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Tracking unfolding and refolding reactions of single proteins using atomic force microscopy methods

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ABSTRACT

During the last two decades single-molecule manipulation techniques such as atomic force microscopy (AFM) has risen to prominence through their unique capacity to provide fundamental information on the structure and function of biomolecules. Here we describe the use of single-molecule AFM to track protein unfolding and refolding pathways, enzymatic catalysis and the effects of osmolytes and chaperones on protein stability and folding. We will outline the principles of operation for two different AFM pulling techniques: length clamp and force-clamp and discuss prominent applications. We provide protocols for the construction of polyproteins which are amenable for AFM experiments, the preparation of different coverslips, choice and calibration of AFM cantilevers. We also discuss the selection criteria for AFM recordings, the calibration of AFM cantilevers, protein sample preparations and analysis of the obtained data.

1. Introduction

Since its invention in 1986 [1], the AFM has evolved from being a resolution imaging tool to a versatile technique used to manipulate and detect single molecules [2–9] as well as to measure the interaction forces between single proteins [10–12]. Single-molecule techniques harness several different disciplines of science (biology, physics, chemistry, material science and computer science) allowing better analysis and understanding of protein function with unprecedented resolution. Here we will discuss the study of the unfolding and refolding pathways using single molecule force spectroscopy (SMFS) techniques. This technique was developed during the 1990s and allows doing experiments at the single-molecule level under physiological relevant conditions. One of the main advantages of this technique is that it allows tracking the structural dynamics during protein folding reactions and can capture transient folding intermediates and misfolded states that cannot be identified by bulk studies. Another advantage of single-molecule AFM is the relatively easy sample preparation. One of the most important applications of SMFS has been done in the field of protein folding/unfolding [2–5,7,8,13–29]. SMFS has been also used to investigate enzyme catalysis as well as the effects of osmolytes and molecular chaperones on protein folding [30–33]. In this paper, we describe SMFS operating principles, practical implementation and we will discuss a few noteworthy examples.

2. Basic principles of single-molecule force spectroscopy methods

2.1. SMFS instrumentation

The AFM is composed of two main parts: a XYZ stage scanner (sometimes separated into Z and XY stages) and an optical head (Fig. 1A). A small cantilever plays the role of microscopic force sensor, a thin and flexible piece of silicon (about 200 μm in length and 10 μm in thickness) that ends with a small pyramid-shaped stylus. A laser beam is shined on the back of the cantilever in order to track the cantilever bending when it contacts the sample (Fig. 1). The force is measured using Hooke's law, $F = k_c \Delta x$, where Δx represents the cantilever deflection, k_c spring constant (stiffness of the cantilever). The optical signal bouncing off the end of the cantilever is then amplified in such a way that a small bending of the cantilever, of only a few nanometers, results in a large change in the photovoltage of the detector. The SMFS method can measure forces in range of 1 to more than 1000 piconewtons and changes in the length of proteins with nanometer and millisecond resolution [3,4,24,26,29,34]. The most common SMFS modes are the length-clamp (Fig. 2A), which yields a force–extension curve and the force-clamp, which gives an extension–time curve (Fig. 2C).

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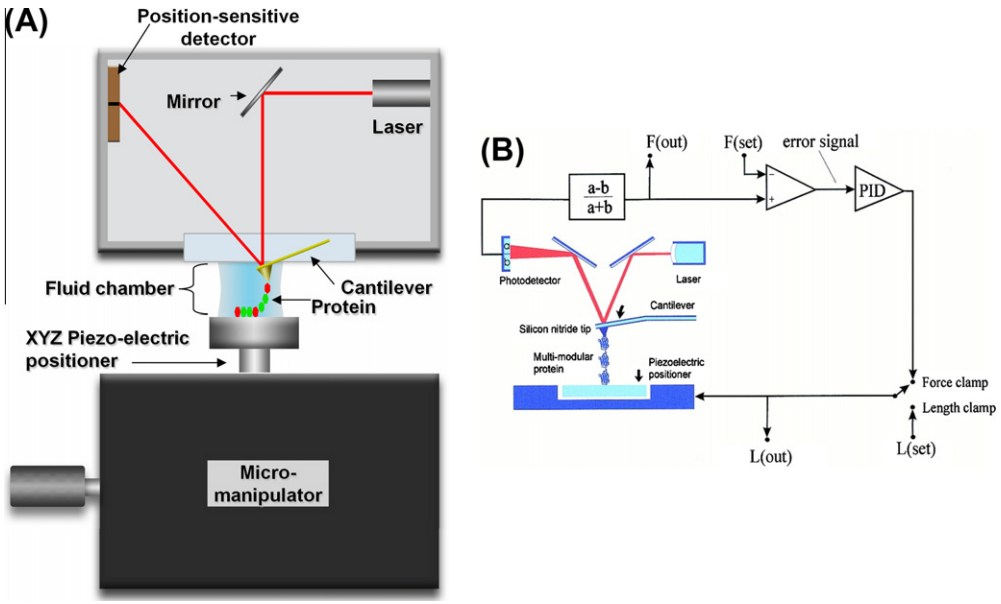

Fig. 1. Schematic diagram of the AFM apparatus and associated control electronics. (A) The AFM consists of two main parts: the scanner (micromanipulator) and an optical head. The center point of the system is a small cantilever that functions as a microscopic spring. The cantilever is brought into contact with the sample and its bending is detected by shining a laser on its back; the light bounces off and is captured by a split photodetector (split into two regions: "a" and "b" photo-signals). A tiny deformation of a few nanometers causes a large alteration in the photovoltage of the detector due to optical amplification of the signal. The photovoltage is then converted into a force signal. The AFM is very sensitive it can measure forces in the range of pico-newtons and distances of only few angstroms. (B) Two modes can be used to stretch single molecule: length-clamp or force-clamp modes. The standard length-clamp mode allows the control of the position (L) and measurement of the resulting force (F) which is calculated from the laser deflection $(a-b)/(a+b)$. The force-clamp mode measures force and then compare it with a set value thus generating an error signal that is fed to a proportional, integral and differential amplifier (PID) whose output is connected directly to the piezoelectric positioner. Reproduced with permission from [76].

2.2. Length-clamp mode

By moving the sample away from the AFM tip, a stretching force is applied to the protein. The length-clamp (or constant velocity) mode records force–extension curves obtained by pulling a single protein in the z axis (Fig. 2B). The interpretation of force–extension curves is not always straightforward. The recorded force peaks can originate from many sources which include not only the unfolding of protein domains but also detachment of other molecules from any of the two anchoring points or disentanglement of molecules. This problem was solved by using native multi-domain proteins (such as titin, tenascin or spectrin) [4,35,36] or recombinant polyproteins [37–40]. For multi-domain and polyproteins the recorded force–extension curve resembles "sawtooth" pattern which represents the sequential unfolding of individual domains. This periodicity allows unequivocal identification of single molecules. The typically forces required to unfold single proteins are in the range of 50–500 pN (at pulling speeds of about 1 μm/s) [2].

2.3. Force-clamp mode

The force-clamp mode controls the force applied to a protein through a feedback mechanism that maintains the applied force constant and quickly corrects the distance between the coverslip and the AFM tip. The force feedback is based on a proportional, integral, and differential (PID) amplifier whose output is connected directly to the piezoelectric positioner [41,42]. The time response of the feedback and piezo is critical. The frequency response of current PID amplifiers and piezoelectric positioners are limited to 5–10 ms, which in most cases is adequate to study the unfolding and refolding reactions. However, recent advances in piezos and PID amplifiers have pushed the limit in the sub-millisecond range (about 150 μs [24]). These new high-speed force clamp spectrometers allow the study of the recoil dynamics of single polypeptide chains under force [24]. The force-clamp mode allows the precise control of the end-to-end distance of the protein with nanometer resolution [13,41]. For example, when a constant stretching force is applied to a multidomain protein (such as titin) or a polyprotein, the domains unfold stochastically in an all-or-none fashion leading to a stepwise increase of the end-to-end length of the protein (Fig. 2B). Force-clamp SMFS techniques are currently being used to tackle fundamental problems in biology such as protein folding [13,43–45] and chemical mechanisms in enzyme catalysis [23,30,46–49]. Force-clamp SMFS techniques allow the direct measurement of the mechanical stability of proteins (energy barriers) and the kinetics of unfolding and refolding pathways and the location of kinetic barriers [13,41,42,46,50–54].

2.4. Preparation of surfaces – the choice of coverslips

In SMFS experiments, the protein of interest is immobilized on a substrate and then by physisorption (i.e., nonspecific adsorption) the protein is attached to the tip of cantilever therefore being caught between coverslip and cantilever. For immobilization purposes, the protein constructs are commonly engineered with terminal cysteine-tags and then adsorbed onto a gold-coated coverslip; the interaction of gold with thiol groups is quite strong and ruptures at around 1 nN [55]. If the protein of interest has solvent exposed cysteine residues, then terminal histidine tags can be used for immobilization on nickel–nitriloacetic acid (Ni–NTA) functionalized coverslips [56,57]. A potential disadvantage is that the unbinding forces between His-tag and Ni–NTA are around 50 pN and thus are lower than typical unfolding forces of Ig-like domains (>100 pN) but compatible with spectrin, ankyrin or C2 domains (they unfold at forces <50 pN).

2.4.1. Glass coverslips

Glass coverslips (12 or 15 mm diameter, 1 oz, Ted Pella, Inc.) should be cleaned by thoroughly spraying them with 70% ethanol, rinse them with MilliQ water and then sonicate them for 30 min in