A. *Biochemistry* 32, 9701-9708 (1993); Perez-Jimenez, R. *et al. J Biol Chem* 283, 27121-27129 (2008)). PDI a domain consisted of residues 18-134 in human PDI and was produced as described in the methods section. The custom-made atomic force microscope has been described previously (Fernandez, J. M. & Li, H. *Science* 303, 1674-1678 (2004)). Experiments were carried out at pH 7.2 in 10 mM HEPES, 150 mM NaCl, 1 mM EDTA. Before each experiment, reduced DTT was added together with the enzyme. 200 μ M DTT was used with TRX (10 μ M) and TRX C35S (40 μ M). 500 μ M DTT was used with PDI a (120 μ M). The polyprotein substrate was absorbed onto a gold coverslip. Experiments consisted of repeated trials of pressing the tip against the surface at 1000 pN for 0.5 s and subsequently retracting. The pulse protocol was applied when attachment was achieved. Traces showing in the denature pulse step sizes other than 11 nm or 14 nm or contained less than 4 unfolding events were excluded from analysis. Only traces showing equal extension at the end of the probe and denature pulses were included. Step size histograms included all steps >5 nm after the initial elastic extension in each force pulse.

Protein expression and purification

[0194] The (I2732S-75C)₈ polyprotein substrate was prepared as previously described (Wiita, A. P. *et al. Nature* 450, 124-127 (2007)). Human TRX and TRX C35S were expressed and purified as described (Ren, X., Bjornstedt, M., Shen, B., Ericson, M. L. & Holmgren, A. *Biochemistry* 32, 9701-9708 (1993); Perez-Jimenez, R. *et al. J Biol Chem* 283, 27121-27129 (2008)). Human PDI a domain was generated by PCR from Ultimate ORF Clone IOH9865 (Invitrogen) as an *Nde I*-*BamH I* fragment. The insert (amino acids 18-134 in the full length PDI sequence) was cloned into *Nde I*-*BamH I* sites of pET-15b vector (Novagen) containing the N-terminal His₆ tag and expressed in *Escherichia coli* BL21-Gold (DE3) (Stratagene). Cells with the construct were grown in LB media supplemented with 100 μ g/mL ampicillin at 37°C for 10 h (OD_{600nm} ~0.6). Protein expression was induced with 0.5mM isopropyl β -D-thiogalactoside overnight. Cells were lysed by sonication and recombinant PDI a domain

was first purified from cell lysate using Ni Sepharose 6 Fast Flow (GE Life Sciences) and then using gel filtration Superdex 300 column. The fractions containing PDI a domain were pooled together and dialyzed into 20 mM sodium phosphate buffer pH 8 and verified by SDS-PAGE. Protein concentration was determined using Bradford assay.

Single-molecule force-clamp experiments

[0195] The details of the custom-made atomic force microscope have been described (Fernandez, J. M. & Li, H. *Science* 303, 1674-1678 (2004)). Silicon nitride cantilevers (Veeco) with a typical spring constant of 20 pN nm^{-1} was used, calibrated using the equipartition theorem. The force-clamp mode provides a feedback time constant of 5 ms. The buffer used in all the experiments was 10 mM HEPES, 150 mM NaCl, 1mM EDTA, at pH 7.2. Before the beginning of the experiment, reduced DTT was added together with the enzyme. 200 μM DTT was used with TRX (10 μM) and TRX C35S (40 μM). 500 μM DTT was used with PDI a (120 μM), to ensure that the enzyme was in its reduced form, while minimizing the effects by DTT on the substrate. The polyprotein substrate was added in a droplet and allowed to absorb onto a freshly evaporated gold coverslip before the experiments. Every experiment consisted of repeated trials where the tip was pressed against the surface for 0.5 s and subsequently retracted. If attachment was achieved, a pulse protocol was applied until detachment occurred. The oxidative folding force-clamp experiments used a triple-pulse force protocol. The first pulse was maintained at 110-130 pN for a time long enough to ensure complete denaturation of the substrate ($>5 \text{ s}$). The second pulse was set at 0 pN and maintained for the desired amount of refolding time. The third pulse was set at a force identical to the first and maintained until complete denaturation could be ensured ($>5 \text{ s}$). Data using custom-written software in IGOR Pro (Wavemetrics) were collected and analyzed. Traces were selected based on the fingerprint consisting of at least 4 unfolding events in the denature pulse. Traces exhibiting step sizes other than 11 nm or 14 nm in the denature pulse were excluded from the analysis to ensure homogeneity. Only traces showing equal extension at the end of the probe pulse and the end of the denature pulse were included, to ensure that the same protein was stretched in the two pulses. Step size histograms were generated using all steps $>5 \text{ nm}$ detected after the initial elastic extension in each force pulse.

[0196] As DTT was used to keep the enzyme reduced in all experiments, control experiments were performed to verify that the reduction events observed were not caused by DTT. The Ig polyprotein was therefore unfolded in a buffer containing 500 μM DTT, without

adding any enzyme. In these experiments, only 11 nm steps were observed during the first 10 seconds of the pulse. A representative trace is shown in FIG. 26. At longer times, occasional reduction steps were observed, but at a frequency too low for rate estimation. Refolding experiments were also conducted under these conditions, and revealed only 11 nm steps in both *denature* and *probe* pulses (FIG. 27).

EXAMPLE 5

[0197] FIG. 29 shows the results of a functional assay to measure the effect of an inhibitor molecule on enzymatic activity. Using a force-clamp spectrometer, the enzymatic activity of 200 μ M PDI was measured by detecting the cleavage of a disulfide in a substrate I27 protein. Accumulation of hundreds of such events yielded a kinetic rate given as events per second. By adding the inhibitor to the reaction volume, the measured rate decreased in a concentration-dependent manner.

Methods

[0198] A substrate polyprotein composed of eight domains of the 27th domain of human cardiac titin, in which each domain contained an engineered disulfide bond between the 32nd and 75th residues (I27G32C-A75C)₈, was used. In the force-clamp experiments, the first pulse of force (175 pN, 0.3 s) applied to the polyprotein lead to the rapid unfolding of the I27G32C-A75C domains up to the disulfide bond. The individual unfolding events could be registered as steps of 11 nm per domain. After the first pulse, the disulfide bonds become exposed to the solvent, where active PDI enzyme is present. A second pulse of force was then applied to monitor single disulfide cleavage reactions by PDI enzymes, recorded as a second series of steps of 14 nm per domain. Several traces (~20) were accumulated, which were then averaged and fit with a single exponential with a time constant τ . The reduction rate at a given force ($r = 1/\tau$) was thus obtained. Repeating the experiment in the presence of an increasing concentration of an inhibitor molecule allowed for measurement of enzymatic activity as a function of inhibitor concentration. In this manner, the functional efficiency (as opposed to merely binding) of a specific inhibitor could readily be assessed.

EXAMPLE 6

[0199] FIG. 30 presents an illustrative force clamp spectrometer according to some embodiments of the disclosed subject matter. Specifically, the spectrometer includes, for

example, one or more “flip” rotating stages, one or more motorized precision translation stages, a piezoelectric actuator, one or more manual precision translation stages, a 4 quadrants photodiode, microfocusing optics, a precision tilt mirror mount, a prism or a D-mirror, a fused quartz fluid cell (that holds the cantilever), a 10X microscope objective and a USB CCD or webcam.

[0200] REFERENCES

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CLAIMS

1. A force-clamp spectrometer comprising:
a cantilever extending from a cantilever chip;
a coverslip adjacent to the cantilever chip and mounted on a piezo-electric positioner;
a laser configured to focus a laser beam on the cantilever; and
a split photodetector configured to detect a reflected beam;
wherein the reflected beam is formed after the laser beam is reflected on the cantilever.
2. The force-clamp spectrometer of claim 1, further comprising a camera for monitoring the laser beam focal spot.
3. The force-clamp spectrometer of claim 2, wherein the laser beam is aligned after using information from the camera.
4. The force-clamp spectrometer of claim 1, 2 or 3, further comprising at least one flip mount for rapidly and reproducibly switching positions of the camera and of the piezo-electric positioner.
5. The force-clamp spectrometer of any one of claims 1 to 4, wherein a substrate is suspended between the cantilever and the coverslip.
6. The force-clamp spectrometer of claim 5, wherein the substrate is extended by applying a force to the substrate.
7. The force-clamp spectrometer of claim 6, wherein the extension of the substrate is controlled by the piezo-electric positioner and the force is measured based on bending of the cantilever.
8. The force-clamp spectrometer of claim 7, wherein the bending of the cantilever is monitored by reflecting a laser beam on the cantilever and detecting the reflected beam on the split photodetector.

9. The force-clamp spectrometer of claim 8, wherein the difference in signal between two halves of the split photodetector is proportional to a force exerted on the cantilever.
10. The force-clamp spectrometer of any one of claims 5 to 9, further comprising a feedback system configured to adjust the extension of the suspended substrate between the cantilever and the coverslip.
11. The force-clamp spectrometer of claim 10, wherein the feedback system is further configured to allow the force-clamp spectrometer to maintain a desired force exerted on the cantilever.
12. The force-clamp spectrometer of claim 1, further comprising a magnetic-orientable prism configured to steer the reflected beam to the split photodetector.
13. The force-clamp spectrometer of claim 1, further comprising a tilt platform coupled to the laser and configured to focus the laser beam on the cantilever.
14. The force-clamp spectrometer of claim 1, further comprising an additional piezo-electric positioner for positioning the split photodetector.
15. A method of measuring the dynamics of bond formation in a substrate by single-molecule force spectroscopy, the method comprising:
 - a. Placing a sample comprising a substrate in the force-clamp spectrometer of any one of claims 1 to 14;
 - b. Applying a force to the sample, wherein the force extends the substrate; and
 - c. Detecting the presence of a bond(s), wherein the presence is indicative of bond formation.
16. A method of measuring the dynamics of folding and bond formation in a substrate by single-molecule force spectroscopy, the method comprising:
 - a. Placing a sample comprising a substrate in the force-clamp spectrometer of any one of claims 1 to 14;

- b. Applying a force to the sample, wherein the force extends the substrate; and
 - c. Detecting the presence of a first bond(s) and a second bond(s), wherein the presence of a first bond(s) is indicative of substrate folding and the presence of a second bond(s) is indicative of bond formation.
- 17. The method of claim 16, wherein the dynamics of bond formation and substrate folding are measured independently.
 - 18. The method of claim 15, wherein the bond is a covalent bond.
 - 19. The method of claim 16 or 17, wherein the first bond is a non-covalent bond and the second bond is a covalent bond.
 - 20. The method of claim 18 or 19, wherein the covalent bond is a disulfide bond.
 - 21. The method of any one of claims 15 to 20, wherein the dynamics are measured in real-time.
 - 22. The method of any one of claims 15 to 21, wherein the dynamics are identified through a fingerprint signal.
 - 23. The method of any one of claims 15 to 22, wherein the dynamics are measured as a function of a force applied.
 - 24. The method of any one of claims 15 to 23, wherein steps b) and c) are repeated over time.
 - 25. The method of any one of claims 15 to 24, wherein the dynamics include kinetic rates.
 - 26. The method of any one of claims 15 to 25, wherein the method comprises measuring the dynamics of one or more additional substrates.
 - 27. The method of any one of claims 15, 18 and 20-26, wherein the sample comprises a compound that reacts with the bond.
 - 28. The method of any one of claims 16, 17, 19 and 20-26, wherein the sample further comprises a compound that reacts with the first or second bond.

29. The method of any one of claims 15 to 28, wherein the sample further comprises one or more enzymes.
30. The method of claim 29, wherein the enzyme is a protease, esterase, phosphodiesterase, or glycosidase.
31. The method of claim 29, wherein the enzyme is an oxidoreductase enzyme.
32. The method of claim 29, wherein the enzyme is thioredoxin.
33. The method of claim 29, wherein the enzyme is an oxidase enzyme.
34. The method of claim 29, wherein the enzyme is protein disulfide isomerase.
35. The method of any one of claims 29 to 34, wherein the enzyme comprises one or more mutations.
36. The method of any one of claims 29 to 35, wherein the sample comprises an enzyme inhibitor.
37. The method of any one of claims 29 to 35, wherein the sample comprises an enzyme activator.
38. A method of measuring the dynamics of unfolding and bond cleavage of a substrate by single-molecule force spectroscopy, the method comprising:
 - a. Placing a sample comprising a substrate in the force-clamp spectrometer of any one of claims 1 to 14;
 - b. Applying a force to the sample, wherein the force extends the substrate; and
 - c. Detecting the absence of a first bond(s) and a second bond(s), wherein the absence of a first bond is indicative of substrate unfolding and the absence of a second bond is indicative of bond cleavage.
39. The method of claim 38, wherein the dynamics of unfolding and the dynamics of bond cleavage are measured independently.

40. The method of claim 38 or 39, wherein the dynamics of unfolding and bond cleavage are measured in real-time.
41. The method of claim 38, 39 or 40, wherein the dynamics of unfolding and bond cleavage are identified through a fingerprint signal.
42. The method of claim 38, 39, 40 or 41, wherein the dynamics of unfolding and bond cleavage are measured as a function of a force applied.
43. The method of any one of claims 38 to 42, wherein steps b) and c) are repeated over time.
44. The method of any one of claims 38 to 43, wherein the dynamics include kinetic rates.
45. The method of any one of claims 38 to 44, wherein the method comprises measuring the dynamics of unfolding and bond cleavage of one or more additional substrates.
46. The method of any one of claims 38 to 45, wherein the first bond is a non-covalent bond and the second bond is a covalent bond.
47. The method of claim 46, wherein the covalent bond is a disulfide bond.
48. The method of any one of claims 38 to 47, wherein the sample further comprises a compound that reacts with the first or the second bond.
49. The method of any one of claims 38 to 48, wherein the sample further comprises one or more enzymes.
50. The method of claim 49, wherein the enzyme is a protease, esterase, phosphodiesterase, or glycosidase.
51. The method of claim 49, wherein the enzyme is an oxidoreductase enzyme.
52. The method of claim 49, wherein the enzyme is thioredoxin.
53. The method of claim 49, wherein the enzyme is an oxidase enzyme.
54. The method of claim 49, wherein the enzyme is protein disulfide isomerase.

55. The method of any one of claims 49 to 54, wherein the enzyme comprises one or more mutations.
56. The method of any one of claims 15 to 55, wherein the substrate is a polymer.
57. The method of any one of claims 15 to 56, wherein the substrate has a folded structure.
58. The method of claim 56, wherein the polymer is a natural protein.
59. The method of claim 58, wherein the protein is cadherin, selectin, IgCAM, fibronectin, fibrilin or titin.
60. The method of claim 56, wherein the substrate is a nucleic acid.
61. The method of claim 56, 57, 58, 59 or 60, wherein the substrate contains one or more mutations.
62. The method of claim 56, wherein the polymer is an engineered polymer.
63. The method of claim 62, wherein the polymer contains domain repeats.
64. The method of claim 63, wherein the polymer is constructed by chemically ligating a polymer scaffold molecule with a customized linker molecule.
65. The method of claim 64, wherein the polymer scaffold molecule is a protein polymer consisting of repeats of the I27 domain from human cardiac titin.

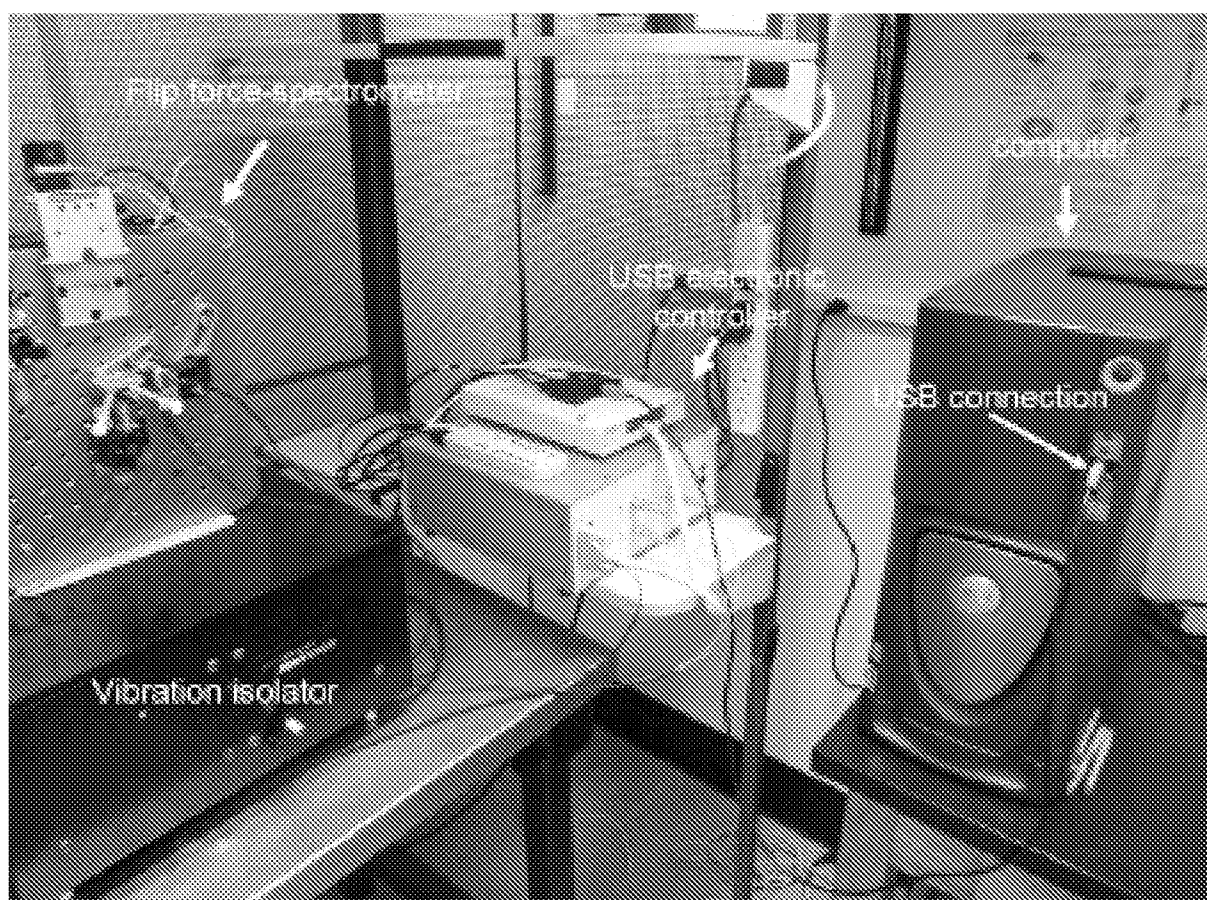


FIGURE 1

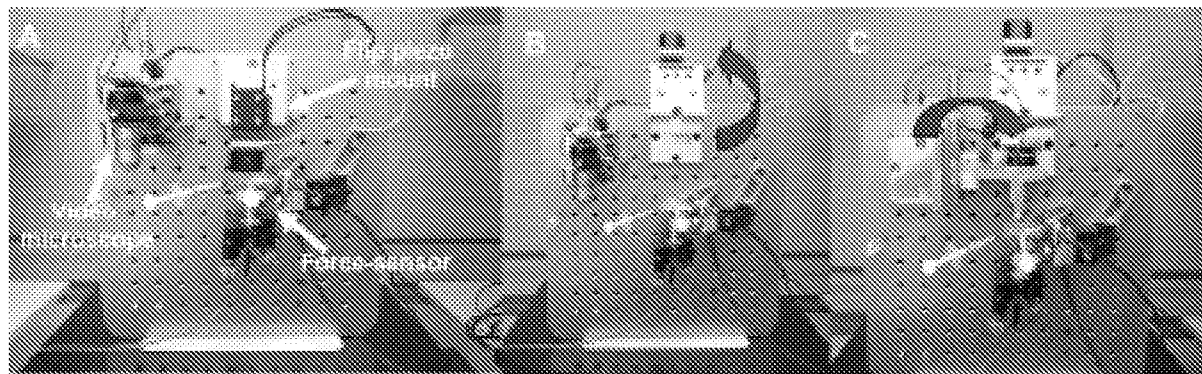


FIGURE 2

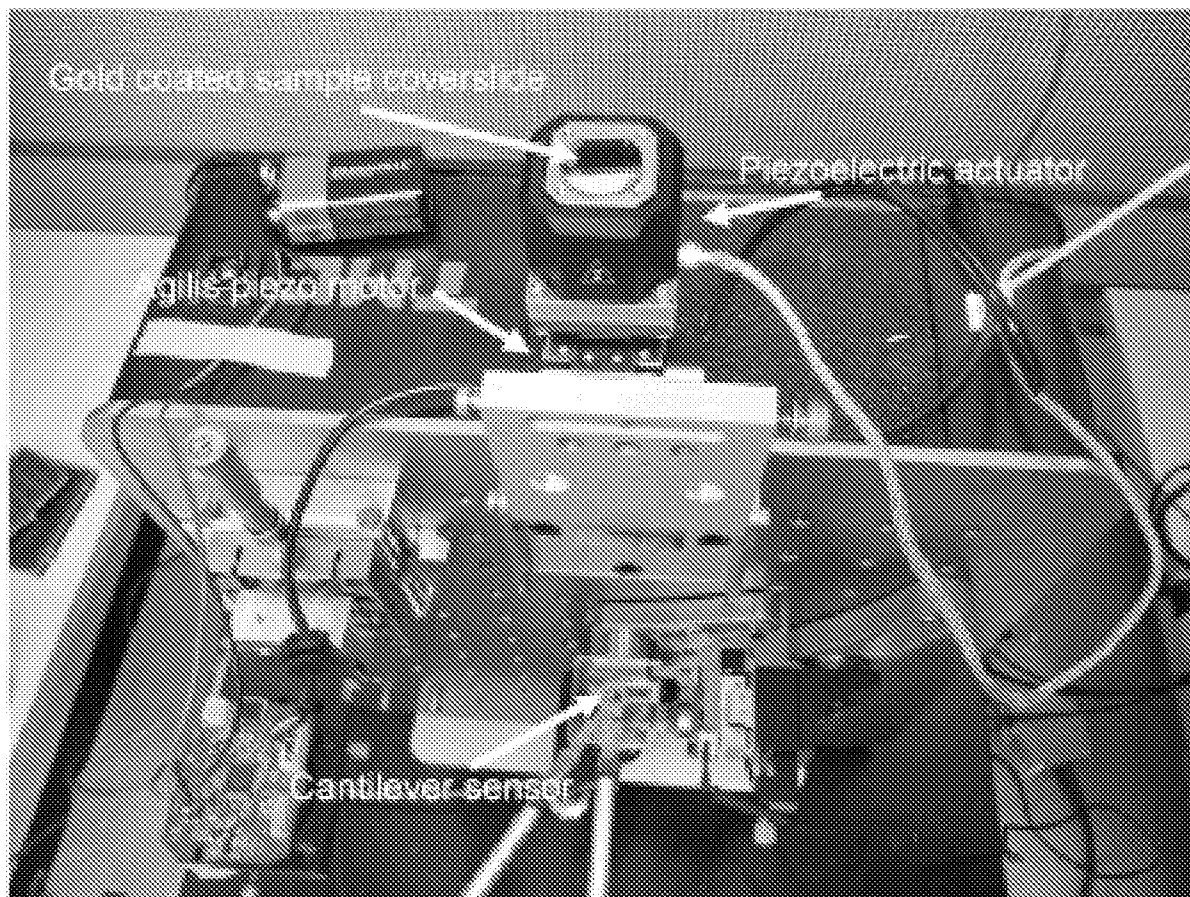


FIGURE 3

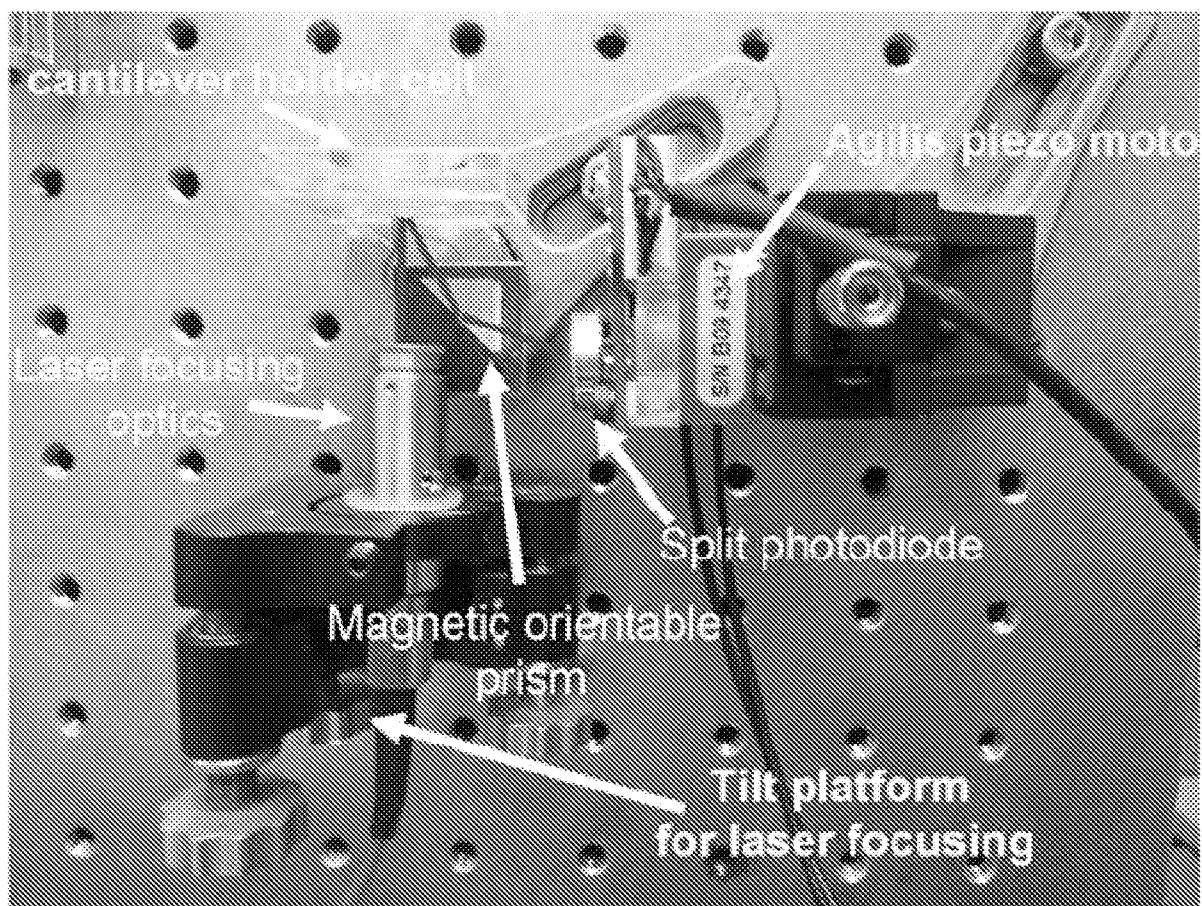


FIGURE 4

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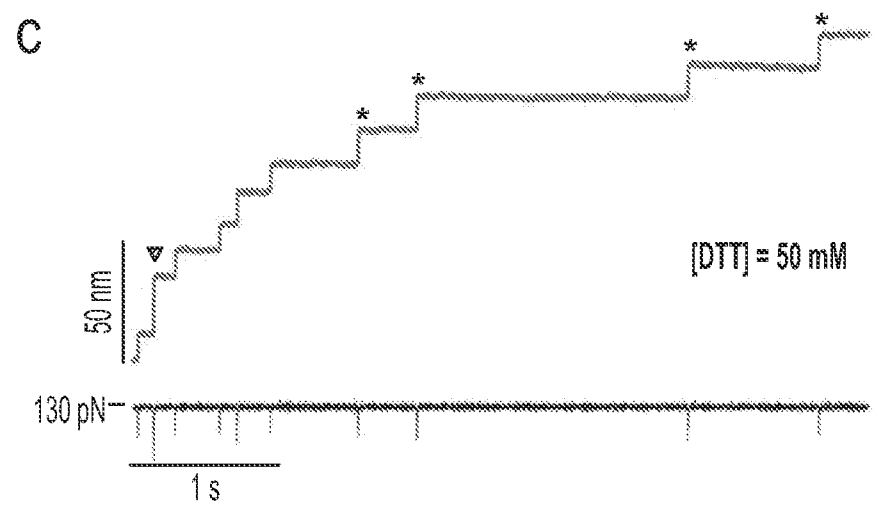
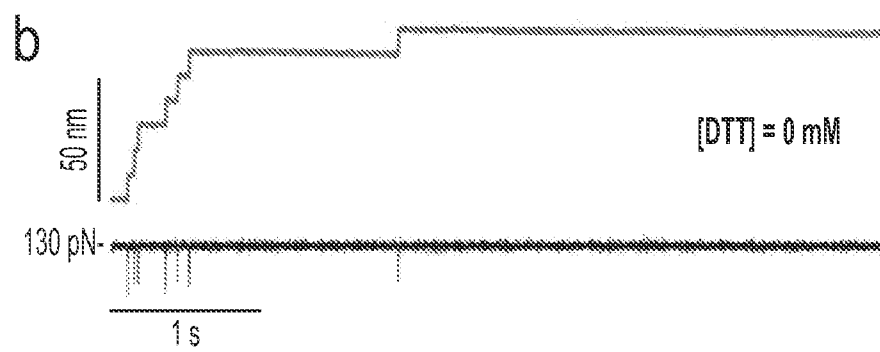
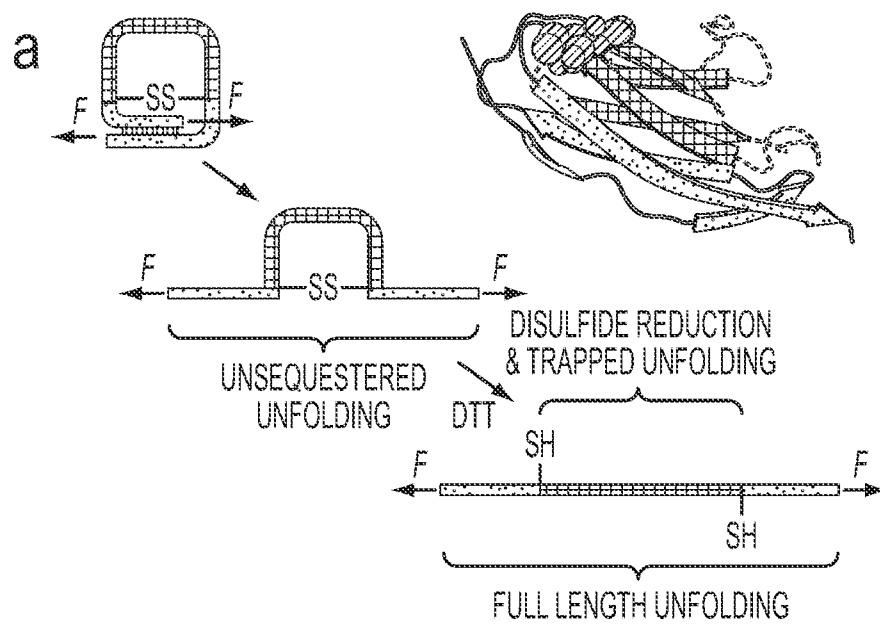


FIG. 5

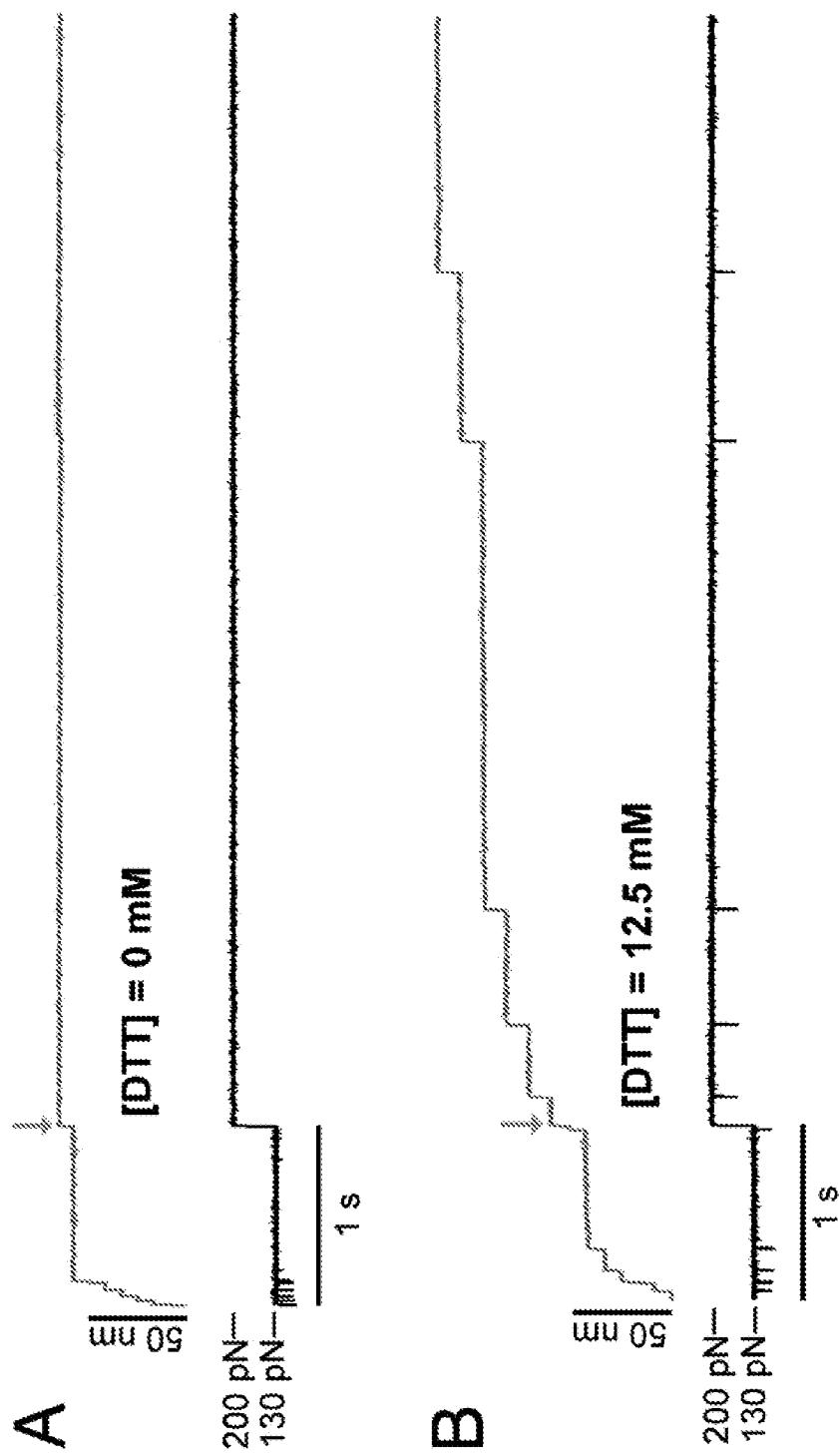


FIGURE 6

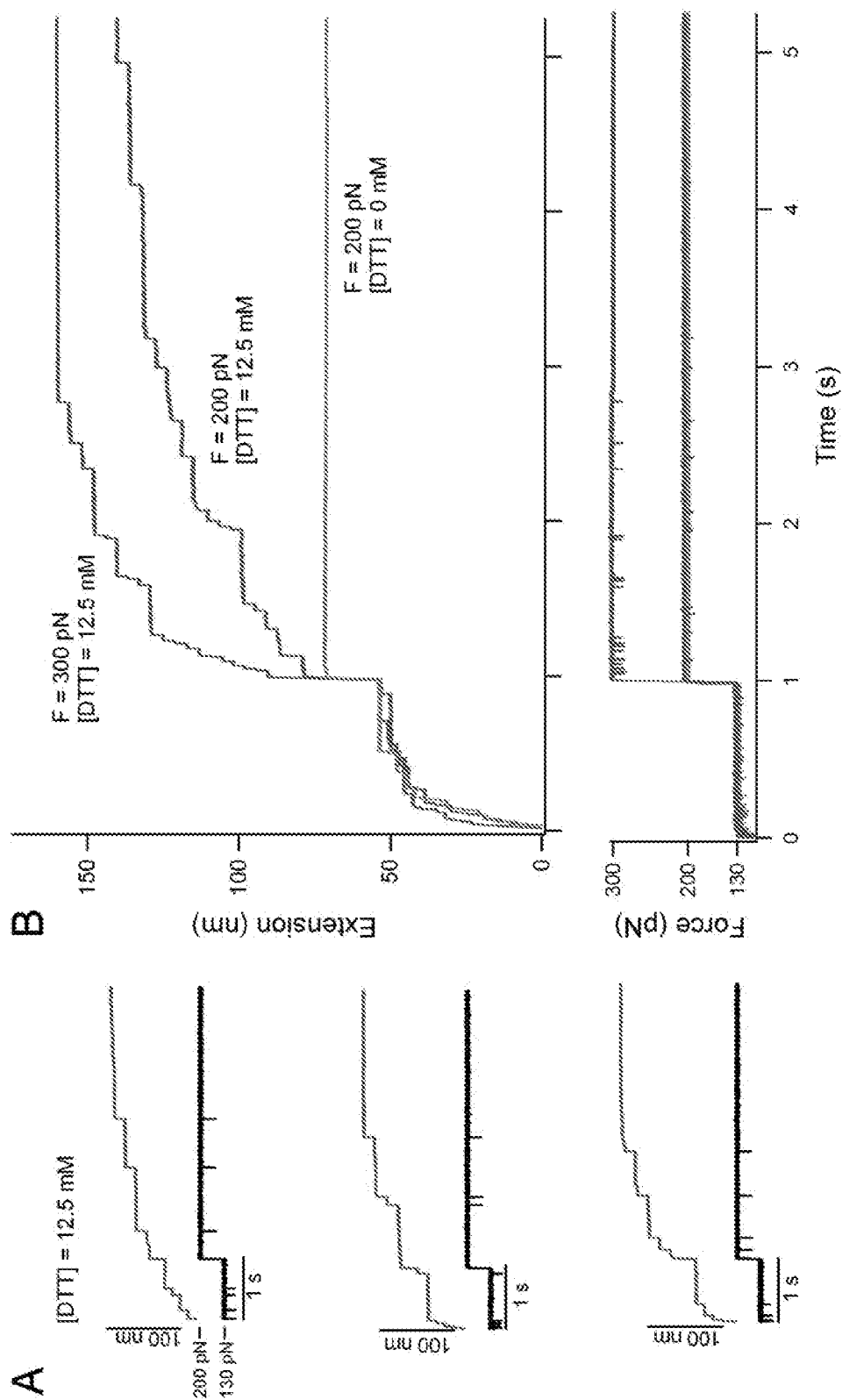


FIGURE 7

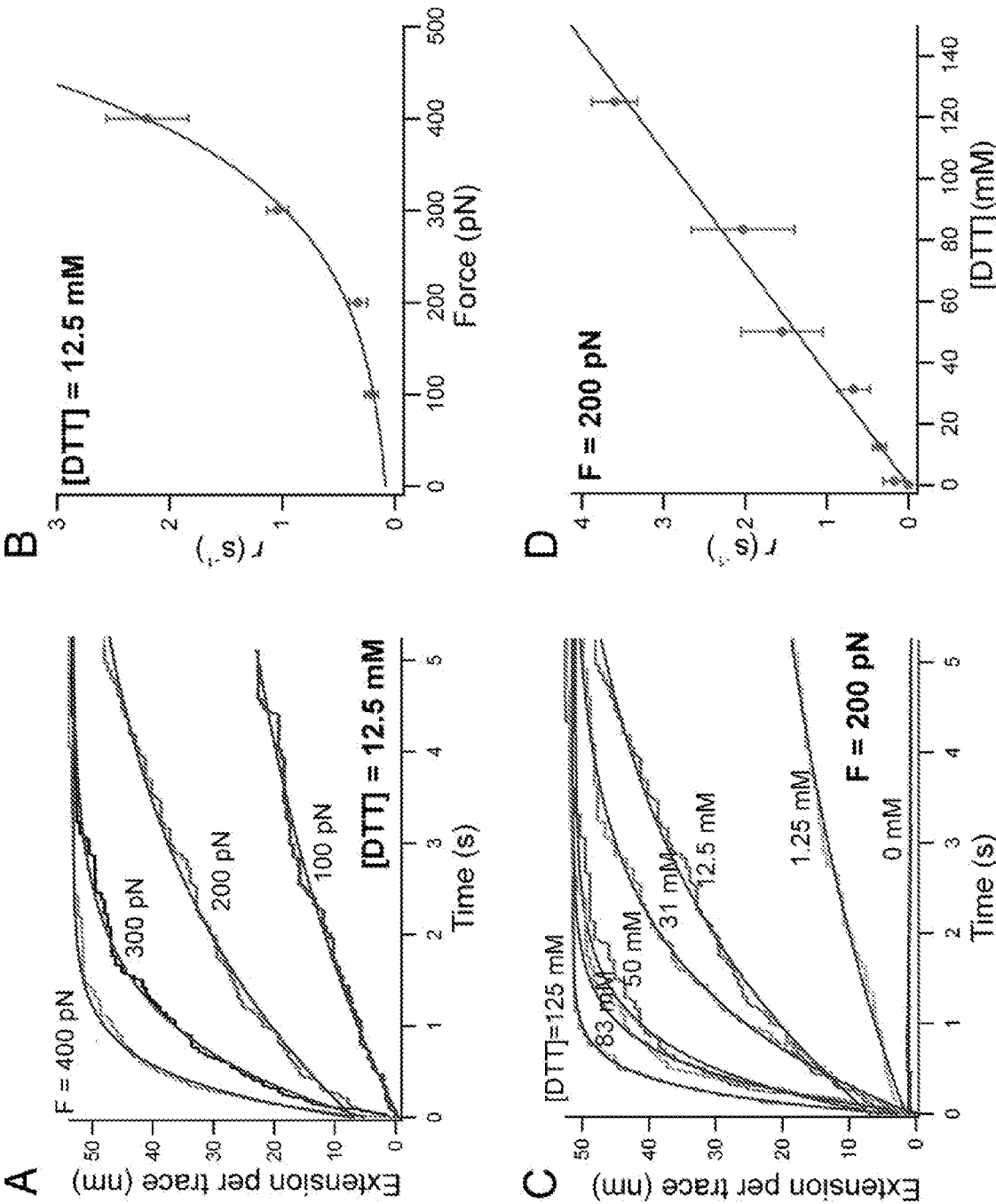


FIGURE 8

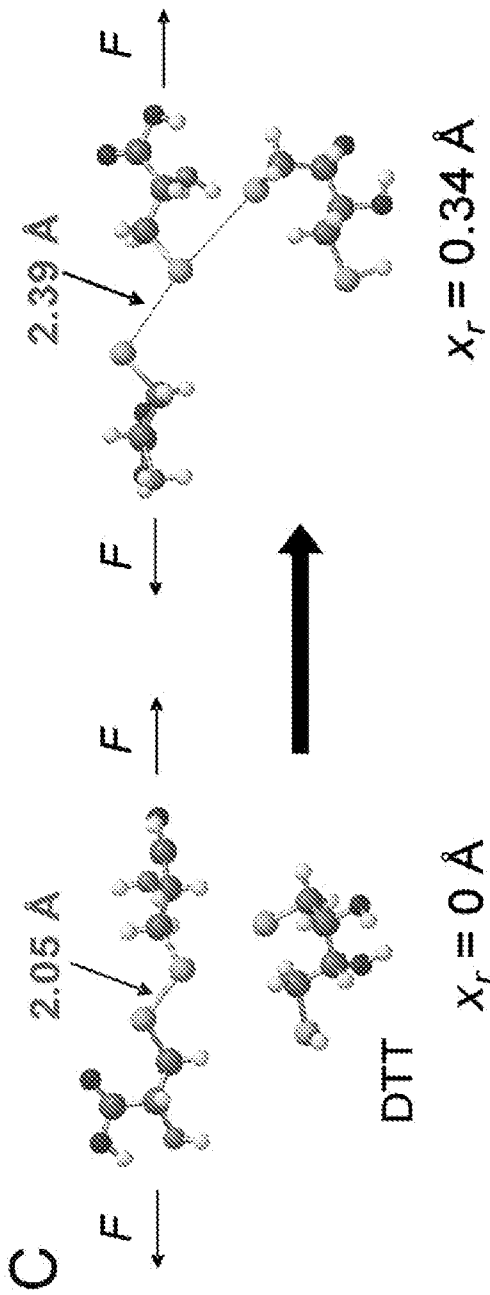
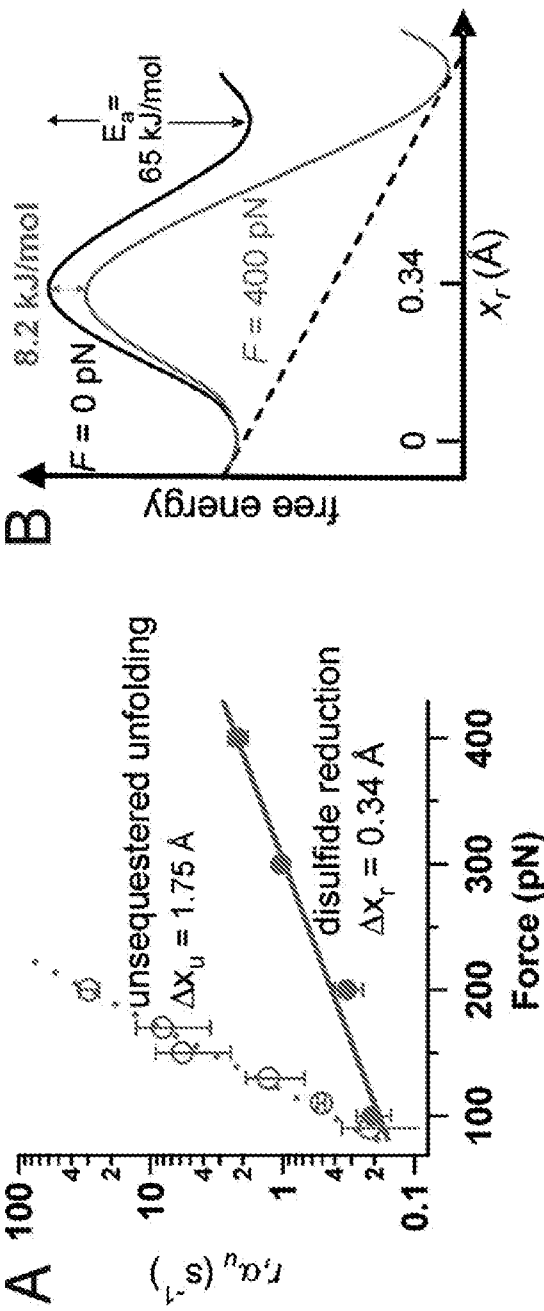


FIGURE 9

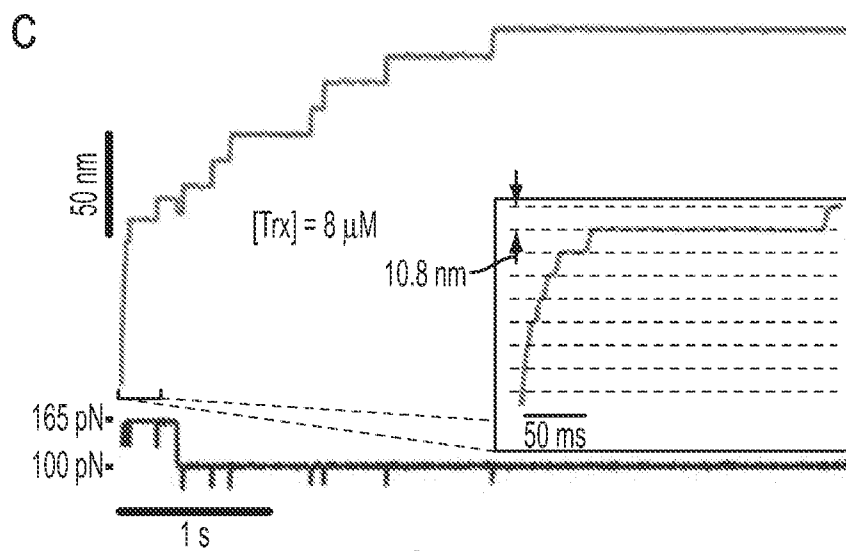
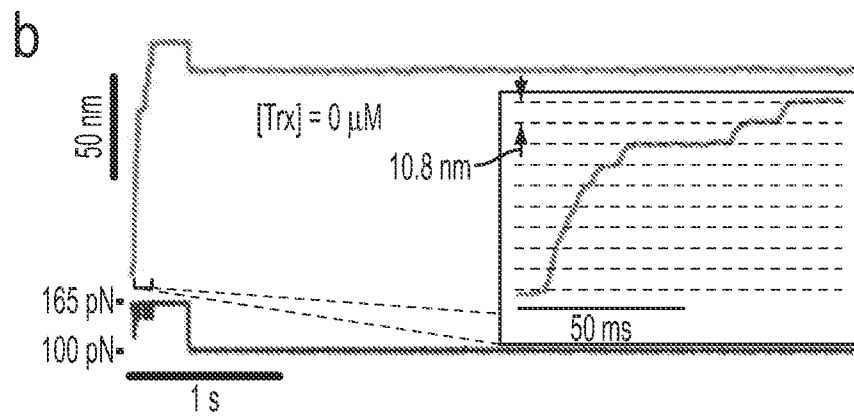
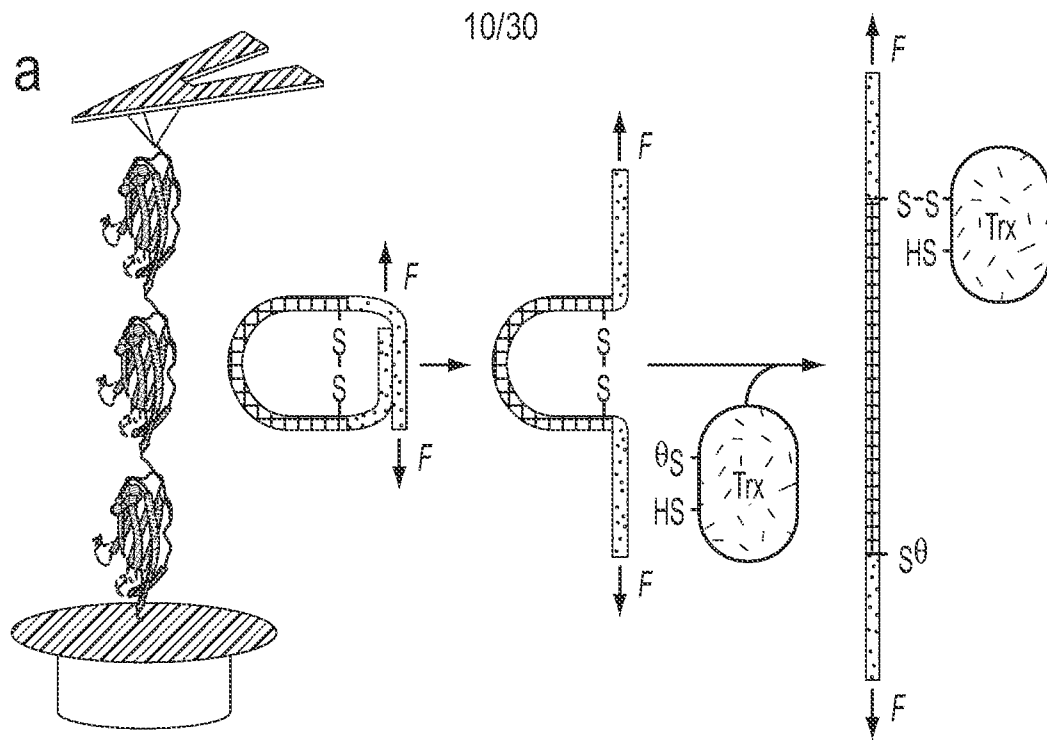


FIG. 10

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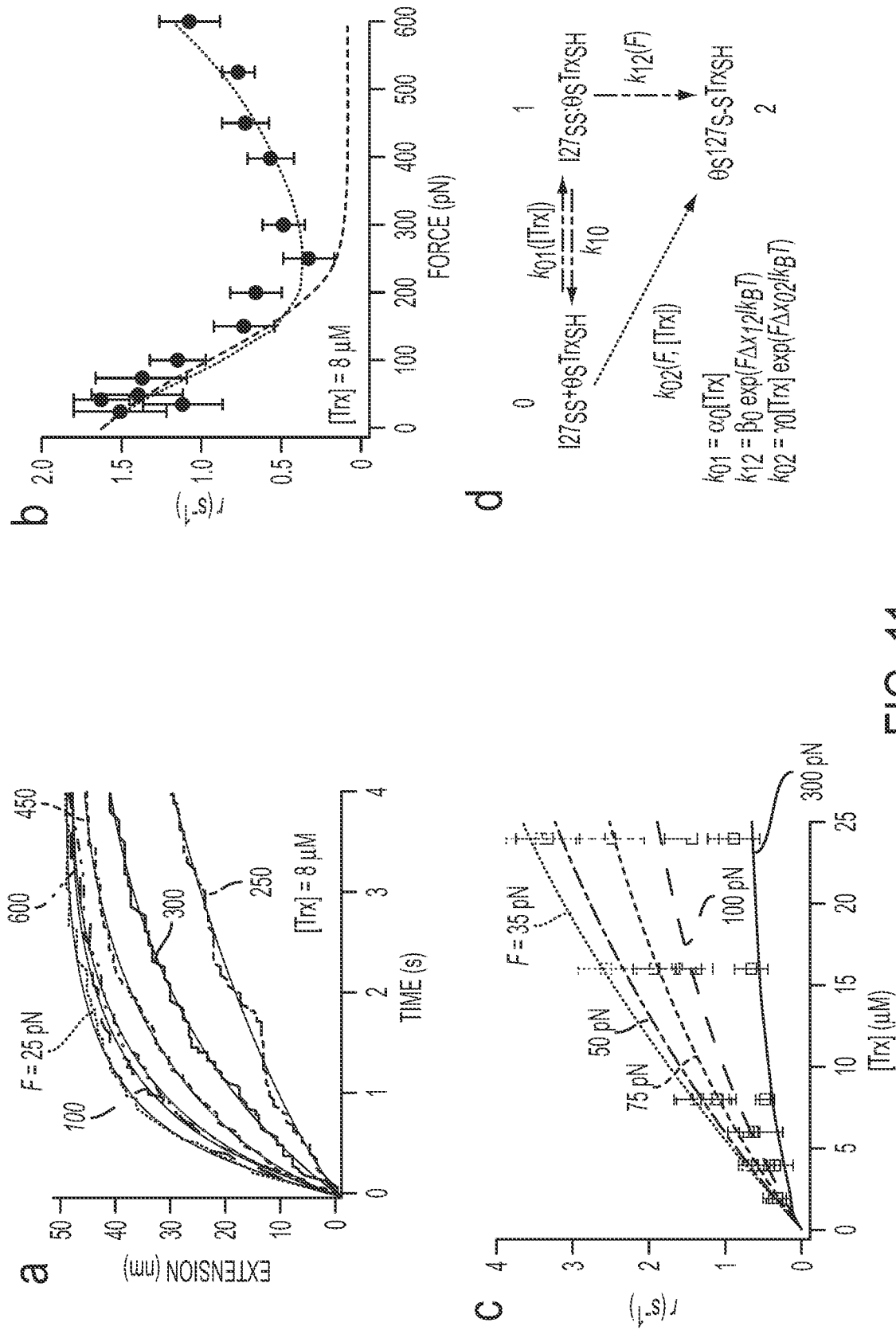


FIG. 11

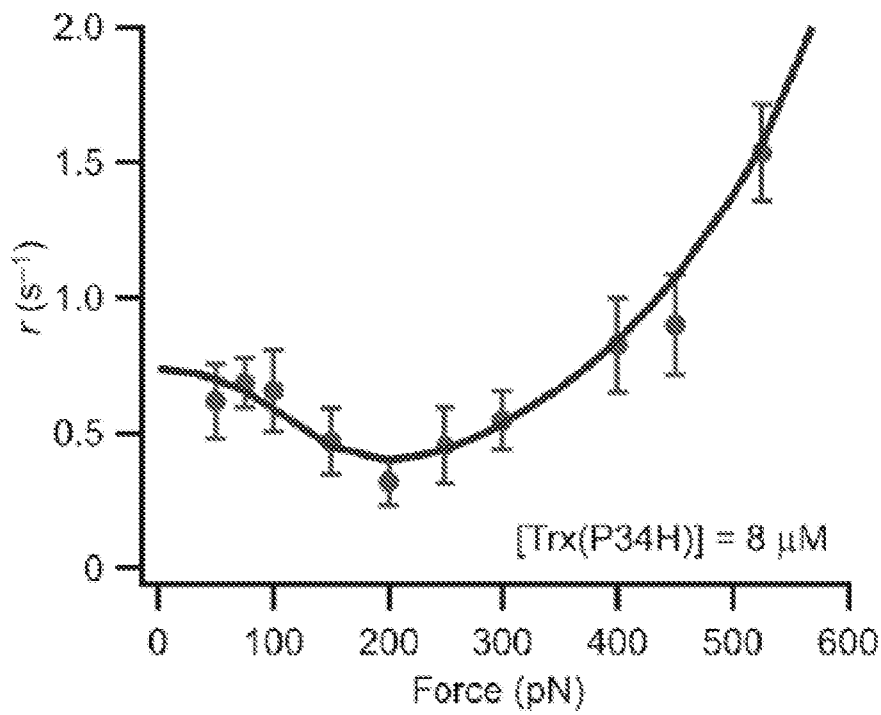


FIGURE 12

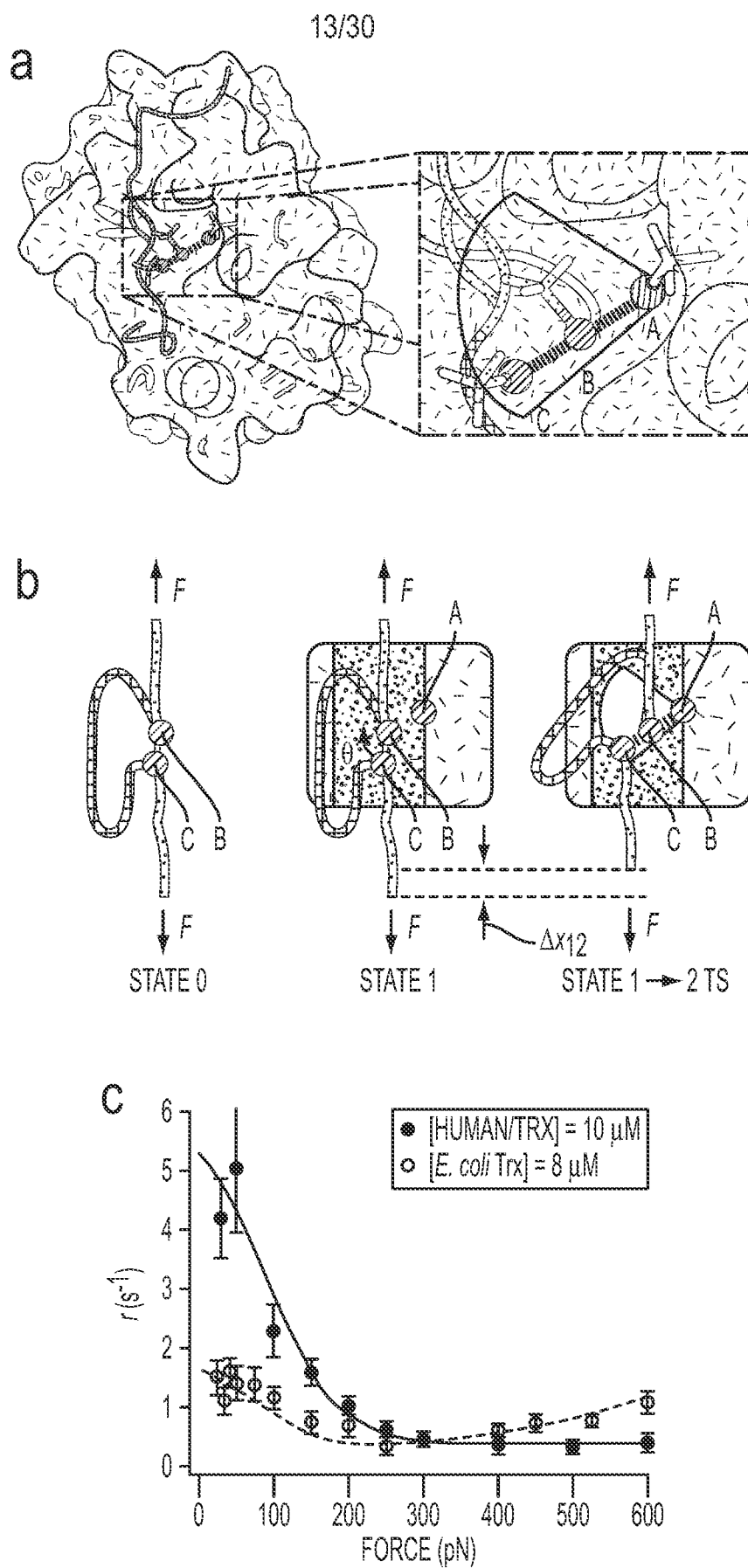


FIG. 13

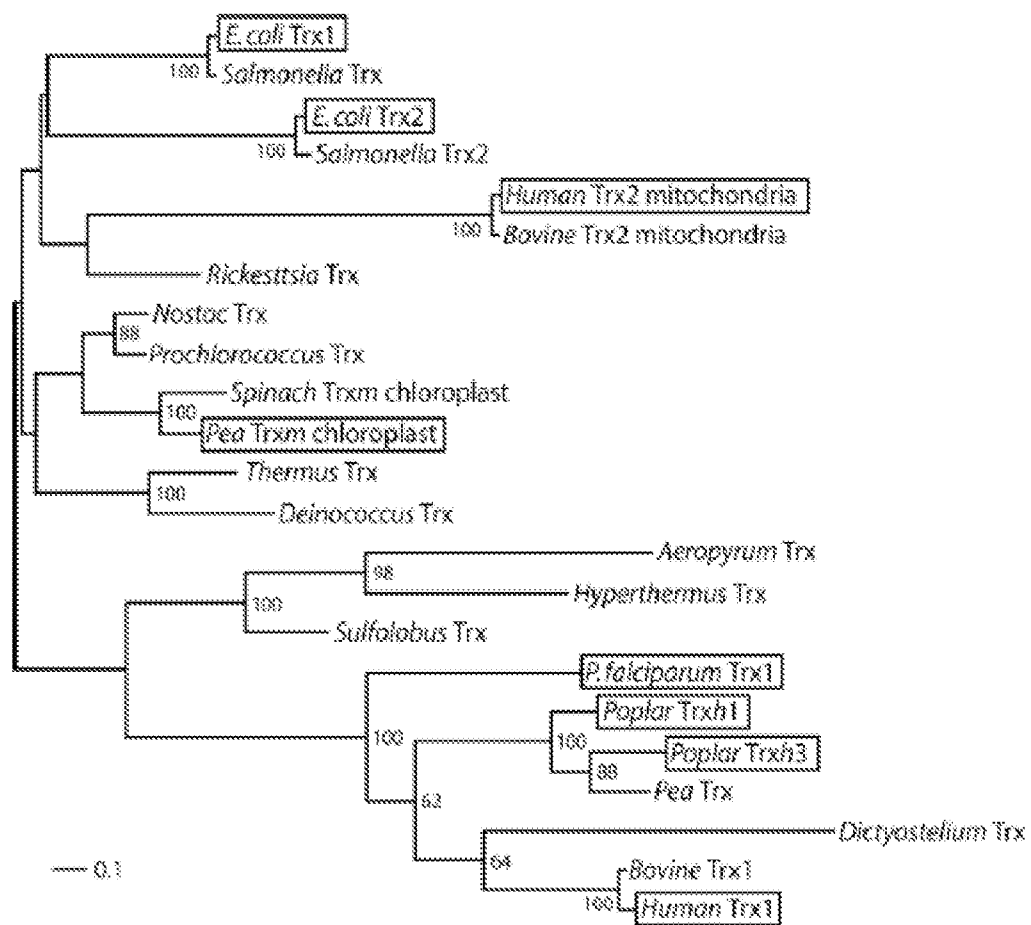
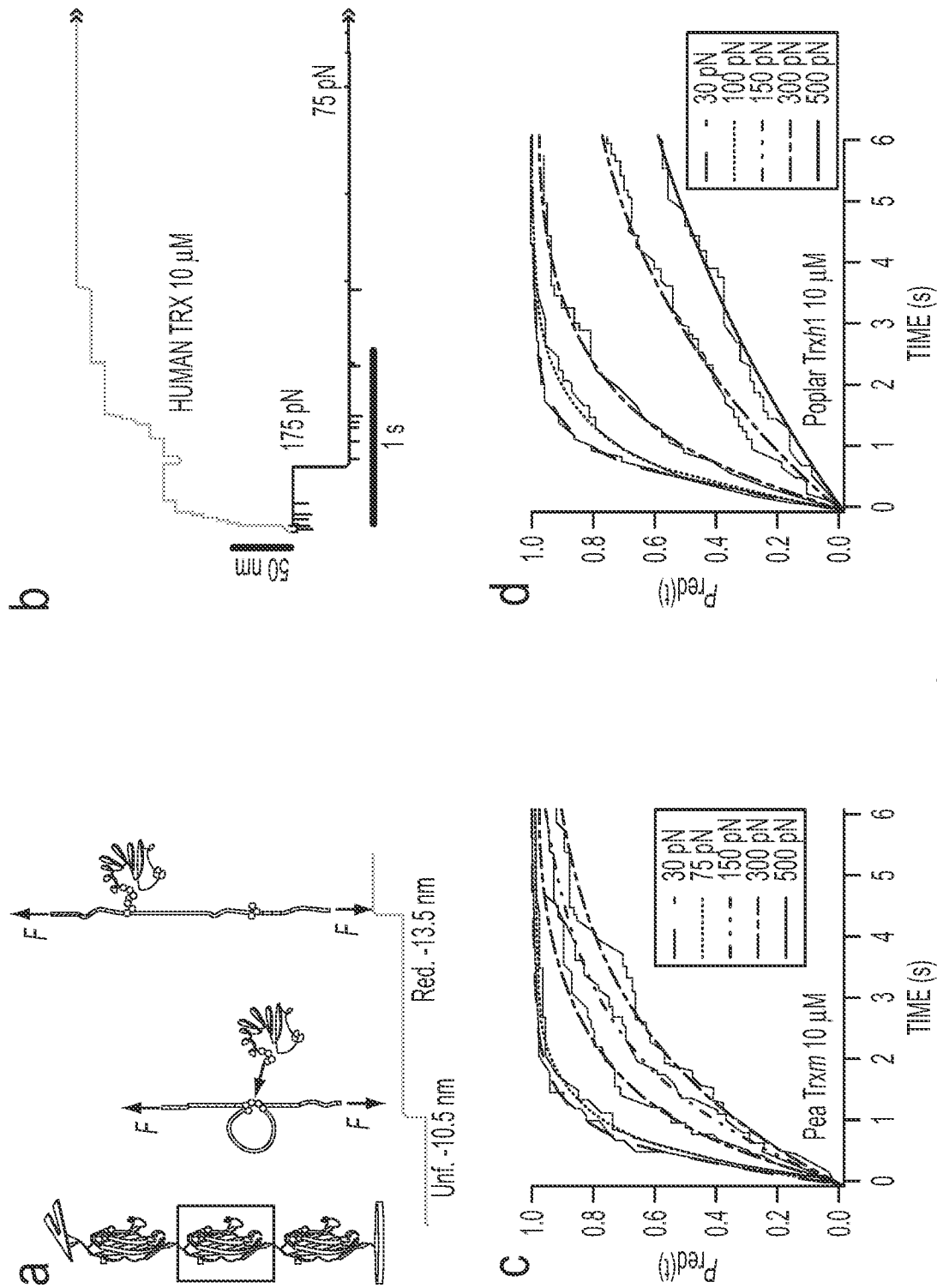


FIGURE 14



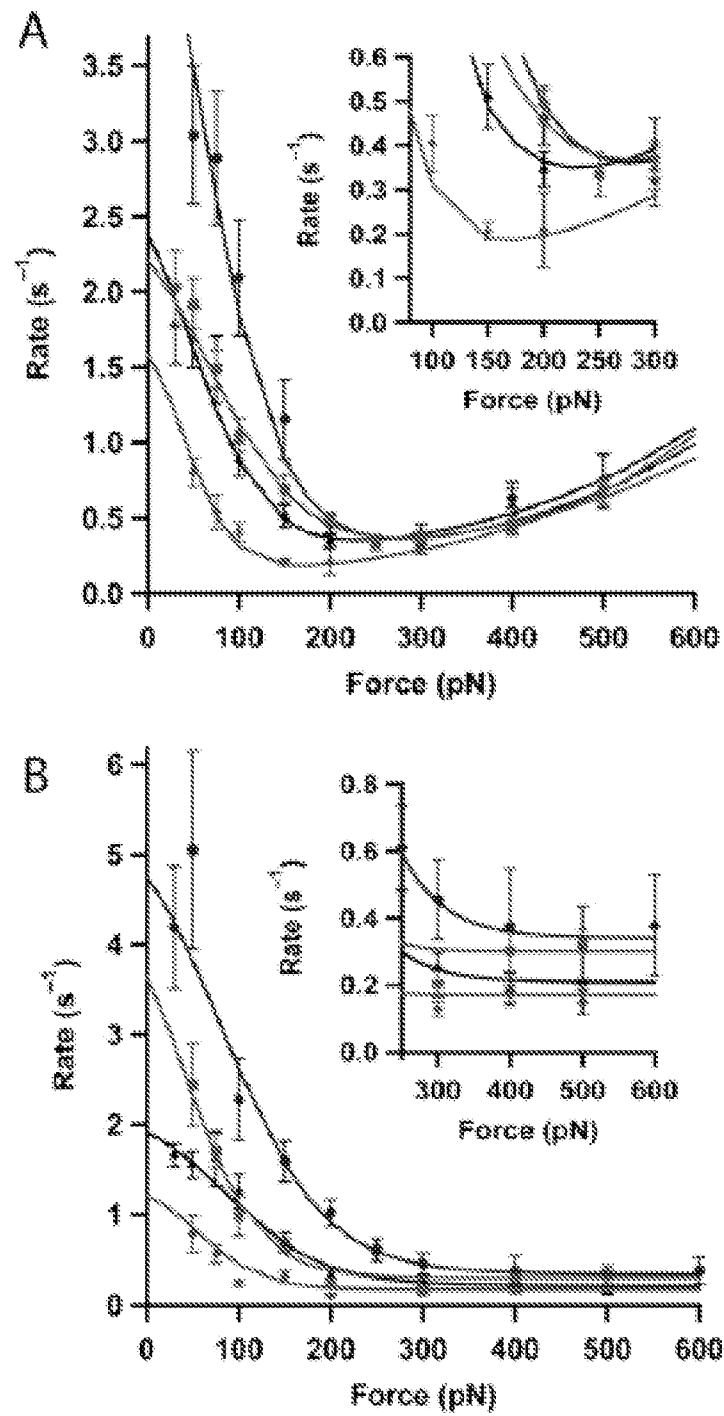


FIGURE 16

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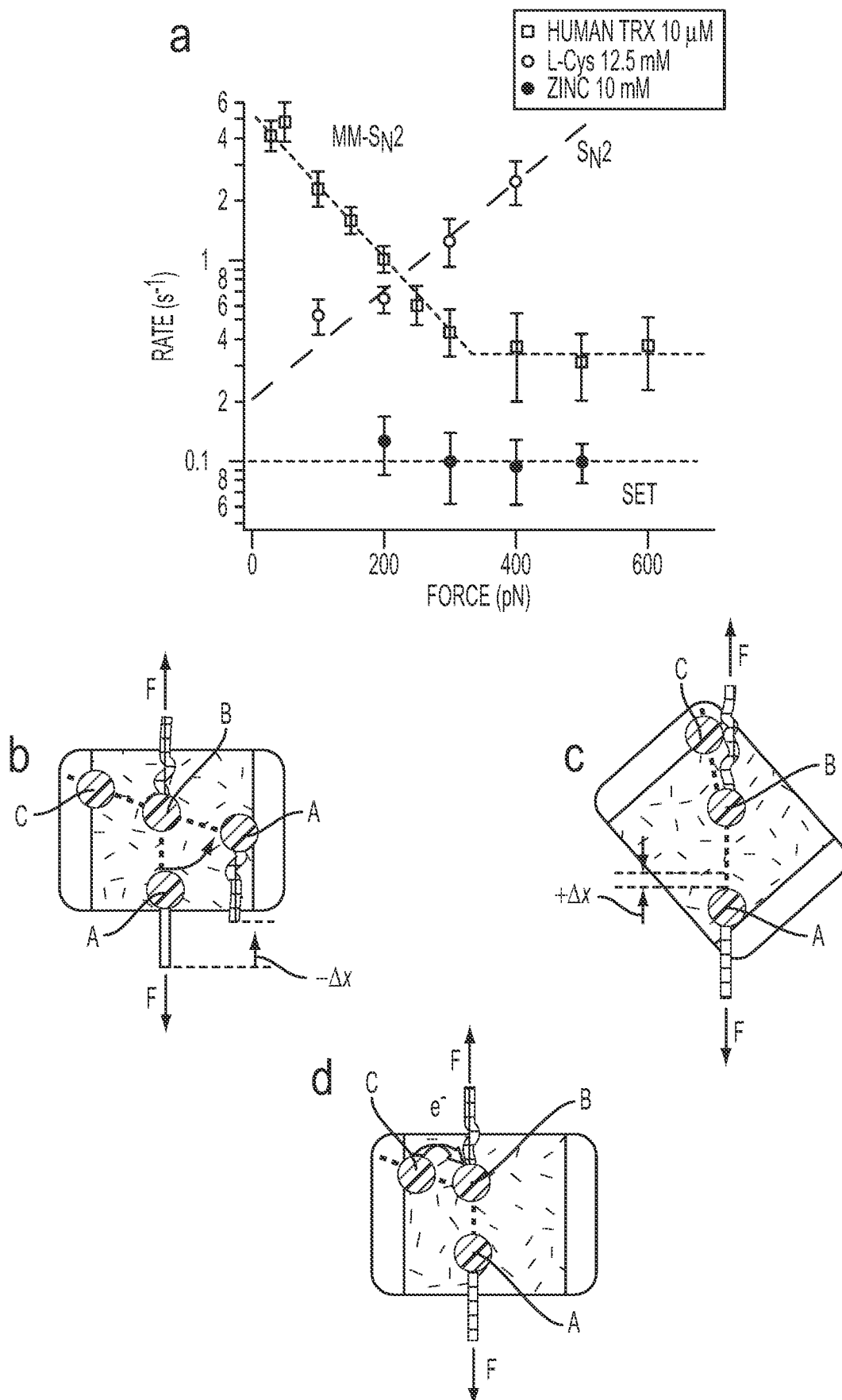


FIG. 17

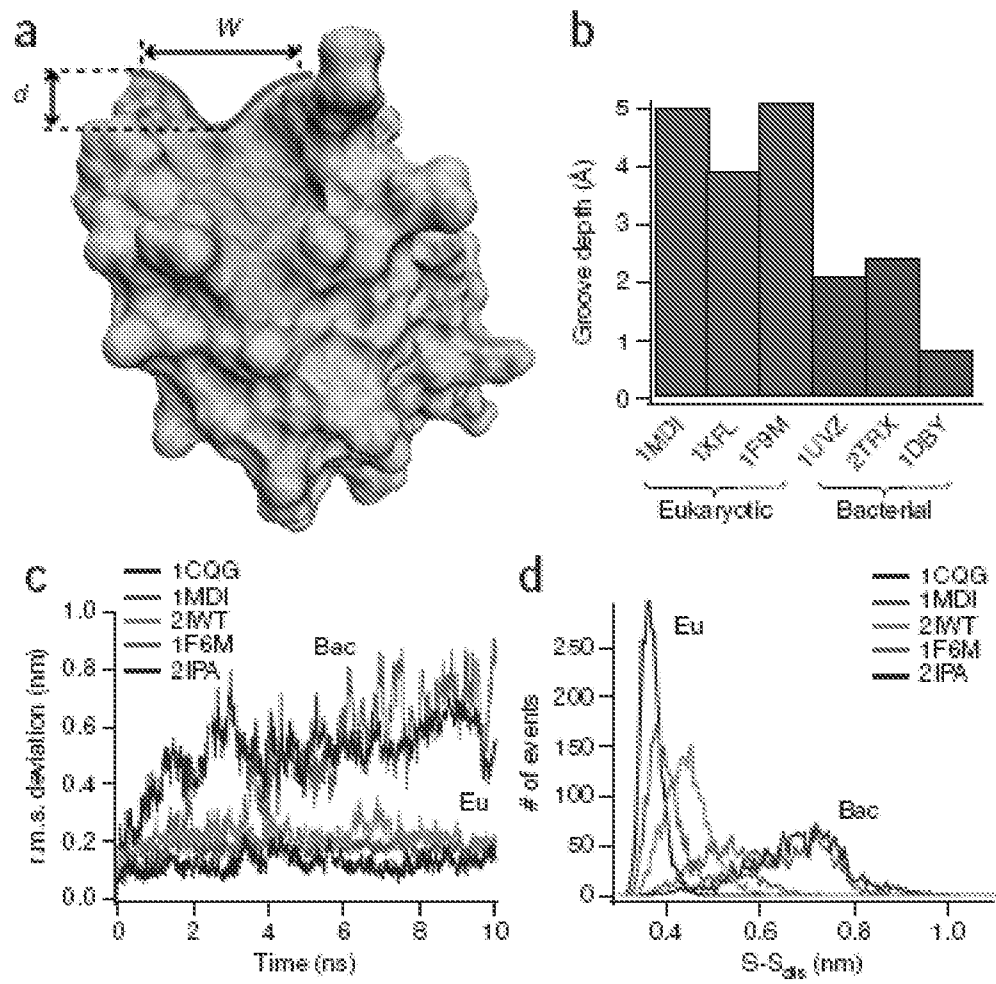
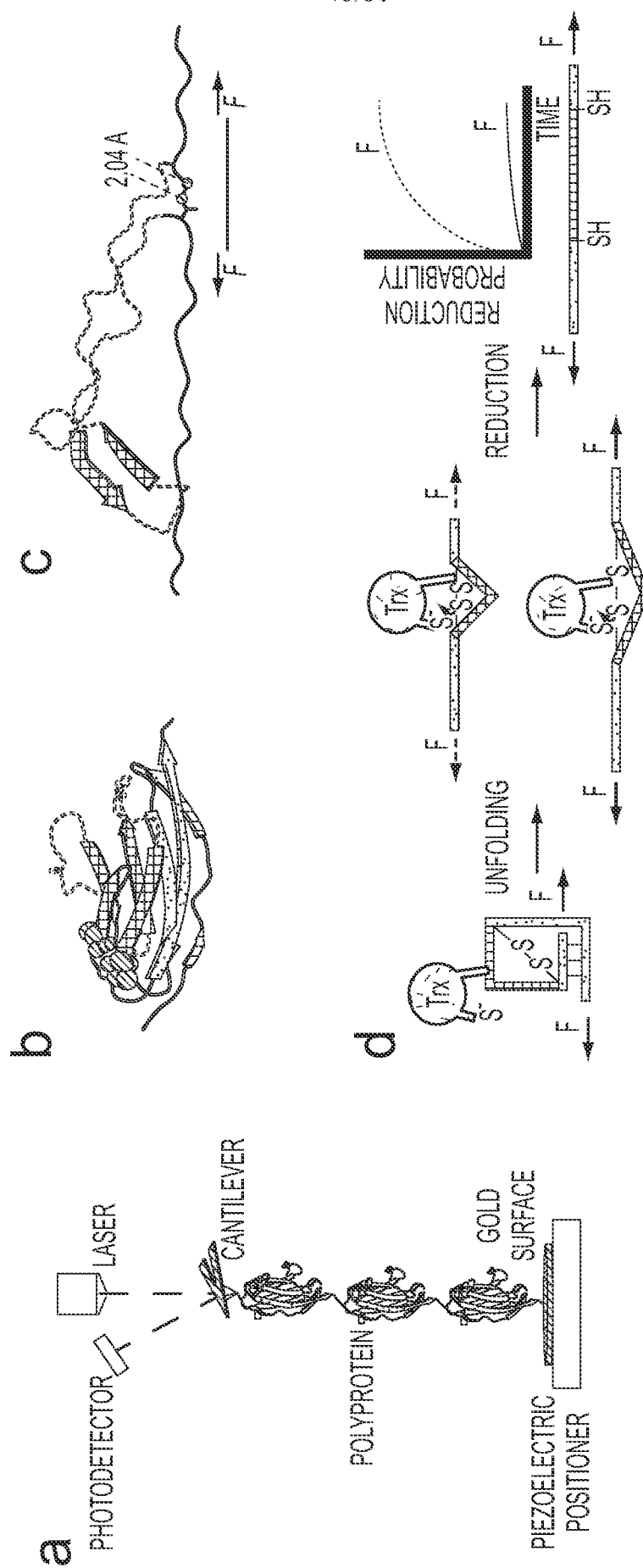


FIGURE 18



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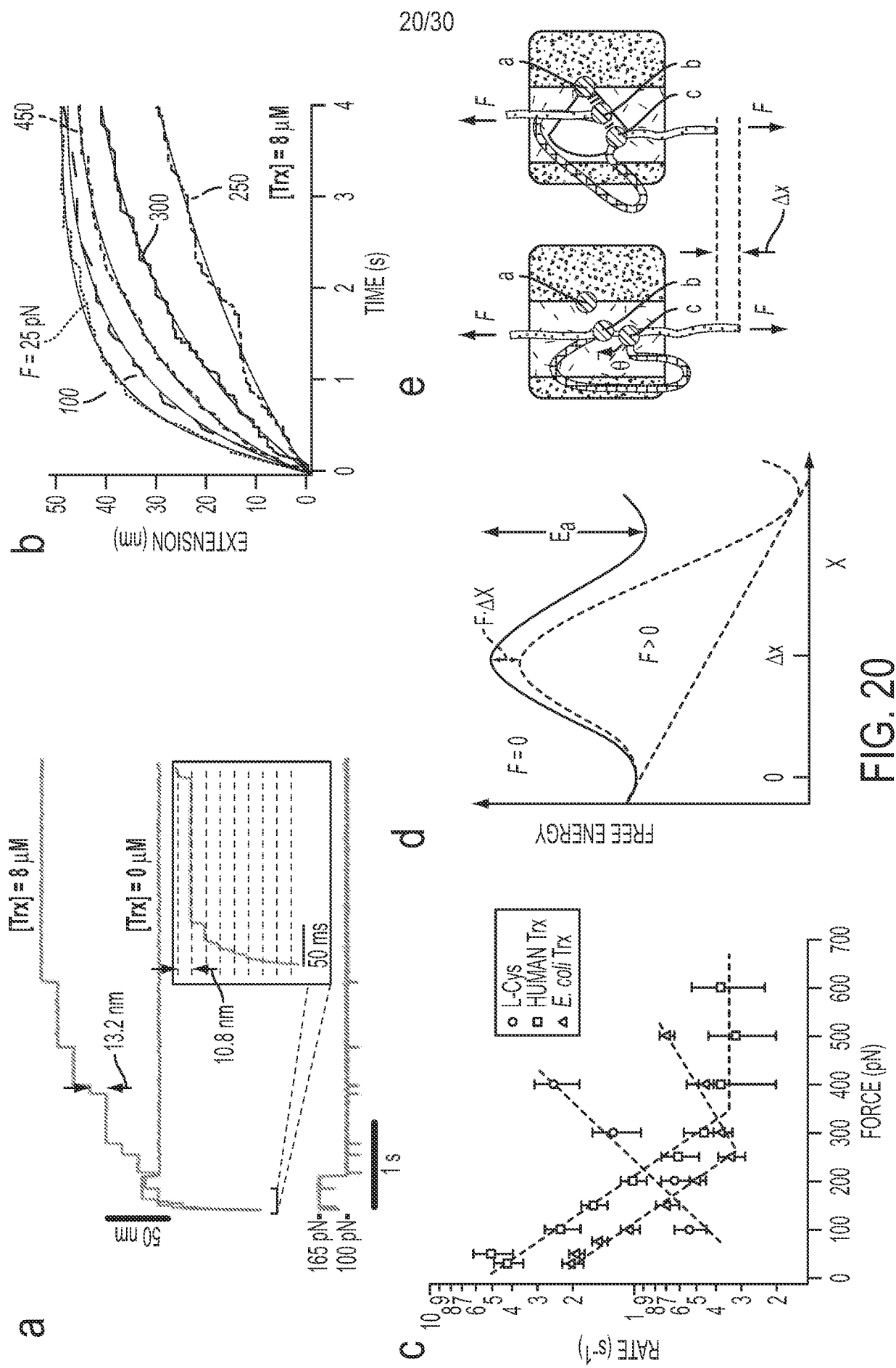


FIG. 20

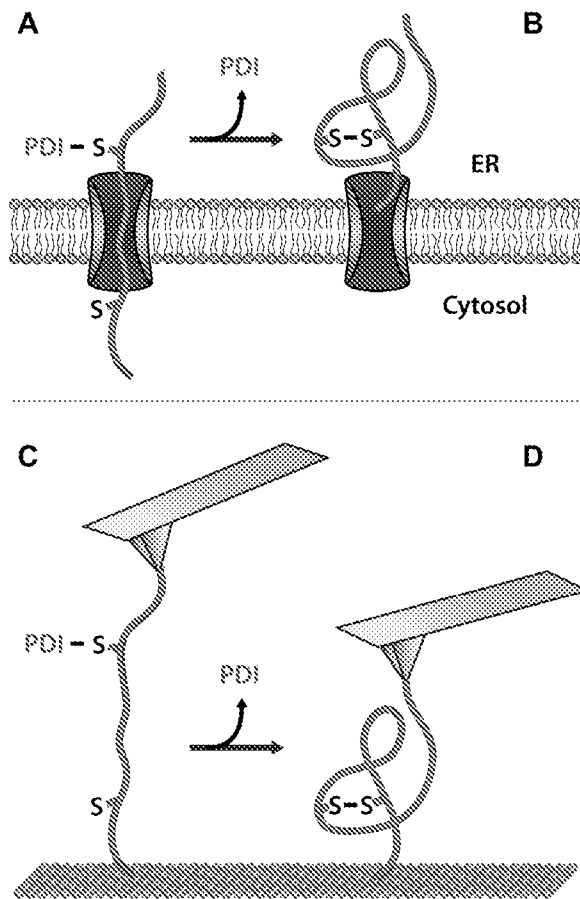


FIGURE 21

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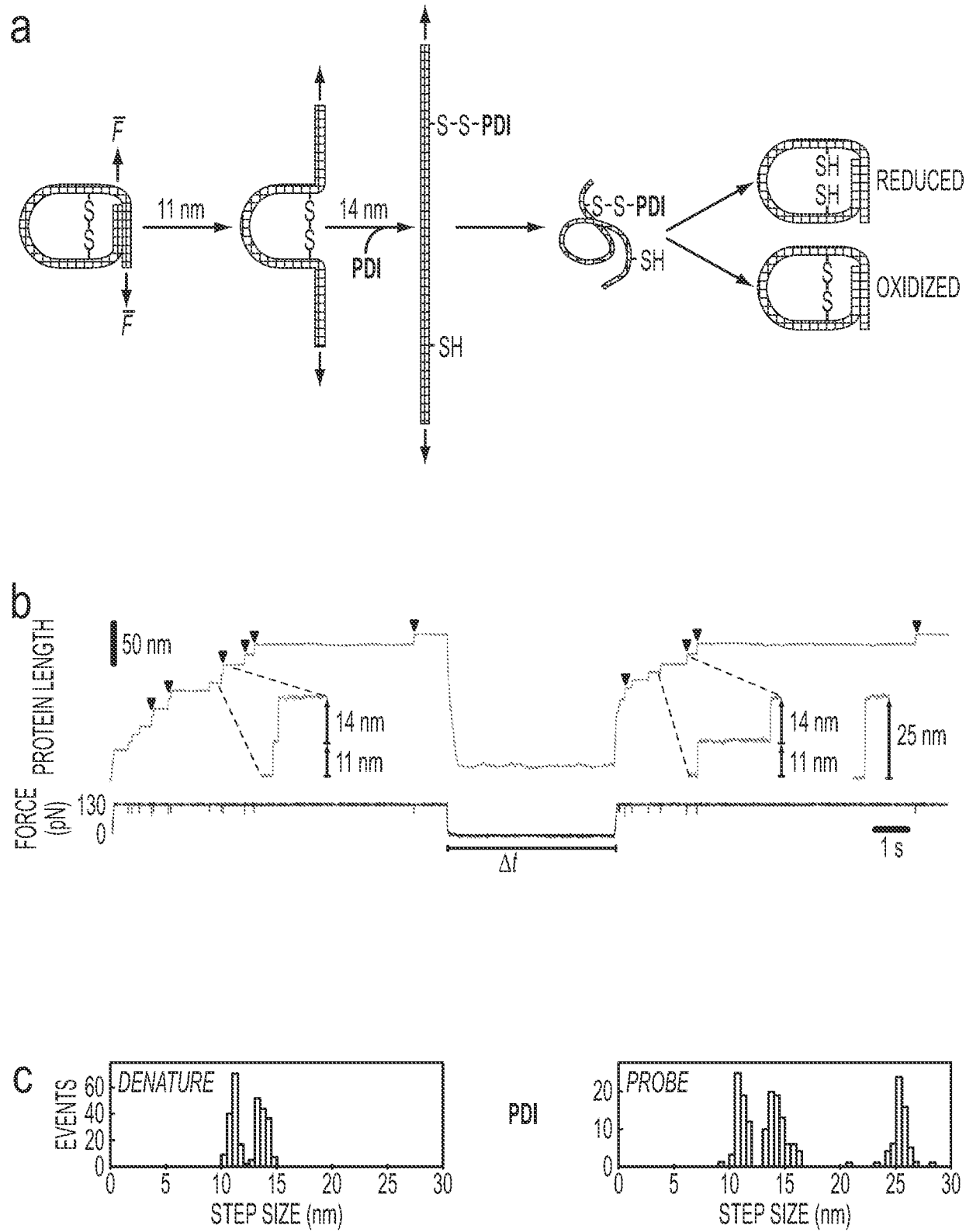


FIG. 22

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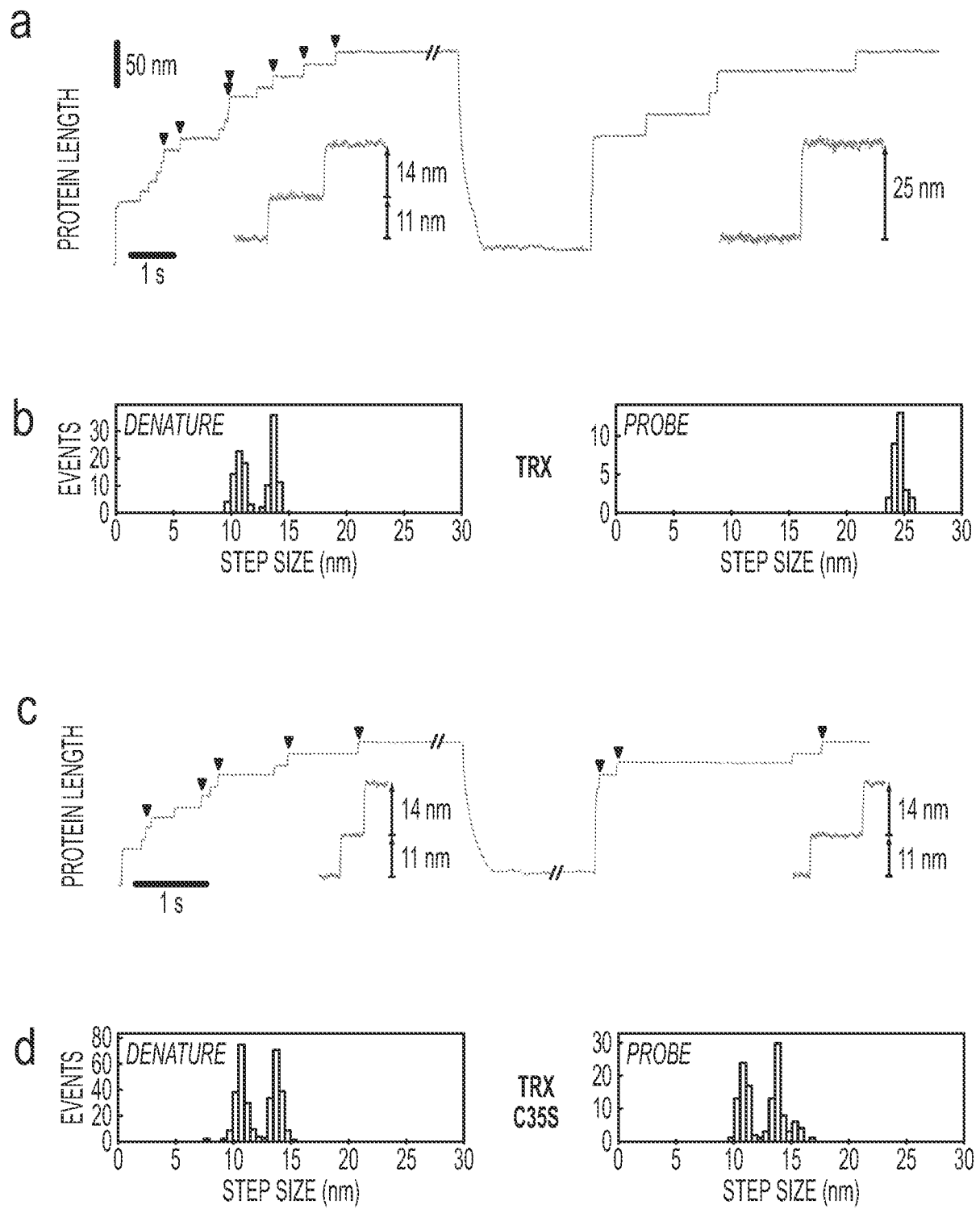


FIG. 23

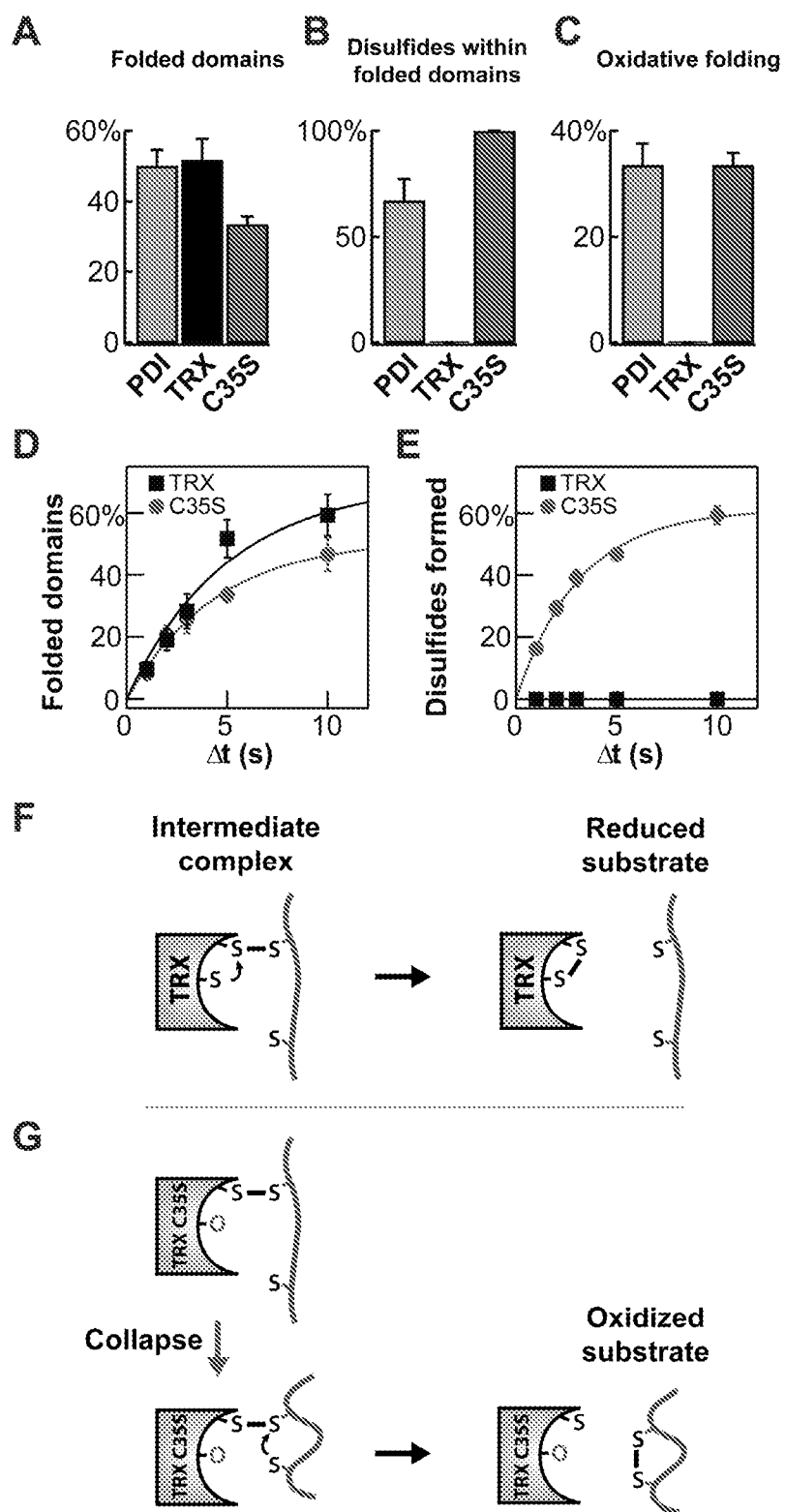


FIGURE 24

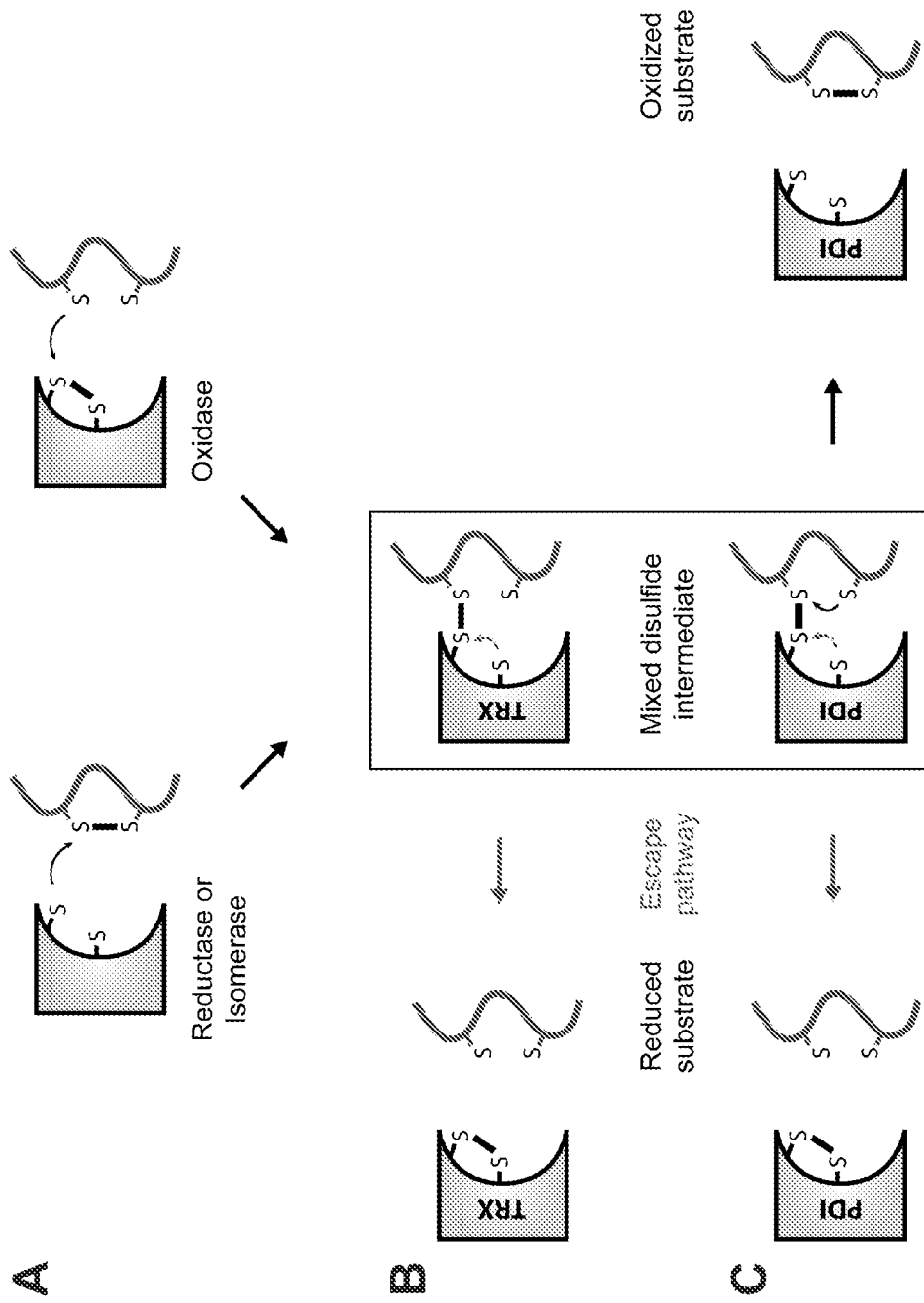


FIGURE 25

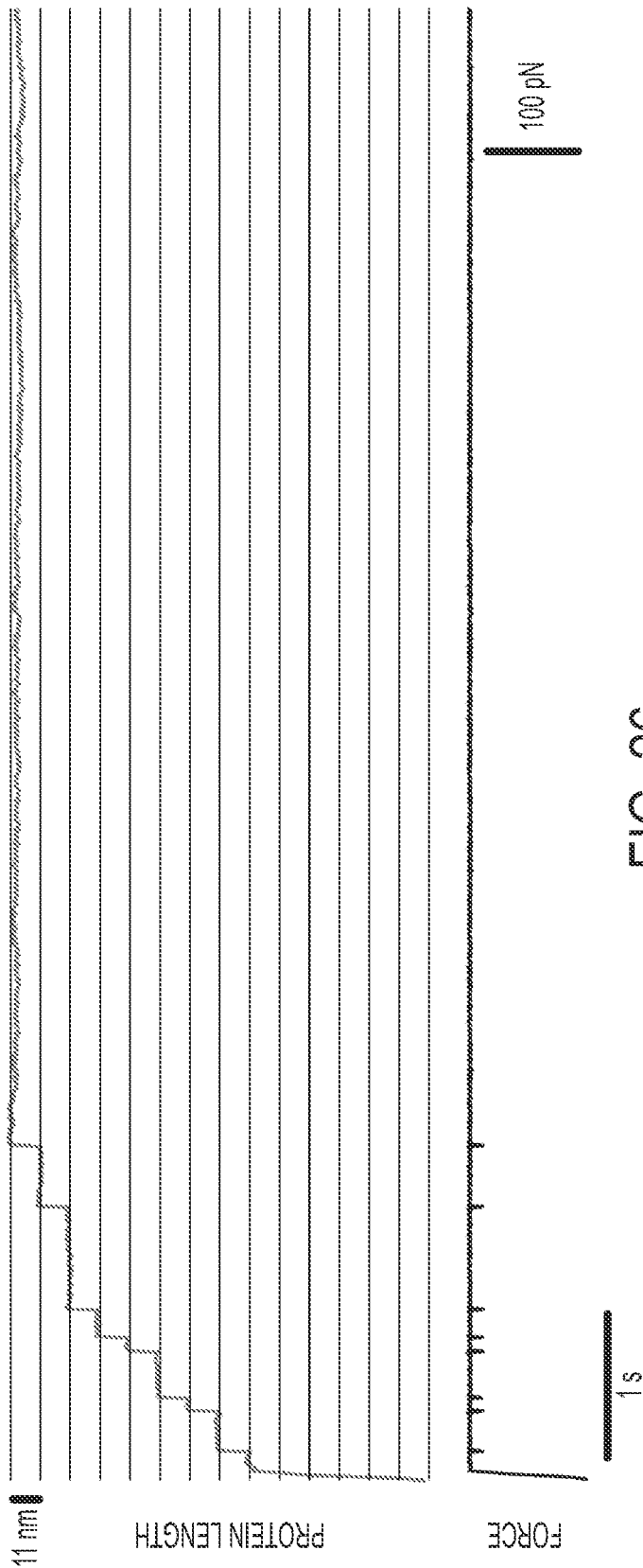


FIG. 26

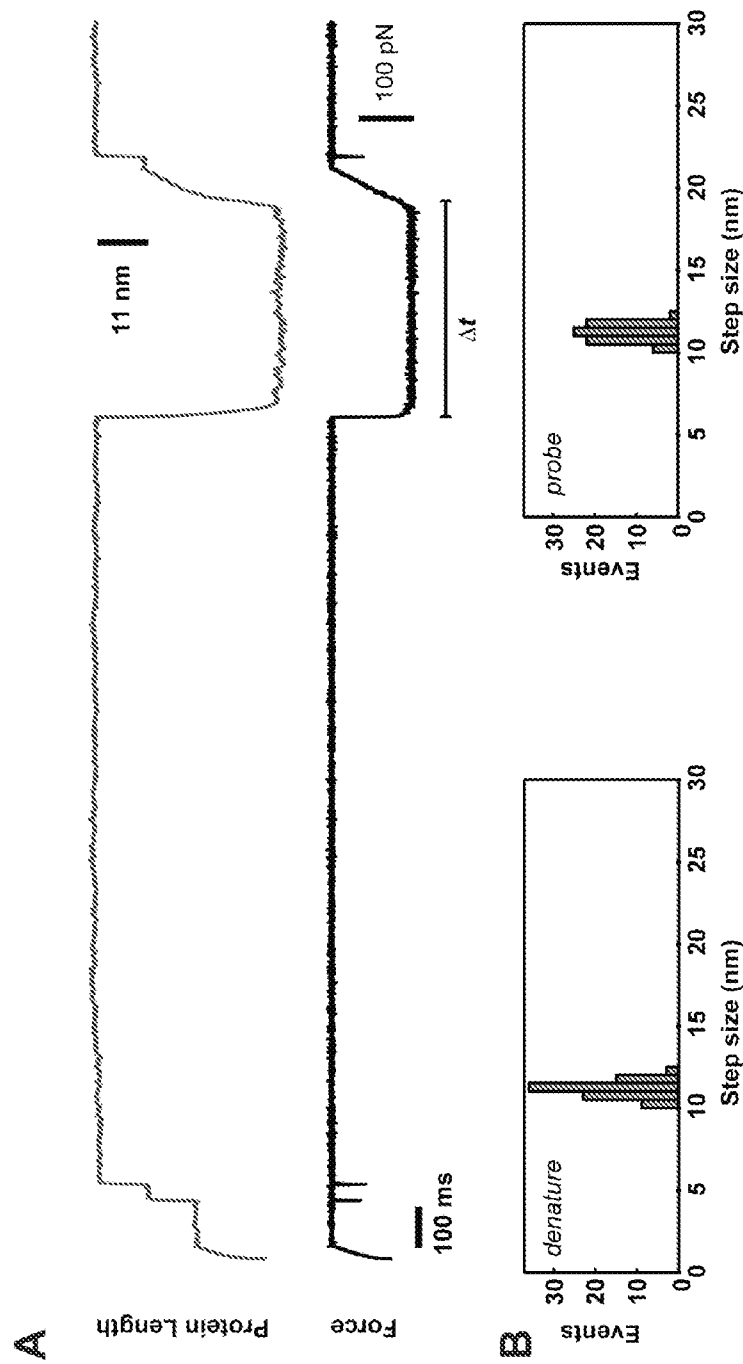


FIGURE 27

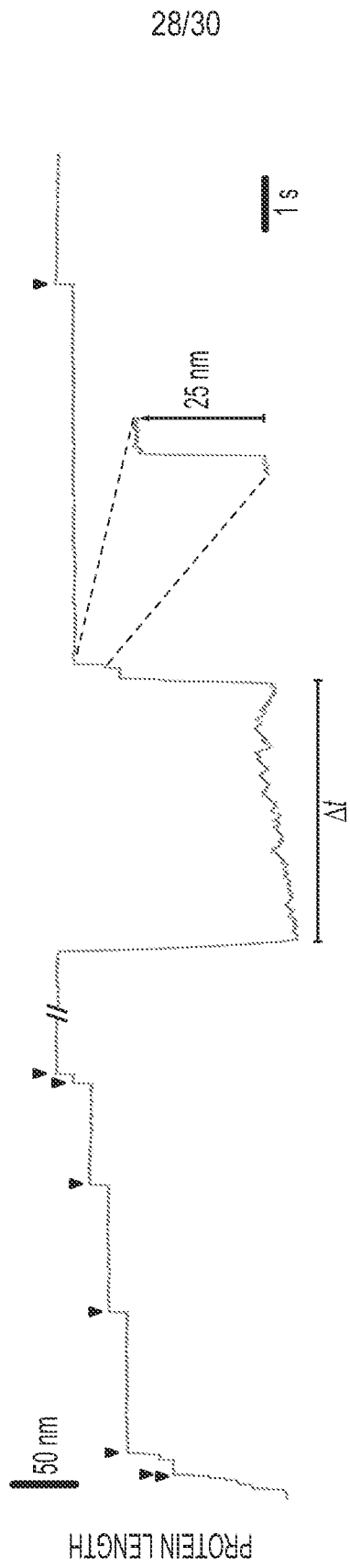


FIG. 28

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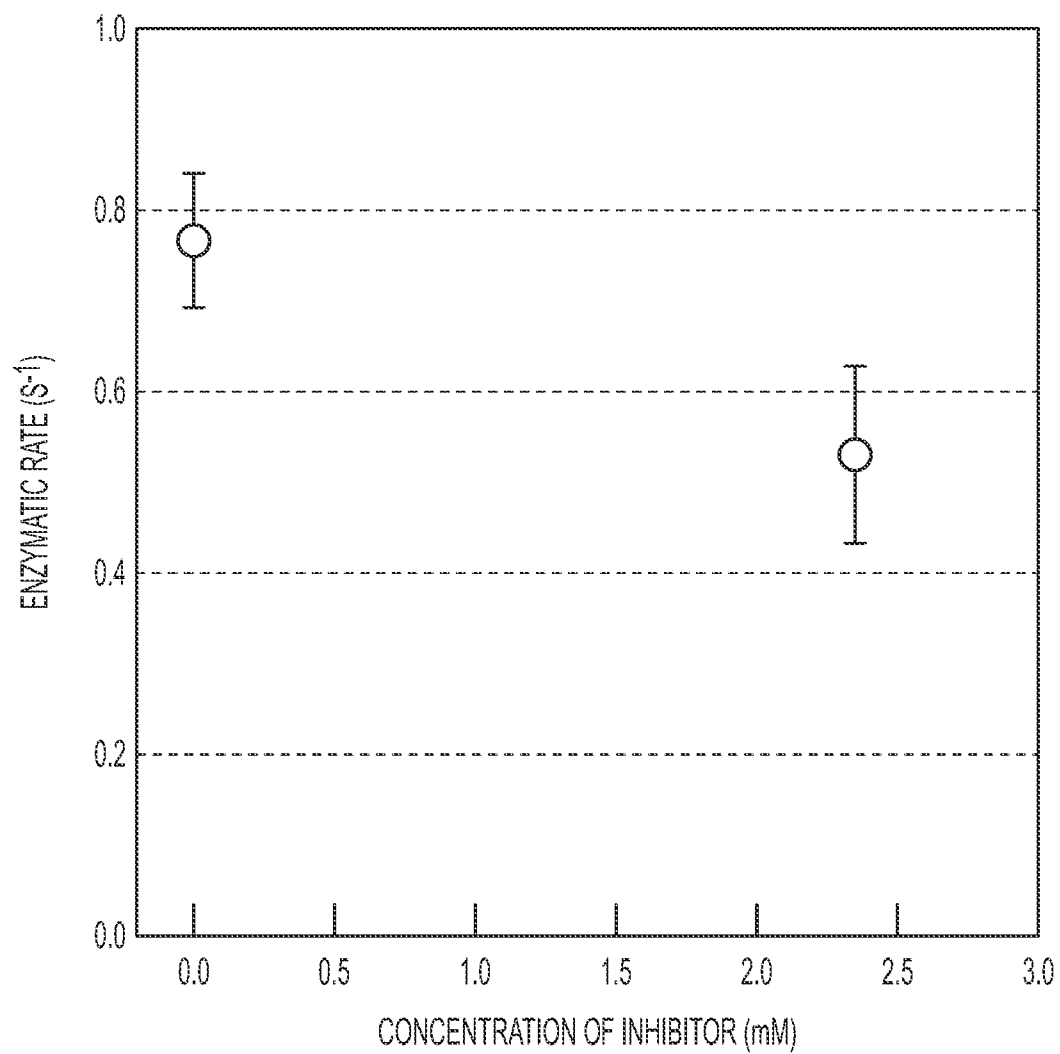


FIG. 29

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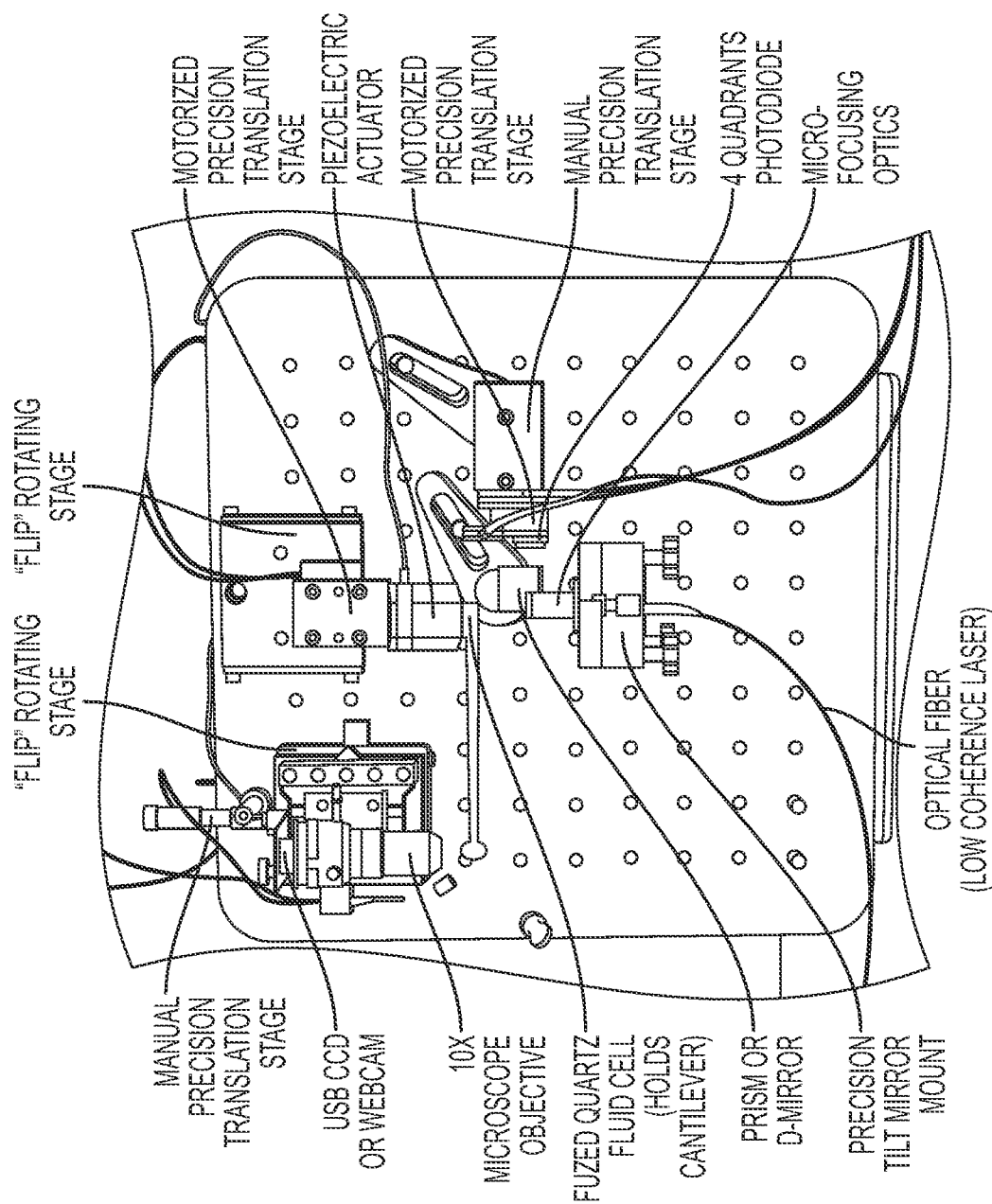


FIG. 30

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/44084

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61B 5/00 (2011.01)

USPC - 600/323

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): A61B 5/00 (2011.01)

USPC: 600/323

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC 600/300, 309, 310, 322, 323; 702/1, 19, 127

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Cantilever, prism, laser, atomic adj force, mount\$3, magnet\$3, beam, tilt, (piezo\$5 piezoelectric), (photodiode photodetect\$3), position\$3, drive, motor, camera

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Oberhauser et al. 'Stepwise unfolding of titin under force-clamp atomic force microscopy', Proceedings of the National Academy of Sciences, January 16, 2001, Vol. 98, No. 2, p. 468-472	1-4, 12-14
Y	US 2007/0180889 A1 (Murayama et al.) 09 August 2007 (09.08.2007) figs. 1-7 and para [0114]	1-4, 12-14
Y	US 6,323,903 B1 (Poulsen et al.) 27 November 2001 (27.11.2001) figs. 1-2 and col 1, ln 67 1 col 2, ln 7	4
Y	US 2004/0042097 A1 (Murnan et al.) 04 March 2004 (04.03.2004) figs. 1-4 and para [0011]	12
Y	US 2003/0015653 A1 (Hansma et al.) 23 January 2003 (23.01.2003) figs. 1-2 and para [0052]	13
Y	US 5,969,345 A (Williams et al.) 19 October 1999 (19.10.1999) fig. 13 and col 6, ln 33-38	14

☐ Further documents are listed in the continuation of Box C.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

04 November 2011 (04.11.2011)

Date of mailing of the international search report

21 NOV 2011

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, Virginia 22313-1450

Facsimile No. 571-273-3201

Authorized officer:

Lee W. Young

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 11/44084

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 5-11 and 15-65
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

专利名称(译)	力钳光谱仪和使用方法		
公开(公告)号	EP2592997A1	公开(公告)日	2013-05-22
申请号	EP2011807541	申请日	2011-07-14
[标]申请(专利权)人(译)	纽约市哥伦比亚大学理事会		
申请(专利权)人(译)	哥伦比亚大学纽约市受托人		
当前申请(专利权)人(译)	哥伦比亚大学纽约市受托人		
[标]发明人	FERNANDEZ JULIO M PEREZ JIMENEZ RAUL KOSURI PALLAV		
发明人	FERNANDEZ, JULIO, M. PEREZ-JIMENEZ, RAUL KOSURI, PALLAV		
IPC分类号	A61B5/00 G01Q60/42 G01N19/04		
CPC分类号	G01N21/01 B82Y35/00 G01N19/04 G01N29/022 G01Q60/42		
优先权	61/364640 2010-07-15 US 61/364208 2010-07-14 US		
其他公开文献	EP2592997A4		
外部链接	Espacenet		

摘要(译)

所公开的主题涉及一种力钳式光谱仪，其能够以恒力模式操作并且允许使用反馈电子器件和软件进行自动数据采集和分析。所公开的主题还涉及使用力钳式光谱仪测量化学反应动力学的方法。所述方法可包括但不限于测量底物折叠和解折叠的动力学，以及键断裂和键形成。