
P16 – Mechanical Unfolding of Proteins Using a Novel Laser-Feedback Controlled Cantilever

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Single molecule force spectroscopy using the Atomic Force Microscope (AFM) can yield important information on the behaviour of single molecules to force. An example of this is the mechanical unfolding of proteins using the AFM in a constant velocity setup. By unfolding a concatenated protein at differing velocities a dynamic force spectra can be built up which allows reconstruction of the energy landscape that the protein traverses during unfolding. This procedure has led to new insights into the determinants of mechanical strength. However, certain factors limit the information that can be gathered by this technique.

In a constant velocity experiment a protein is tethered between a substrate and the AFM tip, and the deflection of the cantilever is used as a measure of the force. The use of a deflecting cantilever is non-ideal for a number of reasons. Firstly, due to the deflecting cantilever the rate at which force is loaded onto protein is non-uniform and the maximum loading rate is limited by the soft AFM cantilever. This limits the dynamic range over which dynamic force spectra can be measured. Secondly, after unfolding of a protein the deflected cantilever recoils, during this time little information can be gained about the dynamics of the protein. Thirdly, the thermal fluctuations of soft cantilevers limit the force resolution.

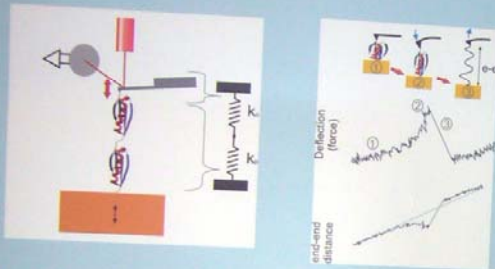
To circumvent these issues a force sensor has been developed that does not rely on the deflection of the cantilever. Instead a secondary laser is used to control the deflection of a cantilever. When the secondary laser is controlled by a feedback loop the deflection of the cantilever can be 'locked', and the laser power required to keep the cantilever locked now becomes a measure of the force acting on the lever. This setup has been applied to constant velocity experiments using Protein L and has revealed features in the energy landscape not detectable in conventional experiments. Additionally new features attributable to refolding intermediates are observed immediately after unfolding.

Mechanically unfolding of protein L with a novel laser-feedback controlled cantilever.

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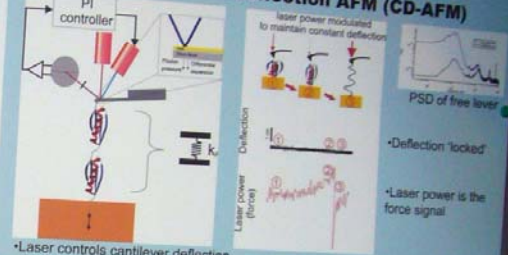
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The problem – constant velocity AFM



- ① Thermal noise of soft cantilevers - limits force resolution and perturbs protein
- ② Compliant cantilever deflects during unfolding – limits maximum loading rate
Compliance of cantilever (and protein construct) leads to non-uniform loading rate
- ③ Recoil of cantilever after unfolding masks important events immediately after unfolding

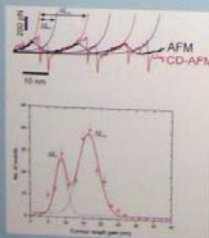
The solution – a laser-feedback controlled cantilever - constant deflection AFM (CD-AFM)



- Laser controls cantilever deflection
 - Feedback loop maintains constant deflection
- ① Thermal noise effectively damped to ~ 1 pN by feedback control
 - ② Cantilever compliance removed – higher loading rates achievable
More uniform loading rate, although protein construct still has an effect
 - ③ No cantilever recoil after unfolding - complete information in region after unfolding

The results – protein L

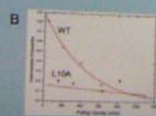
Lack of cantilever recoil allows formation of a folding intermediate



- AFM – single ΔL (~18 nm) consistent with complete unfolding of protein L (not shown).
- CD-AFM – bimodal ΔL distribution
 - ΔL_1 consistent with complete unfolding of protein L
 - ΔL_2 two possibilities;

1. Unfolding intermediate - Metastable intermediate persists after the first unfolding event
2. Folding intermediate - formation of partially refolded intermediate in the period of low force due to the lack of cantilever recoil in CD-AFM

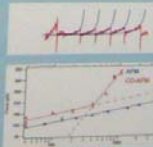
A
Relaxation phase of AFM trace shows no unfolding intermediate even at high bandwidth



B
• Intermediate probability - chance of observing intermediate after each unfolding event.
• For wild type (WT) intermediate probability decays exponentially with pulling velocity, reflecting less time to form partially folded intermediate?
• Variant L10A - has identical unfolding characteristics (i.e. unfolding force) but much slower folding (i.e. $k_f \sim 4$ fold lower).
• Variant has much reduced intermediate probability

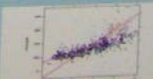
⇒ folding intermediate

Improved dynamic range reveals inner barrier at high loading rates

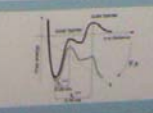


Unfolding force measured at various pulling velocities

- F vs ln(pulling velocity)
1. AFM - single gradient
 2. CD-AFM - a second steeper gradient
 3. AFM and CD-AFM data offset by ~30 pN



- F vs ln(loading rate)
1. Offset disappears – consequence of compliant cantilever
 2. Steeper gradient persists in CD-AFM



- Force acts to tilt unfolding energy landscape by $F \cdot x$
- At higher loading rates landscape is tilted to the extent that inner barrier is exposed
- Observed as steeper gradient in CD-AFM results

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