Electronic Supplementary Material (ESI) for Chemical Communications


# Supporting Information

# Label-free biosensing with single-molecule force spectroscopy

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### 1.1 Modification of AFM probes with ssDNA and Lyz

Silicon nitride AFM probes coated on the back side with a 45±10 nm thick Ti/Au layer (DNP-S10, Bruker Corporation) were cleaned for 30 minutes with freshly prepared Piranha solution ($H_2SO_4$:30% $H_2O_2$, 7:3, v/v) to remove the organic cover on the probes, and then washed with large amount of DI water and ethanol (99%) for several times.

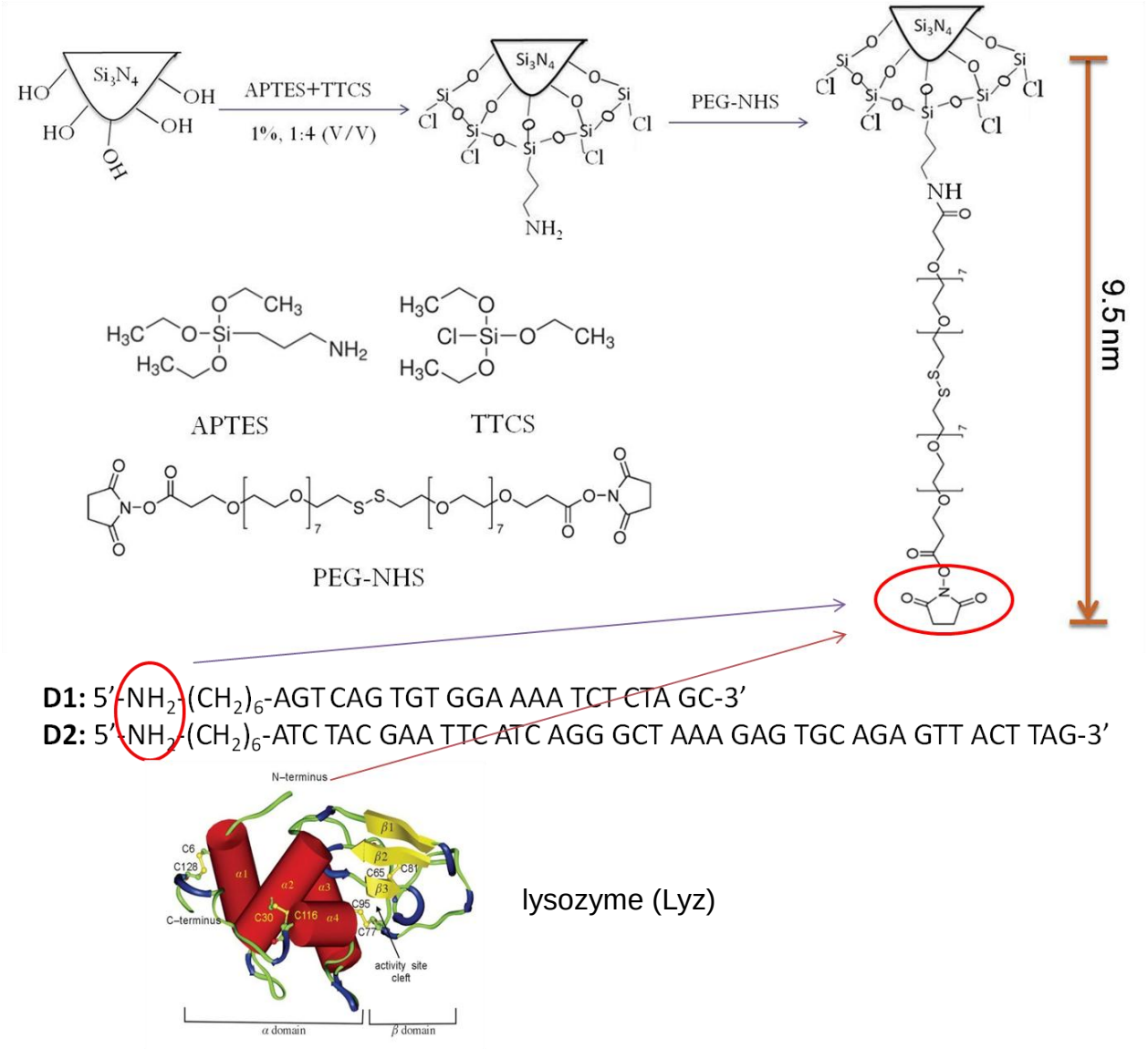



**Figure S1.** Schematic representation of the modification of AFM probes with DNA and Lyz, achieved by a bifunctional PEG-NHS ester linker.

As shown in **Figure S1**, the cleaned AFM probes were then immersed into a mixed solution of 3-aminopropyl triethoxysilane (APTES) and thiethoxychorosilane (TTCS) (1% in toluene, APTES:TTCS,1:4, v/v) for 15 minutes. After washing with DI water and ethanol, the AFM probes were transferred into PEG-NHS ester disulfide (0.1 mg/mL) for 1 h to bind the PEG-NHS ester disulfide onto the AFM probe by covalent interaction between $-NH_2$ and NHS-ester.[1] In the last step, the AFM probes were immersed into either ssDNA (100 nM) or human Lyz (2 ng/μL) for 30 minutes to bind the DNA or Lyz molecules onto the probes. The ssDNA molecules (D1 and D2) (IBA Company, Göttingen, Germany) and Lyz (Sigma-Aldrich) were connected to the AFM probes through the binding of their terminal $-NH_2$ group with another N-succinimidyl ester of PEG-NHS ester linker. The probes were washed with a large amount of DI water to remove non-covalently adsorbed DNA or Lyz molecules prior to the single-molecule force spectroscopy experiments.

### 1.2 Preparation and characterization of graphite and $SiO_2$ surfaces

The preparation of a flat graphite surface was performed by mechanical exfoliation of a wafer of HOPG (ZYB quality, $10\times10$ $mm^2$, NT-MDT company, Russia) using Scotch tape.[2] The exfoliated flat graphite surface was then transferred onto a Si wafer for the next experiments. The preparation of a clean and flat $SiO_2$ surface was done by immerging a single-crystal Si wafer into a freshly prepared Piranha solution for 30 minutes and then washing it with a large amount of DI water and ethanol for several times.

AFM height images of the graphite (**Figure S2(a)**) and $SiO_2$ (**Figure S2(b)**) surfaces were acquired on a NanoWizard 3 NanoScience atomic force microscope (JPK Instruments AG, Germany) in air using the AC mode.

We found that the flat graphite and $SiO_2$ surface are necessary to obtain the single-molecule FD curves. If the substrate surface is not flat, it will be hard to get the single-molecule FD curves with stable plateau force, and it will be difficult to calculate the real tip-sample separation. By the section analysis, we found that the roughness of used graphite and $SiO_2$ surface is in a range of 0.1-0.3 nm with a measuring area similar to SMFS experiments, and therefore is very suitable for

the SMFS measurements.

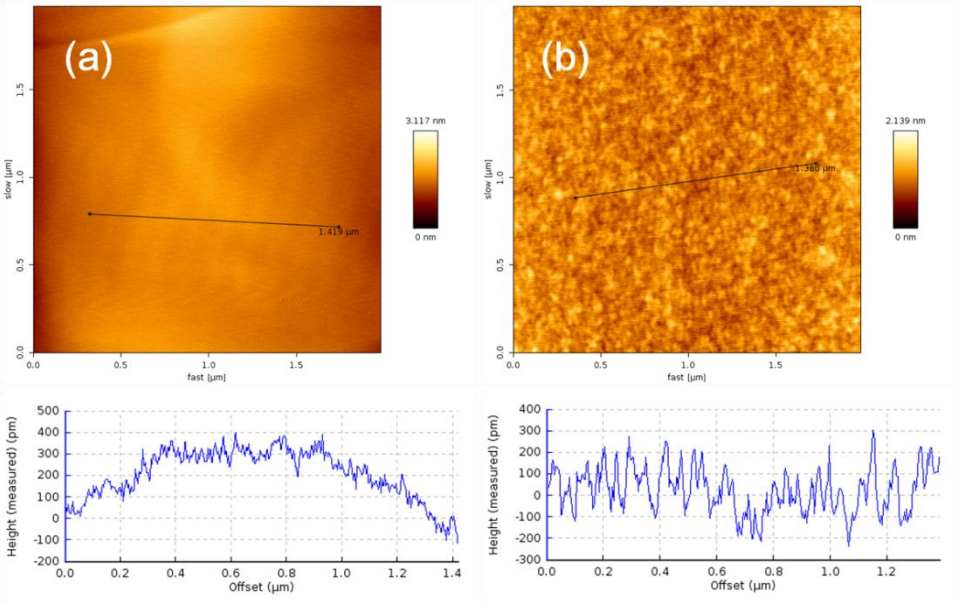



**Figure S2.** Typical AFM height images of (a) graphite and (b) $SiO_2$ surfaces (2×2 $\mu m^2$).

### 1.3 Single-molecule force spectroscopy experiments

For the SMFS measurements, cantilevers with a typical force constant of 0.32 nN/nm were used. All force-distance (FD) curves were acquired on a NanoWizard 3 NanoScience atomic force microscope (JPK Instruments AG, Germany) in liquid cell with the "Force Spectroscopy" or "Force Mapping" modes. For the Force Mapping mode, each data set was comprised of 256 (16×16) individual FD curves taken over a (2×2) $\mu m^2$ area.

Typical parameters for the SMFS measurements were: 1) Z-length: 0.4 µm, 2) peeling speed of 0.5 µm/s and corresponding loading rate of $1.6\times10^5$ N/s, 3) extend time: 0.8 s, and 4) delay time on substrate: 1 s. The aim of this 1 s delay is to favour the interaction of the biomolecules attached to the AFM probe with the graphite or $SiO_2$ surfaces. For the statistical analysis of rupture forces of biomolecules from substrates, at least 3 FD curve sets were measured for each sample, using different cantilevers.

Based on the report by Noy et al.,[3] we set several criteria for selecting satisfying FD curves for the statistical analysis of DNA/graphite rupture forces: 1) the maximum local force fluctuation was less than 50 pN, 2) the maximum plateau slope was less than 25 pN/nm, 3) the

plateau length had to exceed 2 nm, and 4) the tip-sample separation was in the range of 80%-120% of the theoretical length of DNA plus linker. All the data were analyzed with the JPK SPM Data processing (Version 4.2.27).

### 1.4 The effect of salt concentration on the rupture force

This control experiment is performed to identify the effect of salt concentration on the rupture force of the D1 DNA sequence from graphite. All FD curves were collected in a liquid cell with the same experimental conditions as introduced above. The same D1-modified AFM probe was used to measure the rupture force at NaCl concentrations of 0, 20, 50, and 150 mM, respectively. For statistical analysis, three AFM probes with roughly the same spring constant were used to get average rupture forces and standard deviations. The SMFS experiments indicate that the rupture force decreases with increasing salt concentration, as shown in **Figure S3**.

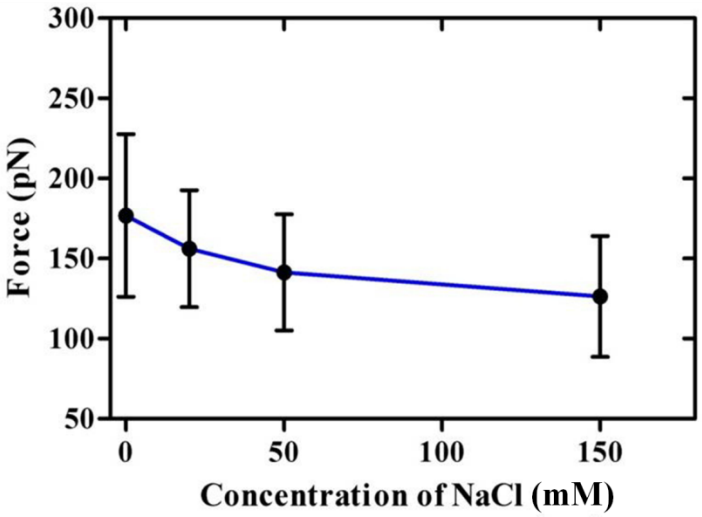



**Figure S3.** Average detachment force of ssDNA (D1 sequence) from graphite as a function of the NaCl concentration.

### 1.5 Single-molecule FD curve between D2 and graphite

The detection of Lyz was performed using a $SiO_2$ surface as the substrate (see main text). The reason for choosing $SiO_2$ rather than graphite (as in the case of DNA) was the too large

interaction force between the anti-Lyz aptamer sequence D2 and the graphite surface. This can be seen from a typical FD curve measured between D2-modified probes and graphite (**Figure S4)**. The detachment force of about 500 pN is larger than the one related to D1 and other ssDNA sequences, probably because of the folded structure of the aptamer.[3,4]

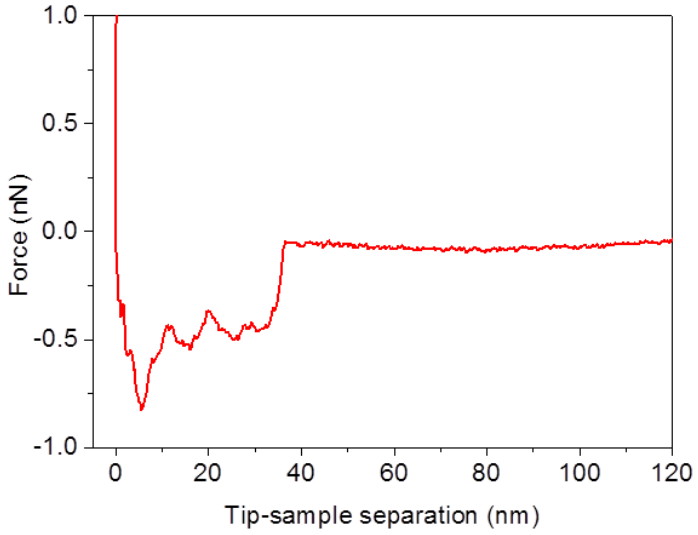



**Figure S4.** FD curve (retract trace) of a D2-modified AFM probe from the graphite surface.

### 1.6 S-S bonds breaking experiment of Lyz

In the single-molecule force spectroscopy experiments of Lyz with the $SiO_2$ surface (see presented FD curves in **Figure 3a** in the main text and in **Figure S5**), we found a characteristic FD fingerprint with multiple force peaks of about 500 pN. Noting that these force values are considerably higher than typical forces required to unfold protein modules (of the order of 100 pN or less[5]), we suggest that the multiple peaks may be caused by the break of intramolecular S-S bonds of the Lyz molecule. To test this hypothesis, we first cleaved the Lyz S-S bonds by reduction with 1,4-DL-dithiothreitol (DTT) and then carried out single-molecule force spectroscopy measurements.[6] In brief, Lyz-functionalized AFM probes were immersed into a DTT aqueous solution (10 mM) for 60 min and then used for force spectroscopy measurements. After the treatment with DTT, we obtained typical FD curves as shown in **Figure S6**. It is

interesting that the strong peaks shown in Figure S5 disappear. The maximum tip-sample separation is in both cases (without or with DTT treatment) about 40-50 nm, which agrees well with the theoretical contour length of the entire Lyz sequence (130 amino acids).

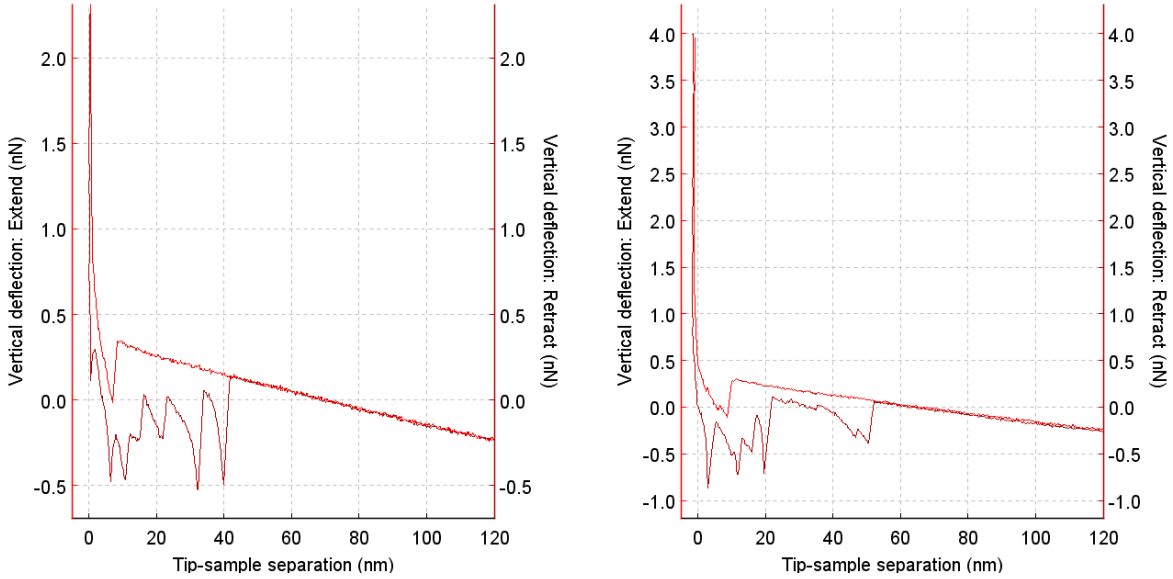
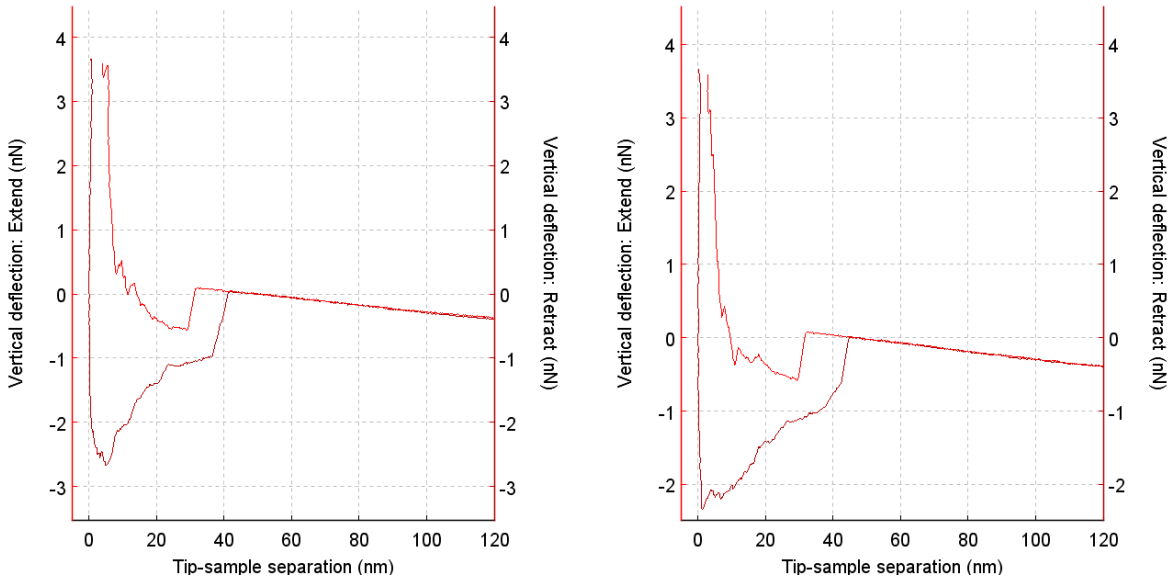



**Figure S5.** Two typical FD curves of Lyz-functionalized AFM probes against a $SiO_2$ surface *prior* to DTT treatment (i.e. with all four intramolecular S-S bonds intact before the experiment).



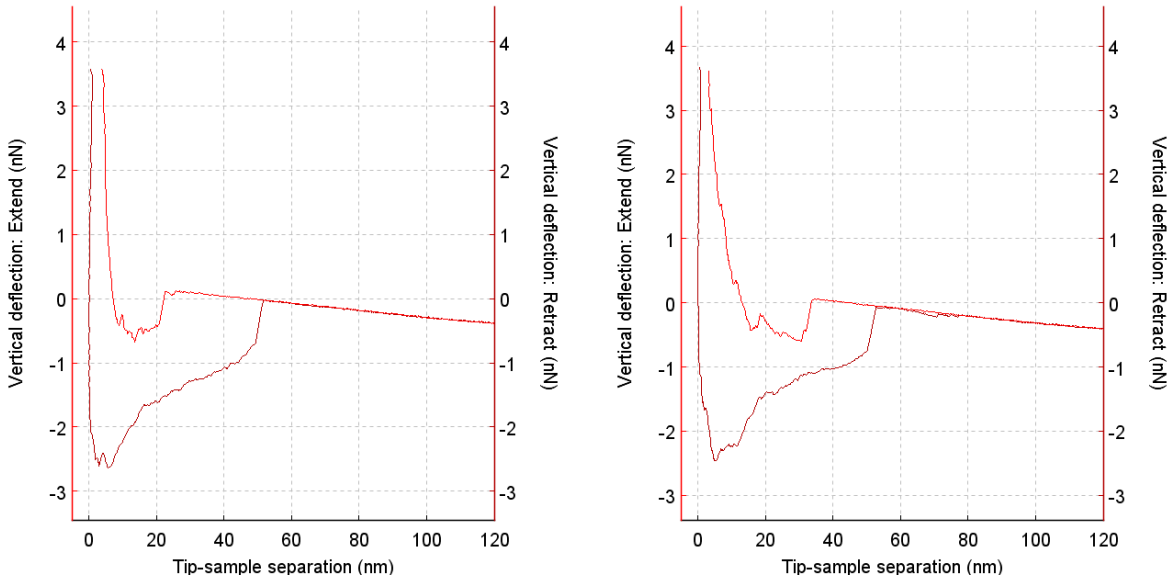



**Figure S6.** Four typical FD curves of Lyz-modified AFM probes against a $SiO_2$ surface *after* DTT treatment (i.e. with cleaved S-S bonds before the SMFS experiment).

### 1.7 Molecular dynamics simulations of stretching and unfolding of Lyz

Results of steered molecular dynamics (MD) simulations of the force-induced unfolding of Lyz in an implicit (Generalized Born) solvent model are presented in **Figure S7.** The simulations have been performed with the AMBER12 program package.[7] An harmonic restraint has been set on the distance between the alpha carbon of the N-terminal end (which is covalently attached to the AFM probe in the SMFS experiments) and the alpha carbon of amino acid 101 in the sequence (chosen as one of the possible anchor points to the surface). In the course of the simulation, the restrained distance has been increased at a constant pulling rate $v_0$=1 m/s to mimic the AFM probe retraction and collect a force-displacement curve.

During the stretching, the opening or unfolding of secondary structure motifs (α-helices and β-sheets) gives rise to only small and broad force peaks which can hardly be distinguished from the background noise (e.g. after 5.5 nm in **Figure S7**). A first strong peak in the force distance curve (at 10.5 nm stretching distance) appears when the stretching force causes evident strain of a first disulfide (S-S) bond in the protein structure (CYS6-CYS127), as visible in the molecular

picture. After manual breaking of this bond and saturation of the S atoms with hydrogen, the pulling protocol has been continued. This manual breaking leads to sudden relaxation of the stretched structure within a time scale of a few ps. After 14.5 nm, a second strong force peak revealed the stretching of a second S-S bond in the sequence (CYS30-CYS115).

In summary, subsequent stretching and (manual) S-S reduction steps lead to a force-distance curve which closely resembles the fingerprints obtained in the SMFS experiments (Figure 3(a) and (b) in the main text, and Figure S5). We note that a quantitative comparison of the force values is not possible at this stage, given that we have limited ourselves to implicit-solvent simulations and modeled the anchoring to the surface via a single amino acids (rather than including an extended surface model), in order to keep the computational time within reasonable limits. Future investigations will address these issues, but go beyond the scope of the present work.

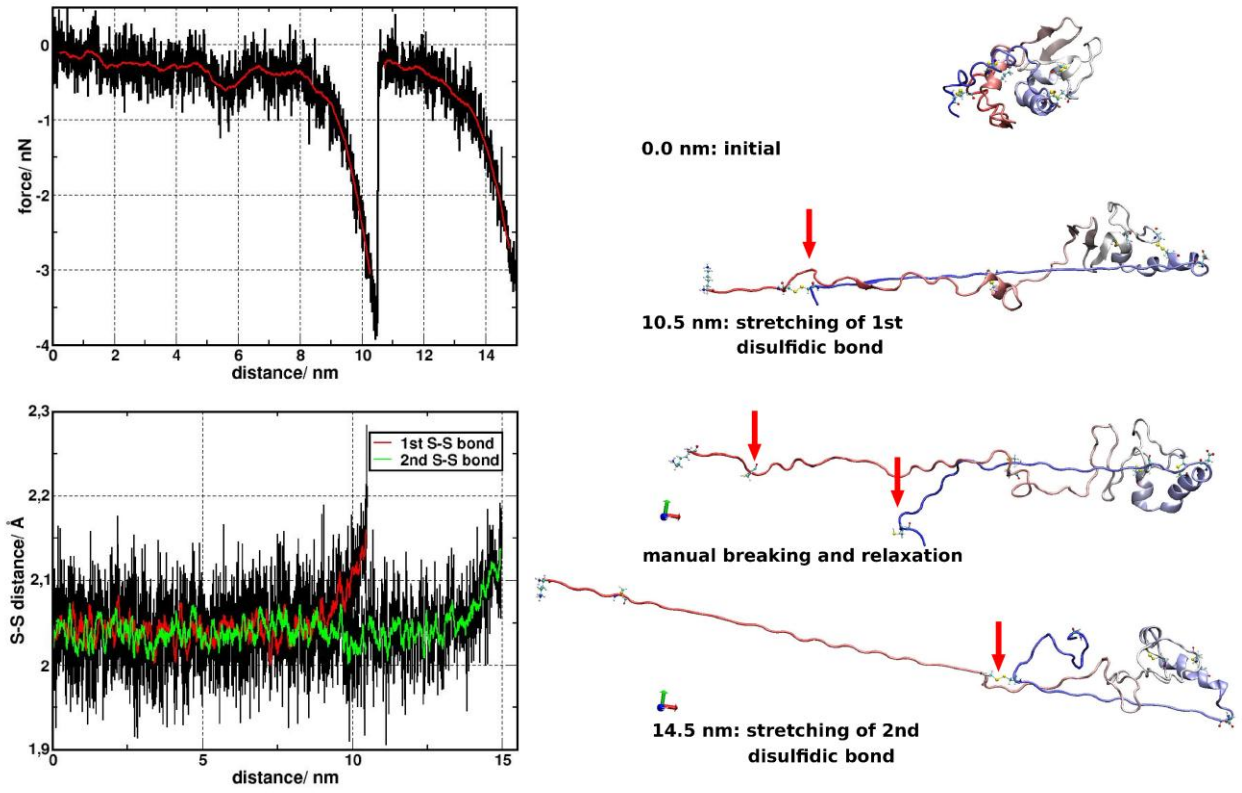


**Figure S7.** (*top left*): force distance curve of one of the possible unfolding pathways of Lyz obtained in a steered MD simulation. The black line shows the force values extracted every ps; the red line is a running average of the computed values every 100 ps. (*bottom left*): evolution of the S-S distances of two S-S bonds in the Lyz structure during the same steered MD simulation, as a function of the stretching length. (*right*): snapshots of the structure of Lyz at significant points during the unfolding. The protein is shown in a cartoon model with an index-based color scheme (red: N-term, blue: C-term). The amino acids to which the distance restraint is applied (LYS 1 and ASP 101) and all cysteine residues are shown as ball and stick models with a name-coloring scheme.

### 1.8 References

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