Archives of Biochemistry and Biophysics 531 (2013) 116–127

Contents lists available at SciVerse ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Review

Atomic force microscopy and force spectroscopy on the assessment of protein folding and functionality

Filomena A. Carvalho [1], Ivo C. Martins [1], Nuno C. Santos *

Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal

ARTICLE INFO

Article history:
Available online 7 December 2012

Keywords:
Atomic force microscopy
Single-molecule force spectroscopy
Protein unfolding
Protein stretching, Intramolecular forces

ABSTRACT

Atomic force microscopy (AFM) applied to biological systems can, besides generating high-quality and well-resolved images, be employed to study protein folding via AFM-based force spectroscopy. This approach allowed remarkable advances in the measurement of inter- and intramolecular interaction forces with piconewton resolution. The detection of specific interaction forces between molecules based on the AFM sensitivity and the manipulation of individual molecules greatly advanced the understanding of intra-protein and protein–ligand interactions. Apart from the academic interest in the resolution of basic scientific questions, this technique has also key importance on the clarification of several biological questions of immediate biomedical relevance. Force spectroscopy is an especially appropriate technique for "mechanical proteins" that can provide crucial information on single protein molecules and/or domains. Importantly, it also has the potential of combining in a single experiment spatial and kinetic measurements. Here, the main principles of this methodology are described, after which the ability to measure interactions at the single-molecule level is discussed, in the context of relevant protein-folding examples. We intend to demonstrate the potential of AFM-based force spectroscopy in the study of protein folding, especially since this technique is able to circumvent some of the difficulties typically encountered in classical thermal/chemical denaturation studies.

Introduction

Proteins acquire their unique functions through specific folding of their polypeptide chain(s). The investigation of their stability or resistance to unfolding is commonly performed by chemical or thermal denaturation [1–3]. However, commonly proteins also respond to the application of an external force to maintain their native structure. As such, mechanical forces can also be employed for protein folding/unfolding studies in a number of different proteins. In fact, mechanical forces are centrally involved in many basic protein-mediated physiological processes. To study the mechanical behavior of these proteins, it is important to use techniques able to detect forces and distances at the single-molecule level, a task for which atomic force microscopy (AFM[2]) is particularly well-suited. This technique has emerged as one of the most versatile and easy to handle for this type of approach.

The concept of a succession of "folding waterfalls", in which we can think of the progression of folded states as the jumps in the water from one waterfall to the next, is a more adequate description for how we can approach the problem of protein folding via AFM and is depicted in the graphical abstract. Thinking in the folded protein as being in a stable level in the succession of waterfalls, AFM allows for the physical pulling of the protein from a lower level to an upper level, allowing the study of intermediate states.

* Corresponding author. Address: Unidade de Biomembranas, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisbon, Portugal. Fax: +351 217999477.
E-mail address: nsantos@fm.ul.pt (N.C. Santos).

[1] Both authors have equally contributed to this work.

[2] *Abbreviations used:* AFM, atomic force microscopy; APTES, 3-aminopropyl triethoxysilane; BCX, β-1,4-endoxylanase from *Bacillus circulans*; DNA, deoxyribonucleic acid; DTT, dithiothreitol; HBHA, heparin-binding hemagglutinin; I1, I1 immunoglobulin domain module of the elastic I-band of titin; I27, I27 immunoglobulin domain module of the elastic I-band of titin; NhaA, *Escherichia coli* Na^+/H^+ antiporter; NVLP, Norwalk virus-like particles; SMD, steered molecular dynamics; SAM, self-assembled monolayer; SMFS, single-molecule force spectroscopy; PEG, polyethylene glycol; TMV, tobacco mosaic virus; TNfnALL, recombinant tenascin fragment; WLC, worm-like chain.

0003-9861/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.abb.2012.11.007

Furthermore, this mechanical movement in the folding pathway allows for, if desired, the protein to be maintained in a specific conformation (as if water would be frozen in a specific point of the waterfall), allowing for a controlled study of the protein folding and of specific protein structures otherwise not easily detectable by conventional methods. Before proceeding on this topic, a short introduction to certain aspects of protein folding and structure is important to our discussion of the use of AFM in folding/unfolding studies.

Protein folding

Protein folding, although thoroughly researched, is not yet fully understood [2–10], with its mechanism(s) remaining one of the most elusive problems in biology [4–12]. It is thus not the aim of this article to provide a detailed description of the research in the field of protein folding. Rather, it is to discuss AFM-based methodologies in the context of protein folding studies.

Protein folding can be defined as the self-organization of proteins into three-dimensional units, frequently under the eventual coordinated action of chaperones [2,3,11]. It is generally accepted that the progress of polypeptide chains into their native three-dimensional structure is determined by the primary amino acid sequence [2,3,11], as originally proposed in the Anfinsen's dogma [12,13]. It is also this primary amino acid sequence that promotes the characteristic secondary structure and determines the progress into the classical tertiary structure. Different secondary structural elements exist. The most prevalent are the α-helix and β-sheet organizations [11], which were predicted from essentially theoretical consideration [14–17], before the first protein structures were known [18–20]. Random coil (which designates the lack of a characteristic regular organized structure) and β-turns (which connect adjacent domains) are also commonly found [2–11].

Protein folding is an extremely fast process that therefore cannot possibly depend on a purely random-search mechanism, as stated by the Levinthal's paradox [21]. All possible conformations can not be explored, given the extremely limited time span of the protein folding process [21]. As such, a sequential and highly cooperative reaction is believed to take place, in which a certain polypeptide chain under a given condition has a restricted number of thermodynamically available conformations and progresses from one to the next [4,5,9,21–23].

In the final rearrangement of the primary amino acid residues sequence of soluble proteins into the typical tridimensional structure, hydrophobic residues are generally buried inside the protein, while the more hydrophilic portions of the protein are exposed to the solvent. Therefore, as proteins fold, solvent–protein interactions are normally broken and substituted by intra-molecular interactions [24,25]. The interaction of the hydrophobic side chains between themselves is highly favorable in an aqueous environment [26]. Based on these considerations, the folding of a number of individual proteins has been successfully explained [23,27,28]. However, up to now, it is not fully understood how the process of protein folding occurs (*i.e.*, how from a specific amino acid sequence a given stable three-dimensional structure is formed). Some suggestions on the possible mechanism of protein folding have been put forward, namely the framework [29–31], the molten-globule [32] and the nucleation–condensation, which presents the former two models as extreme variations of a nucleation–condensation mechanism [3,33,34].

Integrating current understanding of protein folding, its mechanism can be illustrated in energetic terms as a folding funnel [4–6,10,22,23,35–37], such as the represented in Fig. 1. This concept represented a theoretical breakthrough in the understanding of protein folding [4,5,10,37]. In short, the funnel concept states that as the protein folding reaction proceeds, the entropy of the polypeptide chain (and the surrounding solvent) decreases as more intricate and compact structures are formed that are richer in intra-molecular interactions and thus have lower entropy. In this model, the rough surfaces with local small energy wells correspond to local energy traps in which transiently stable conformations accumulate, with the deeper energy minima promoting the forma-

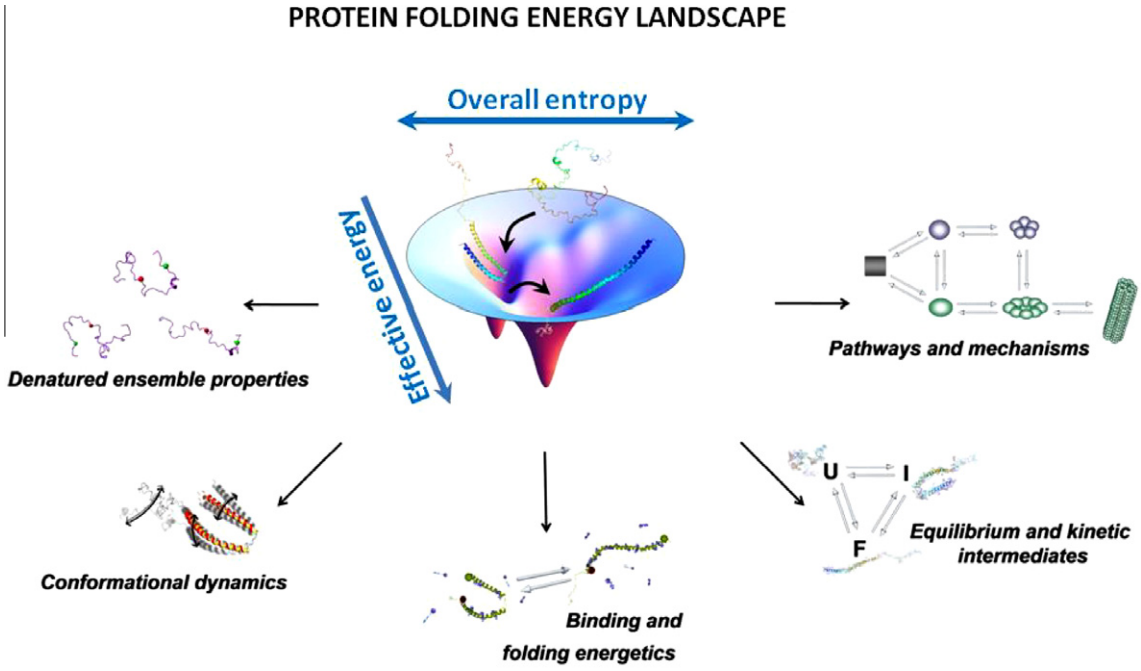

Fig. 1. Current understanding of the protein folding funnel concept. Protein folding is governed by the search of energetically favorable conformations (U, I and F stand for unfolded, intermediate and folded protein structures, respectively). Folding/unfolding classical thermal and chemical denaturation studies have been essential to understand a number of protein-mediated biological processes. However, novel mechanical-based approaches, such as AFM based force spectroscopy, are required to progress further in the understanding of protein folding. Adapted from reference [35].

tion of more stable intermediate conformations [4,5,10,23,37]. However, in spite of this wealth of information, a detailed understanding of the protein folding process demands additional experimental data. The use of AFM-based single-molecule evaluation of proteins would certainly further benefit our understanding of their folding mechanisms.

Atomic force microscopy

Atomic force microscopy belongs to a family of microscopy methodologies based on the scanning of surfaces, collectively named scanning probe microscopy. It measures the interaction force between a sharp tip and a surface. The tip may be dragged across the surface, or may vibrate as it moves along it. The interaction force depends on the nature of the sample, the probe tip and the distance between them. The AFM not only allows the construction of images of surfaces but also the study of molecular interactions and their mechanical forces [38].

Topographical imaging involves scanning or tapping the tip mounted under a flexible cantilever across the surface (Fig. 2). A laser beam is reflected off the back of the cantilever, and small changes on the deflection of the cantilever are detected with a position-sensitive photodiode. This photodiode commonly has four quadrants, with the laser initially pointed at the center of them. Upon the scanning of the sample, any small deflection of the cantilever will change the position of the reflected laser, distinguishing between vertical and lateral deflections of the tip. The system electronics processes the deflection signal and determines the topological height changes on the surface of the sample [39].

AFM has the capacity of imaging, in air or in liquid, non-conductive and conductive surfaces beyond the light diffraction limit, reaching atomic and molecular resolution. Lateral resolution is limited by the particular sample conditions required but it may go from, roughly, 1 nm (and in some settings even bellow that) to molecular scale up sizes of around 100 μm (depending upon the resolution of the XYZ scanner stage) [40]. It is thus a powerful tool, as it is able to measure a large range of orders of magnitude. The vertical resolution limit goes to subatomic resolution (<1 Å). Detailed aspects of the AFM instrumentation can be found at several books and review articles [40–44].

AFM has been developed mainly as an imaging technique [44]. Nevertheless, the AFM can also be used to quantify the interaction between the tip and a specific spot of the sample, taking advantage of the piconewton sensitivity of the method. This approach is usually termed "force spectroscopy" (even not being in fact a spectroscopy, as it is not based on the interaction of radiation with matter).

AFM-based force spectroscopy

AFM-based force spectroscopy allows the measurement of inter- and intramolecular interaction forces required to separate the tip from the sample, with piconewton resolution. Due to their force detection sensitivity and spatial resolution, AFM enables the detection of specific interaction forces at the single-molecule level, having been used for that purpose in the study of interactions between proteins and/or peptides and their ligands [45–50]. In force spectroscopy measurements, it is not mandatory for the tip to scan the sample surface, as it may be just brought into contact with the surface and retracted. In this mode, the cantilever moves in the vertical direction (z axis), toward the surface, and then in the opposite direction. During this procedure, the cantilever deflection can be recorded as a function of the vertical displacement of the piezoscanner. The result is a cantilever deflection *vs.* scanner displacement curve, which can be converted into a force–distance curve (Fig. 3) by applying the Hookes's law of elasticity:

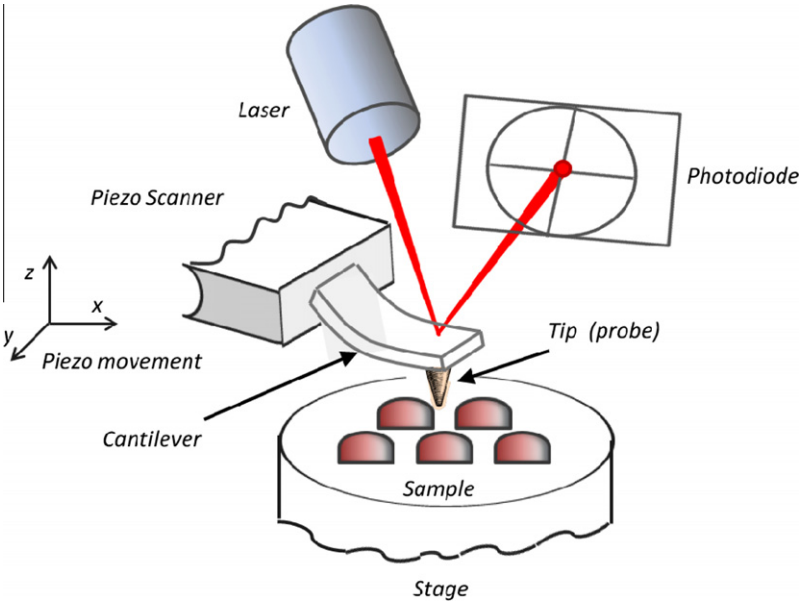

Fig. 2. Scheme of an atomic force microscope. The main components of an atomic force microscope are the microscope stage, the electronics controller and a computer. The microscope stage is constituted by the XYZ scanner (which has the responsibility of moving the AFM tip relative to the sample, or the sample relative to the tip, depending on the AFM model), sample holder and force sensor. AFM stages, especially those for using with biological samples, usually include an integrated optical microscope to view the sample and tip. All these components are supported onto a vibration isolation platform, which reduces the background noise and increases the resolution of the measurements. All the electronic components of the AFM are usually in a modulus interfacing the microscope stage and the computer. The electronics are used to generate the signals that drive the XYZ scanner at the microscope stage, as well as to digitalize the signals coming from the AFM, which can then be simultaneously displayed and recorded by the attached computer. According to the parameters set via the computer, the feedback signals coming out and going back into the AFM stage can be controlled by the electronic box. Specific computer software is used to acquire the AFM data. Usually, the computer also has software to process and analyze the obtained images. The feedback controller feeds the signal from the force transducer back into the piezoelectric, in order to maintain a constant force between the sample and the tip when necessary. Adapted from reference [39].

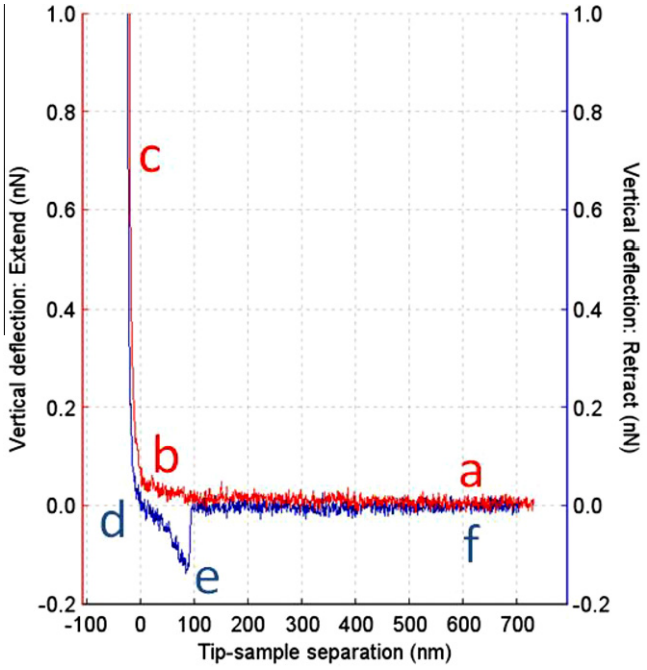

Fig. 3. Typical force–distance curve obtained by AFM-based force spectroscopy, showing a single adhesion event. The red line is the approach curve and the blue line is the retraction curve. The cycle begins with the tip away from the cell surface (a), on its neutral position (0 pN), from which it starts moving down towards the surface, reaching the contact point (b), where the cantilever starts bending upwards, and indents through the surface until a certain defined trigger force is reached and the movement of the tip stops (c). There is a waiting time before starting the retraction curve. Afterwards, the tip and cantilever begin the upward movements away from the sample, in the opposite direction, reaching the contact/adhesion point (d), where the force is again 0 pN. If no binding adhesion occurs between the molecules attached on the tip and the cell surface, the tip continues its upward movements, at 0 pN of force, and reaches back the neutral position at a defined z distance point. If a bond is formed between the tip and the sample, as the cantilever moves upward, it bends down to negative values in force (e). In some surface distance point, the bond breaks and the cantilever jumps back to its neutral position (f). This completes the force–distance cycle. Adapted from reference [39].

$$F = -k\Delta x \quad (1)$$

where F is the force, k the spring constant of the cantilever and Δx the length of the deflection of the cantilever. force–distance curves can be recorded either choosing single well-defined points of the x, y image or at multiple locations, which can be used to generate a "force–volume" image. The design of a force–distance curve depends on the tip, sample and medium composition. The curves obtained for samples in air vary from those obtained in liquid medium. In air, as the cantilever approaches the surface, the initial forces are too small to achieve a deflection and therefore it remains in the same position on the ordinate axis. At some point, the attractive forces (van der Waals and capillary forces) exceed the value of the spring force of the cantilever and the tip deflects, jumping into contact with the surface. After the contact point is established, as the tip continues to be pressed on the sample, both the deflection of the cantilever and the repulsive contact force increase. Afterwards, when the tip begins to retract from the sample, it often remains in contact with the surface. At some point, the upwards movement of the cantilever will overcome the tip-sample interaction force and this bond is broken, releasing the tip from the sample.

The curves in liquid medium follow the same principle as described above for air, although the approach curve has a different profile. It presents a gradual increase in strength and, thus, it is more difficult to establish the point where the tip and sample come into contact, since the initial compression of the surface causes a minimum deflection in the cantilever. When the tip is removed from the surface by the repulsive forces, there is a delay on the response of the system, due to the elasticity of the sample and/or long–range forces [51,52].

Different types of forces can be studied using AFM-based force spectroscopy, both attractive and repulsive. Van der Waals interactions are the main type of force present upon the approaching of two hard surfaces in the absence of long–range interaction [53]. If the approach curve has a smooth and exponentially increasing repulsive force, electrostatic or polymer-brush forces may be present [51,52]. On AFM force curves, some material properties of samples can be investigated by relating the applied force with the depth of indentation as the tip is pressed against the sample. Viscoelastic properties of some biological systems have been determined using AFM force curves, including cartilage, gelatin gels, glial cells and epithelial cells (for review, see [54]).

The central basis of adhesion forces, on the retraction curve, is the development of a capillary bridge between tip and sample. This capillary force varies if the measurements are made in air or in liquid [53]. When a polymer connects the tip to the surface, a negative deflection of the cantilever at a given distance away from the sample is obtained. The polymer extension sets the differences on the distance of the observed deflection. The polymer is stretched until inter- or intramolecular bonds are broken and/or the polymer detaches from the tip or the surface. After that, the cantilever returns to the zero-deflection position [52,53,55]. Another type of study can be conducted when measuring the unbinding events between specific receptor–ligand pairs [55]. This binding/unbinding force is usually characterized by a stepwise deflection of the AFM cantilever, returning afterwards to the zero-deflection line. These measurements can be associated to a single (un)binding event or to the sequential unbinding of multiple receptor–ligand interactions. More difficult to interpret are the curves that combine polymer extension and adhesion events. Examples of all these types of force curves are detailed in [52].

To calculate the quantitative parameters associated to the forces shown above, polynomial functions are commonly used [52]. For instance, in polymer extension studies there are several models that can be applied, such as the worm-like chain (WLC) model [56] or the freely-joined chain model [57]. To quantitatively analyze the force curves, some specific requirements are also necessary, such as the accurate calibration of the spring constant of the cantilevers used. Cantilever stiffness depends on its shape and on its material properties [58]. The most commonly used calibration method is the thermal noise method, introduced in 1993 by Hutter et al. [59].

Single-molecule force spectroscopy

Single-molecule force spectroscopy (SMFS) is an extremely powerful tool for detecting and localizing single molecular recognition events, and for exploring the energy landscape of molecular interactions.

For having the best conditions of the recognition studies, tips are commonly functionalized with one or a small amount of probe molecules. These molecules can recognize a specific target molecule on the sample surface. When analyzing the stretching of biomolecules, and in order to measure specific and strong interactions between tip and sample, it is necessary to specifically attach to the tip the biomolecule under study. The attachment should be firm enough to avoid reallocations, but it should maintain some autonomy of the molecule to change its conformation during or before the interaction. In force measurements, both the tip on the cantilever and the surface can be chemically modified to form specific and strong bonds. The simplest method to attach molecules to the tip is

via their non-specific adsorption. However, the covalent attachment methods enumerated below usually yield better performances.

Tip functionalization protocols commonly use commercially available silicon nitride (Si_3N_4) or silicon tips. The most common approaches used for this starting step of tip functionalization are direct amination by silanization or "etherification" using trichlorosilane groups, to form organosilane layers (such as formation of amino-terminated monolayers on the silicon surface of the tip with 3-aminopropyl triethoxysilane (APTES)) [45,60]. The "etherification" can be also performed using ethanolamine hydrochloride. The most accepted process is the immobilization of thiol-based self-assembled monolayer (SAM) on gold-coated tips [38,61]. The second step of the functionalization protocol is the introduction of a linker/spacer group between tip and ligand of interest. These spacers facilitate the receptor–ligand recognition and the signal transduction from the recognition event to the cantilever. The most commonly used spacer is polyethylene glycol (PEG), but other structures, such as glutaraldehyde [62,63] or carbon nanotubes [64], can also be used. A high variety of heterobifunctional PEG linkers is commonly used [65]. More details about these methods can be found at some reviews [38,66].

Force spectroscopy technique has been used to study a large number of biological applications such as indentation, adhesion and unfolding studies. Cells elasticity by indentation studies, in terms of the Young's elastic modulus, can be measured by using the tip to indent the cell, via the Hertz model [67,68]. This is a very powerful tool for *in situ* characterization of interfaces of heterogeneous polymer systems and measurements of the elasticity of biological structures, on different types of cells and organelles [52,54,69].

Common examples of adhesion studies involve interactions between antibodies and antigens or other ligand–receptor pairs. Adhesive forces can be measured and mapped over the surface, with dynamic force spectroscopy studies, enabling the calculations of the thermodynamic and kinetic parameters of the binding [70,71]. Examples of this approach include measurements on different types of cells, such as endothelial cells, erythrocytes and bacteria [72,73]. In general, different ligand–receptor forces have been measured at the single-molecule level by AFM-based force spectroscopy. Examples of such experiments include avidin/streptavidin, antibodies, DNA, carbohydrates, lectins, cadherins, integrins, selectins and bacterial adhesins (for specific references see [74]). Force spectroscopy studies of adhesion complexes widened up their range of applicability when the adhesion measurements started to be done directly with a living cell immobilized on an AFM cantilever, enabling the direct measure of cell–cell interactions [69]. This methodology is particularly useful to probe adhesive properties of suspended cells, such as erythrocytes or leukocytes, allowing the characterization of multiple and single-molecule rupture events [69].

Unfolding studies

After attaching a molecule both to the tip and to the sample surface, the unfolding, stretching or adhesion of single molecules can be studied. Molecules with a long chain, such as proteins or DNA, can be stretched between the tip and the substrate surface. With this process, parameters such as stiffness, persistence length and inter- or intramolecular transitions can be studied [75].

Integrated on molecular interactions studies, SMFS has also been often applied to protein folding/unfolding studies. AFM-based protein studies are especially suited for the group of proteins commonly referred to as "mechanical proteins", a group of polypeptides and proteins that respond to force application under physiological conditions [76,77]. Protein folding/unfolding studies are usually performed on multi-modular constructs, in which protein domains serve as linkers [78–80]. There are two main advantages on using this type of AFM studies. The first one is that AFM tip functionalization processes become unnecessary to pick up and stretch one molecule at a time, as the protein serve as handles for the pressure-induced AFM tip–protein bond [76–80]. The second advantage is that different protein modules unfold one at a time. This leads to a simplification on the interpretation of the data from the force curves. Usually, these force curves have a recognizable series of peaks, each representing the unfolding of a single protein module [80]. The mechanical fingerprint of proteins constructs can be parameterized using models of the polymer elasticity theory. This theory estimates that the increase in the length (x) of a polyprotein when one domain unfolds can be fitted with the WLC model of entropic elasticity [51,76]. This model predicts that the stretch force (F) can be related to the fractional extension (x/L) of the chain by the following equation:

$$F(x) = \frac{k_B T}{A}\left(\frac{1}{4(1-x/L)^2} - \frac{1}{4} + \frac{x}{L}\right) \tag{2}$$

where A is the persistence length (a measure of the chain's bending rigidity), k_B is the Boltzmann constant, T is the absolute temperature and L is the contour length. Fitting this model to a force distance curve with successive force peaks reveals that each unfolding event increases the contour length of the polyprotein by a constant value, ΔL.

For the purpose of studying protein folding, the more common and intuitive force–extension AFM mode is frequently used. Force extension can be roughly described as pulling and extending a molecule while measuring the force applied and distance achieved. The unfolding process of proteins caused by an external mechanical force can be observed as a process in which the increasing external force lowers the activation barrier between the folded and unfolded state. Thus, the unfolded force measured is logarithmically dependent of the retraction speed and the dynamic force spectra may be plotted [71,81–83]. The faster the retraction is, the faster the unfolding process, as expectable due to the fact that force-induced unfolding has a lower transition energy barrier between the folded and the unfolded state (with thermal induced variation easily overcoming the remaining energy barrier in the timespan of the experiments) [57,81,82,84]. This allows deriving from the speed dependence of the unfolding forces a final unfolding rate constant, α, according to the equation [81,82]:

$$\alpha(F) = \alpha_0 \exp\left(\frac{F\Delta x}{K_B T}\right) \tag{3}$$

where α_0 is the unfolding rate without any external force applied and Δx is the distance to the unfolding transition state. An unfolding probability of the protein pulled at constant velocity, P_u, can then be determined as $P_u = \alpha\Delta t$, using Monte Carlo simulations (where Δt is the polling interval) [57,76,85,86].

A classical example of the use of AFM–force spectroscopy combined with Monte-Carlo simulations is the study of the mechanical properties of the first proximal Ig domain of the elastic I-band part of the muscle protein titin, which contains two modules with a known structure: I27 and I1. I1 possesses a 100 residues long β-sandwich structure with seven antiparallel β-strands [86–90]. The use of hetero-polyproteins constructs composed of four tandem repeats of I27–I1 dimer has allowed, by using information regarding the well-characterized I27 domain from human cardiac titin, to mechanically fingerprint and identify single molecule stretching events [86–90]. The classical saw-tooth pattern is seen (Fig. 4A), with each single saw-teeth corresponding to the mechanical unfolding of a single Ig domain of the stretched polyprotein

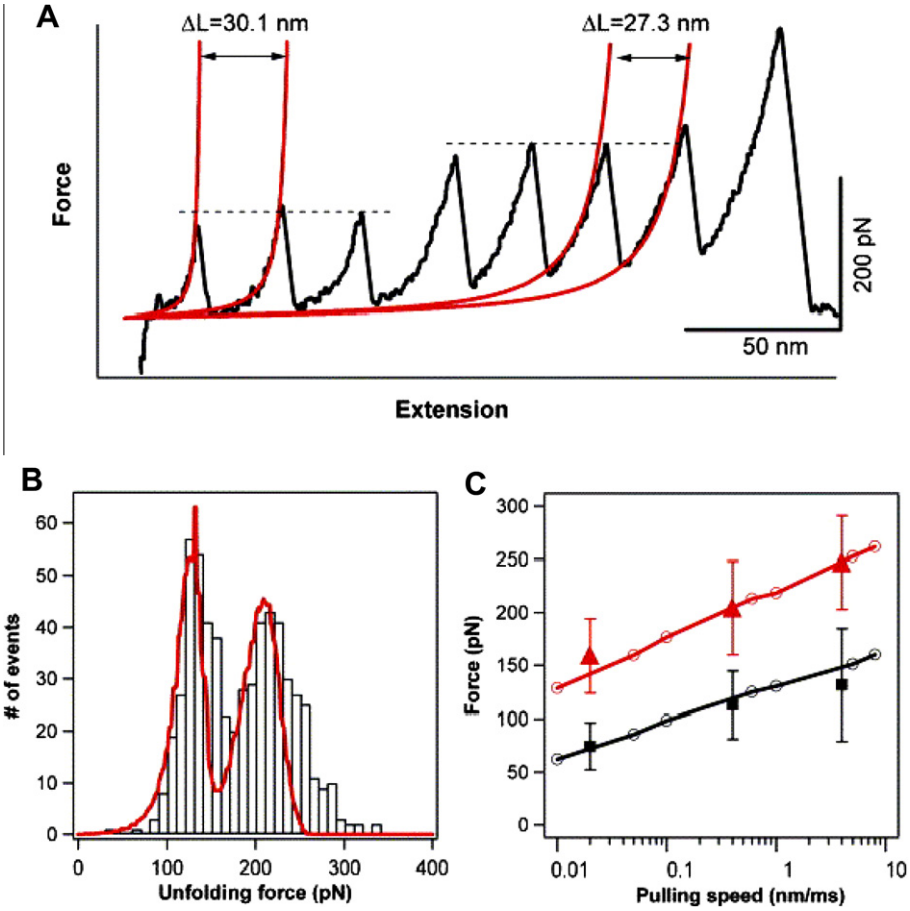

Fig. 4. Studies of titin using AFM force spectroscopy and the polyprotein Ig domain chimera I27–I1. (A) Stretching single $(I27–I1)_4$ polyprotein chimera results in force–extension curves with a characteristic saw-tooth pattern; (B) Histogram of the unfolding forces for the polyprotein; (C) Plot of pulling speed dependence for the two levels of unfolding forces observed, one corresponding to the I27 and the other to the I1 sections of the polyprotein chimera. Reprinted with permission from reference [86].

[84,85]. A frequency histogram of unfolding forces of the polyprotein could be derived (Fig. 4B), showing two separate peaks, each corresponding to the mechanical unfolding of I1 and I27 domains. Thus, in an elegant non-chemical and non-thermal dependent manner, the unfolding of these two domains could be studied in a single experiment, characterizing in detail the stability of each individual domain. Following, the pulling speed dependence for the unfolding forces could be plotted (Fig. 4C). Finally, all this together allowed to, via Monte Carlo simulations (as a rough estimate with intrinsic low sensitivity [85,91]), derive the mechanical unfolding rate constant at zero force and the distance to the transition state.

AFM-based force extension studies can be combined with other techniques, such as steered molecular dynamics (SMD) simulations, allowing to capture the intermediate non-native protein structures that fit with the measured AFM data. This has been performed for barstar, an 89 amino acid residues protein involved in the intracellular inhibition of the extracellular ribonuclease barnase (Fig. 5) [92,93], a well-studied protein [94,95]. Thus, combining both methods takes one step further the power of AFM-based force extension protein folding analysis in a simple and elegant manner. Importantly, since the thermal or chemical-induced unfolding of many proteins is irreversible *in vitro*, AFM force–extension studies are sometimes the only adequate method to study protein unfolding, as it was the case of β-1,4-endoxylanase from *Bacillus circulans* (BCX) [96]. Single-molecule AFM force–extension studies allowed to directly measure the folding and unfolding kinetics of BCX *in vitro*, employing what can be defined as mechanical denaturation. Thus, AFM-based studies pave the way for a general methodology to characterize folding kinetics of proteins that are not suited for traditional equilibrium methodologies, given their irreversible denaturation profile.

Other large sized proteins, which tend much more frequently to aggregate and precipitate irreversibly in the traditional approaches of studying folding, have been successfully studied via AFM. One such example is the AFM–force spectroscopy study of fibrinogen, a large blood plasma protein (~340 kDa) that is activated by thrombin to form the fibrin fibers found in blood clots [97]. The forced unfolding of engineered linear oligomers of fibrinogen produced well-defined saw-tooth patterns matching those of the globular protein ubiquitin [98], while being, as expectable [97], distinct of myosin II, a muscle protein [99]. This demonstrates the power of AFM to study different proteins in a very simple and reproducible approach.

Two other related and yet different AFM methods for studying protein folding are also popular: force-clamp and force-ramp. In force-clamp spectroscopy, a protein molecule is held at a constant stretching force. Then, the protein is allowed to fold and unfold, while that constant mechanical force pulling it apart is maintained. This allows to measure folding/unfolding as a function of time at different stretching forces, which is useful in the characterization of molecular motors [100,101]. Force-ramp studies can be defined as a variation of the later, where the force is quickly varied over time, in contrast to a constant force-clamp experiment where each of the protein molecule modules are allowed to unfold slowly and separately.

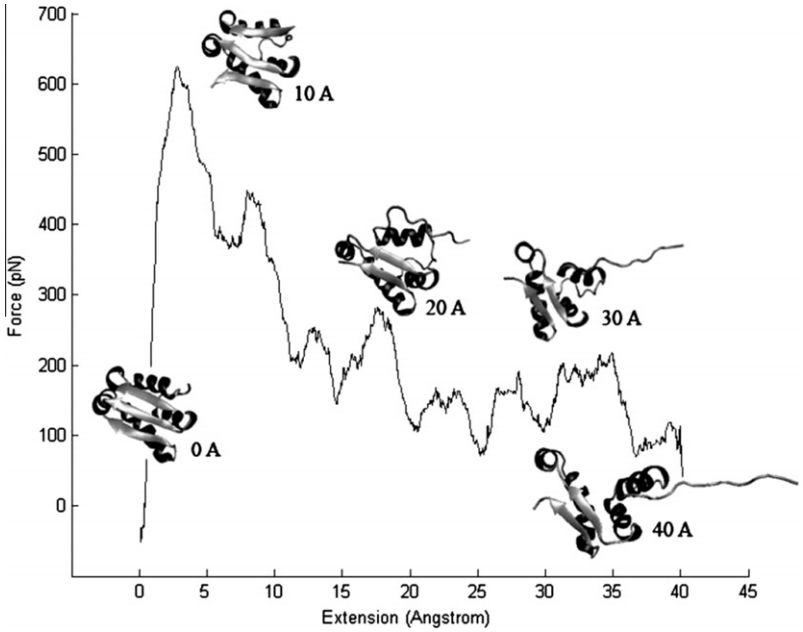

Fig. 5. Combining AFM force–extension studies with steered molecular dynamics (SMD) simulations can provide unique glimpses at the folding/unfolding mechanism. Here, force–extension curves of the mechanical unfolding of barstar, a small 89 residues protein, combined with constant-velocity SMD simulations at 1 ms^{-1} show that stretching barstar from its N- and C-termini results in the peeling, instead of shearing, of the C-terminal β-strand. Snapshots of the structure of barstar along the mechanical unfolding pathway are shown at different extensions. Reprinted with permission from reference [93].

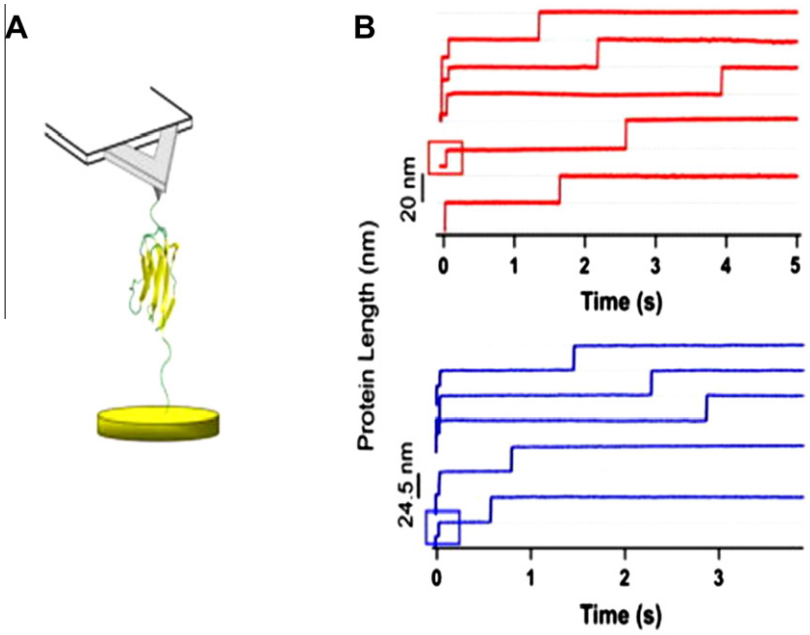

Fig. 6. Force clamp measurements may be employed to follow the unfolding of individual protein monomers. (A) Experimental setup schematics. (B) Experimental length versus time recordings of ubiquitin (red), stretched at 110 pN, and I27 (blue), stretched at 150 pN. Both show well-defined steps corresponding to the monomer length (ubiquitin monomer being 20.0 nm and I27 monomer 24.5 nm). Reprinted with permission from reference [92].

The obvious main advantage of AFM methodologies over traditional force–extension studies is the possibility of expressing the folding/unfolding parameters as a function of the applied force, with the main readout being a staircase-like graph of protein length versus time, where each step corresponds to an individual molecule folding/unfolding event (for each of these events there is a small indentation due to the force feedback). It is therefore of an easier interpretation. Similarly to the force–extension, an accurate unfolding probability can be determined. However, with the force-ramp, a relatively small number of curves can generate an accurate unfolding probability of a given molecule at different forces, since the force-ramp directly measures the force dependence of the unfolding probability of a modular protein [76]. In fact, force-clamp spectroscopy is a sensitive technique that can be employed to yield a molecular fingerprint of the unfolding process, having been used to study the folding/unfolding rate kinetics of different proteins, including ubiquitin and I27 monomers (Fig. 6) [92]. It can be said that force-clamp spectroscopy at the single-

monomer level reproduces the kinetics of unfolding and refolding measured using polyproteins, which proves that there is no mechanical effect of tethering proteins one to another, at least in the case of ubiquitin and I27 [92].

Studying folding/unfolding via mechanical forces is of special interest in the case of modular proteins, which frequently undergo stretching forces within the context of their physiological role. That is the case of tenascin, an extracellular matrix protein involved in regulating cell–matrix interactions, where single-molecule force-ramp spectroscopy has allowed the characterization of the unfolding/folding kinetics of a recombinant tenascin fragment, TNfnALL (Fig. 7) [102]. This fragment has been derivatized onto a cantilever and studied regarding the folding/unfolding kinetic parameters, demonstrating the applicability of AFM-based force-ramp spectroscopy in manipulating and handling proteins and peptides in folding studies.

Since the overall intrinsic properties of membrane proteins resemble those of soluble ones (with small but yet important differences present) [103], it is not surprising that force–extension studies have also allowed the characterization of the unfolding profile of membrane proteins incorporated in the membrane in a physiological or quasi-physiological manner, an experimental setting of great biological relevance (as compared to, for example, chemical denaturation in a simplified buffer condition) [99,100]. Single-molecule membrane protein unfolding studies were first conducted with bacteriorhodopsin from native purple membrane patches isolated from *Halobacterium salinarium* [104,105], via differential and sucrose density gradient centrifugation after lysis of the cells by dialysis against distilled water [106]. The specific nature of force–distance spectra was determined at high resolution (Fig. 8) by applying forces of 0.5–1 nN for 1 s, allowing the bacteriorhodopsin polypeptide to adsorb to the AFM tip, after which it was pulled away from the membrane. The findings obtained revealed a curve with a profile similar to those described above for the force–distance spectrum of soluble globular proteins. It was also clear from the data that the protein could be unfolded up to a distance of 75 nm. In addition, by looking at each individual force–distance spectrum, it was possible to infer the properties of intermediates along the unfolding pathway; namely, their length, most likely shape and likelihood of distinct structural elements unfolding, separately or in tandem.

Single-molecule force spectroscopy has also been used to study the folding in the membrane of the *Escherichia coli* Na^+/H^+ antiporter NhaA [107]. It was possible to assess folding intermediates of a single NhaA polypeptide forming structural segments such as the Na^+-binding site, transmembrane α-helices, and helical pairs (Fig. 9). The folding rates of structural segments were determined (ranging from 0.31 s^{-1} to 47 s^{-1}), providing a detailed insight into a distinct folding hierarchy of an unfolded polypeptide into the native membrane protein structure. In spite of the direct comparison of the stability parameters obtained via AFM with the data obtained via other techniques being not always easily feasible [98], this shows once again the great potential of AFM-based force spectroscopy.

It can be said that, overall, the adaptation from AFM-based protein folding studies with soluble proteins to studies with mem-

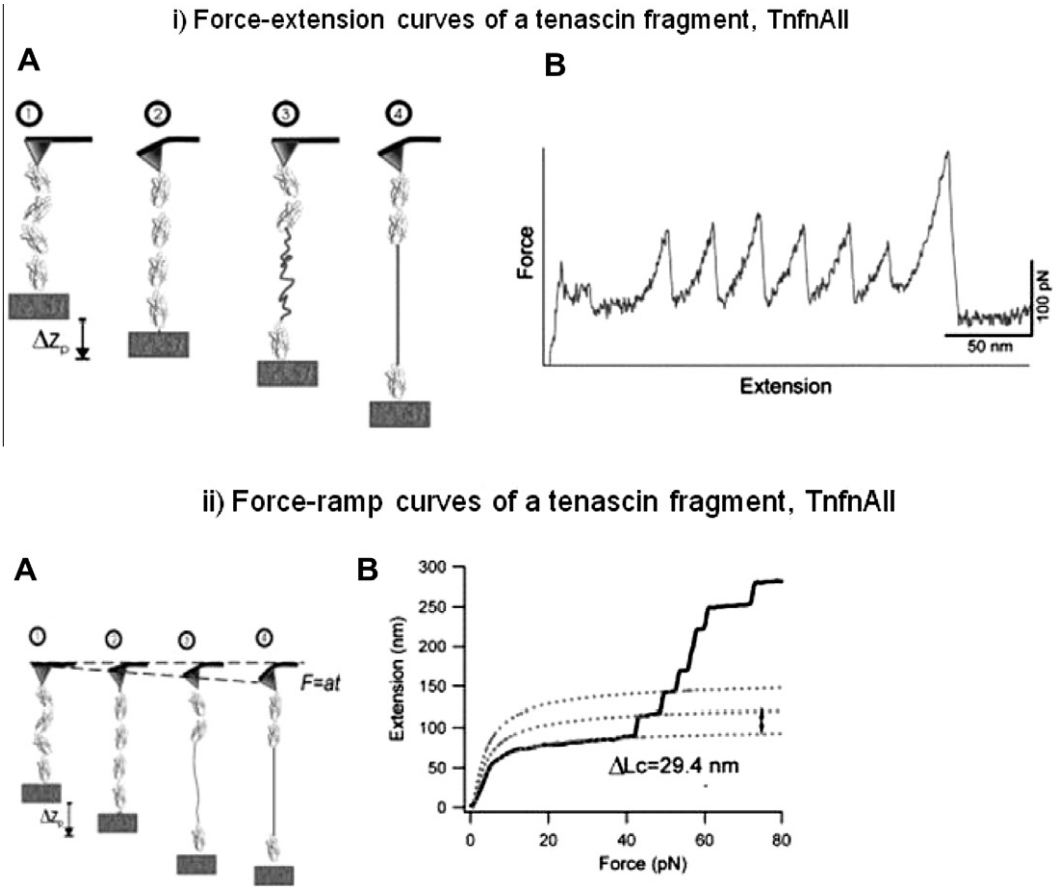

Fig. 7. Unfolding of tandem modular TNfnALL protein. (i) Force extension measurements with (A) showing a schematic representation of the experiment, where the protein is pulled at a constant velocity, leading to a characteristic saw-tooth pattern (B), due to the sequential mechanical unfolding of each individual domain. (ii) Force-ramp measurements with (A) showing a scheme of the experiment, where the stretching force, *F*, increases linearly ($F = at$, where a is the ramp rate and t is the time), resulting in the staircase-like curve (B) as a consequence of the mechanical unfolding of individual domains. Adapted from reference [102].

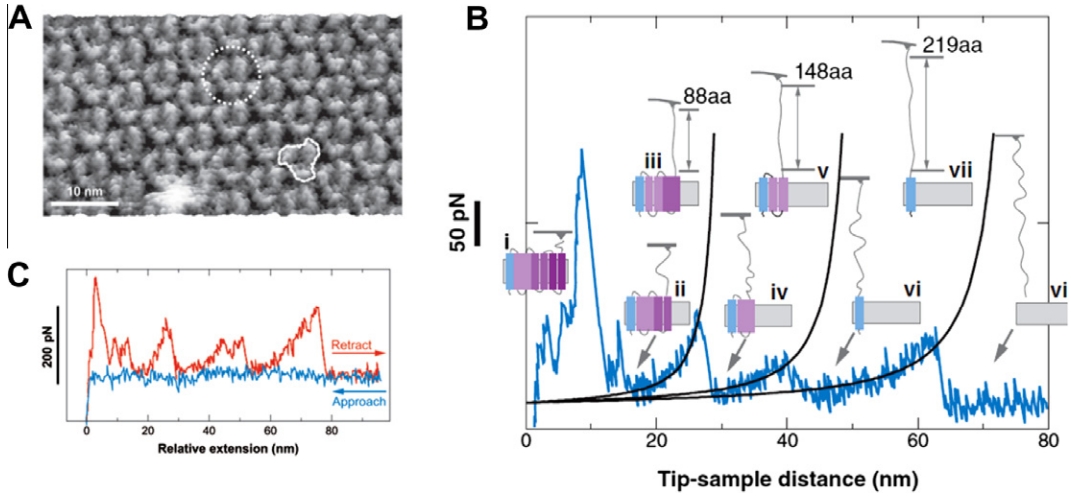

Fig. 8. AFM-based force spectroscopy controlled unfolding of a membrane protein. (A) AFM topographic image of the cytoplasmic purple membrane surface of *Halobacterium salinarium*, showing bacteriorhodopsin, a membrane protein forming trimers at the membrane surface. (B) Single-molecule AFM force–extension studies of bacteriorhodopsin allowed the characterization of the force–distance profile of the protein unfolding in the biological relevant membrane context. (C) The unfolding pathway described by each force–distance spectrum is scrutinized. The roman numerals indicate intermediates along the unfolding pathway. Whereas some structural elements unfold individually, others have a certain probability to unfold with other elements. Adapted from reference [104,105].

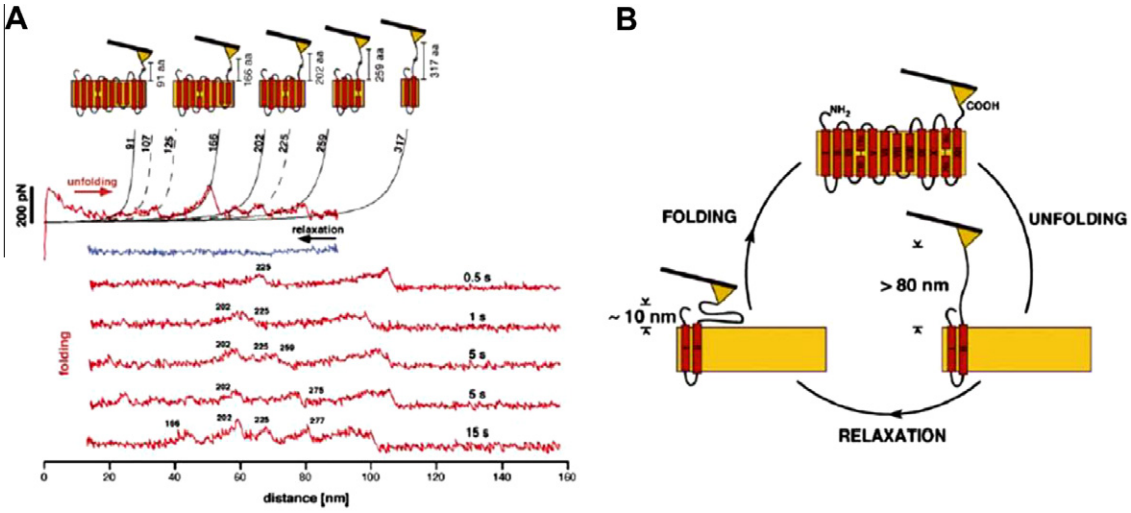

Fig. 9. Single-molecule unfolding and refolding of the NhaA Na^+/H^+ antiporter from *E. coli*. (A) Force–distance unfolding/refolding curves of NhaA. The worm-like chain model fits most force peaks. The major unfolding pathway is also depicted. (B) Scheme of a refolding experiment. Initially, the intact NhaA molecule was unfolded, after which the tip was separated from the membrane (up to 80 nm), so that the last helical pair remained anchored in the membrane. Then, the stretched polypeptide was relaxed as the tip was brought into proximity (10 nm) of the surface. After a specified delay, the polypeptide was repeatedly pulled, while recording the force–distance data. Reprinted with permission from reference [107].

branes present is considerably straight-forward. The setting is indeed very similar to membrane-free unfolding studies by AFM, proving the versatility of AFM-based single-molecule spectroscopy.

It is also important to stress that AFM-based studies of protein unfolding have been directly compared with chemical denaturation methods, revealing similar results. For example, by engineering a protein made of tandem repeats of identical Ig modules, AFM unfolding data were obtained and compared with similar data obtained via classical guanidinium chloride chemical denaturation [85]. The unfolding rates obtained by the two methods are essentially the same, with the unfolding rate constant extrapolated to 0 M denaturant being $4.9 \times 10^{-4}\ s^{-1}$, very similar to that obtained from the Monte Carlo simulation of the AFM data ($3.3 \times 10^{-4}\ s^{-1}$). AFM data therefore readily extrapolates to the unfolding rate obtained by chemical denaturation. Furthermore, the transition state for unfolding appears at the same position on the folding pathway when assessed by either method. As such, mechanical unfolding of a single protein by AFM can be directly compared with traditional unfolding experiments to obtain additional data. Importantly, novel non-kinetics AFM-based data for the study of protein (un)folding are now available [108]. Most importantly, AFM-based measurements have the key advantage of being able to measure (un)folding processes at the single-molecule level.

A number of studies involving AFM-based force spectroscopy and virus capsid proteins have been developed in recent years. For example, we have studied the dengue virus capsid protein interaction with different biological ligands relevant for *in vivo* assembly [46,47,109,110], while others have directly aimed at the study of the stability of the full viral particle capsid

[111–113], showing the potential of AFM in solving similar but yet diverse biological problems. One interesting study relates to Norwalk virus-like particles (NVLP) structural and mechanical stability [113]. These particles contain a protruding domain, which if deleted yields smooth mutant particles, as shown by AFM, implying that the protruding domain drastically changes the viral capsid properties by increasing the stiffness of NVLP and thus stabilizes the viral nanoparticle. AFM studies with the tobacco mosaic virus (TMV) also demonstrated that individual TMV particles flexibility can be measured via AFM [111,112]. TMV particles were shown to be semi-flexible rods (rather than hard rods), with a length and width of, respectively, 301 and 14.7 nm. The average height was also assessed via AFM (roughly 16.8–18.6 nm), which therefore reveals that the capsid particles are able to support the pressure force applied onto them by the AFM tip without being crushed. Importantly, the values agree perfectly well with X-ray diffraction and electron microscopy measurements [114]. AFM is thus suited for the analysis of mechanical properties such as elasticity and stiffness, all determinant factors in viral capsids stability.

In an interesting related study, it was shown that actual virus, such as TMV, can even be used to estimate the AFM tip apex radius [112]. Thus, not only the stability of virus capsids can be determined via AFM-based approaches but also shows that once that is known, this knowledge can cross-feed itself in a benign positive feedback loop, being an excellent example of how AFM-based techniques can still be much improved in the future via unconventional approaches. That study concluded that TMV particles are a well-adapted calibrator for AFM tips on the imaging of single isolated biomolecules.

Up to now, we have been discussing the ability of AFM-based force spectroscopy to monitor protein (un)folding, taking into consideration only the contribute to the measured "folding force" that is given by intramolecular non-covalent bonds. Nevertheless, SMFS can also be used to monitor the strength of covalent bonds, such as disulfide bonds, essential for many proteins structure and function. One early example of it was an AFM study of the rupture force necessary to break apart covalently bound polysaccharide molecules, anchored between a surface (with alternative binding chemical groups) and an AFM tip (and then stretched until the covalent bond under study is broken) [115]. With this approach, it was possible to measure, among other bonds, the force required to break apart a sulfur-bond (1.4 ± 0.3 nN). A variation of this approach was adapted to measure the intramolecular disulfide bonds contribute to protein stability, namely with the elastic I-band of titin, containing the I1 immunoglobulin domain [86]. SMFS was used to prove that hat the formation of a disulfide bridge in I1 is a relatively rare event in solution, even under oxidative conditions, contrasting with previous assumptions based on its X-ray structure [116], as well as on the similar I27 domain. Going further on this topic, SMFS was employed in AFM force-clamp experiments to directly study the effect of force on the kinetics of thiol/disulfide exchange in the previously mentioned I27 domain of titin [117]. Essentially, a constant stretching force was applied to a single engineered disulfide bond in the I27 protein domain, after which the rate of reduction by dithiothreitol (DTT) was measured. It was clear from the data that the reduction rate depends linearly in the DTT concentration and varies exponentially with the applied force (increasing 10-fold over a 300 pN range). The data fit well with a mechanism whereby the disulfide bond is lengthened by 0.34 Å at the transition state of the thiol/disulfide exchange reaction. This AFM data suggests that thiol/disulfide exchange in proteins is a force-dependent chemical reaction and that mechanical force plays a role in disulfide reduction. All this together, on one hand, highlights the power of AFM-based measurements on the study of protein features. On the other hand, it also shows the importance of the study of protein properties in the context of the application of physical forces.

It is important to point out here that AFM-based force spectroscopy studies allow for determining not only the intramolecular forces playing key roles in protein folding and stability, but also intermolecular forces between homo-oligomers that are driving the final mature protein formation. One well-known example of such a study is with the heparin-binding hemagglutinin (HBHA) [118], a virulence factor for *Mycobacterium tuberculosis* pathogenesis. Briefly, single-molecule atomic-force microscopy was employed to measure individual homophilic HBHA–HBHA interaction forces. The analysis of the force curves produced using AFM tips derivatized with HBHA bound to the tip via the protein N-terminus showed that they could be fitted to a model whereby the formation of dimers or multimers of HBHA occurs in a manner consistent with the unfolding of α-helical coiled–coil structures. However, force curves with AFM tips derivatized with the protein bound via the lysine-rich C-terminal suggested that no such oligomerization and formation of specific coiled–coil interactions occurs. Importantly this was validated with data on live mycobacteria, suggesting the occurrence of such HBHA dimer/oligomer formation. These data support HBHA mediated bacterial aggregation via the observed multimer formation. AFM can thus also be used to study specific protein oligomerization, giving information regarding the protein arrangement into a three-dimensional oligomeric structure (and thus function), which in turn allows for the understanding of events such as bacterial aggregation mechanisms.

Conclusion

Integrating a detailed mechanistic understanding of the protein folding process with the three-dimensional structural data accumulated over the last decades will yield great progress. Currently, as further described elsewhere [119–131], it is already possible to produce predictions regarding several features of a polypeptide chain, with a variable degree of accuracy. Of the features of interest, the accurate prediction of the tridimensional structure of peptides and proteins *in silico* remains one the most promising and simultaneously elusive problem. Several reasons concur to this present situation, one of the most important being our incomplete understanding of the molecular mechanisms that mediate protein folding. Moving further on this topic requires additional experimental data, ideally on single protein intra- and inter-molecular interactions in their real biological contexts and/or in the presence of biological ligands. Hence, the high resolution ability of atomic force microscopy and compatibility with biologically relevant experimental settings makes it especially suited to solve these important biological and biomedical questions in the context of folding/unfolding studies of single proteins in physiological conditions. Given the versatility of the force–extension, force-clamp and force-ramp modes, this technique may yet be combined with single-molecule protein recognition and protein–ligand interaction studies. This puts forward the key advantage of being able to track the effect of the interaction directly on the protein structure. Thus, the use of AFM-based force spectroscopy in folding studies (measuring intramolecular interactions), combined with protein–ligand interaction studies, paves the way for further advances in the field. AFM-based force spectroscopy measurements are becoming increasingly common and easier to handle, which also allows for their wider use in several other areas. This is of special importance for biomedical studies, since a detailed understanding of the molecular mechanisms involved in health and disease are crucial for the future development of novel protein-targeted drugs and treatments.

Acknowledgments

This work was supported by Fundação para a Ciência e a Tecnologia – Ministério do Ensino e Ciência (FCT–MEC, Portugal) and by Fundação Calouste Gulbenkian (Portugal). I.C.M. also acknowledges FCT–MEC fellowship SFRH/BPD/74287/2010. The authors declare that there is no possible conflict of interest involved.

References

[1] A.S. Politou, D.J. Thomas, A. Pastore, Biophys. J. 69 (1995) 2601–2610.
[2] T.R. Jahn, S.E. Radford, Arch. Biochem. Biophys. 469 (2008) 100–117.
[3] A.R. Fersht, Nat. Rev. Mol. Cell. Biol. 9 (2008) 650–654.
[4] J.D. Bryngelson, P.G. Wolynes, Proc. Natl. Acad. Sci. USA 84 (1987) 7524–7528.
[5] J.D. Bryngelson, J.N. Onuchic, N.D. Socci, P.G. Wolynes, Genetics 21 (1995) 167–195.
[6] P.G. Wolynes, J.N. Onuchic, D. Thirumalai, Science 267 (1995) 1619–1620.
[7] P.G. Wolynes, Proc. Natl. Acad. Sci. USA 94 (1997) 6170–6175.
[8] P.G. Wolynes, Philos. Trans. R. Soc. A Math. Phys. Eng. Sci. 363 (2005) 453–464.
[9] L.S. Itzhaki, P.G. Wolynes, Curr. Opin. Struct. Biol. 18 (2008) 1–3.
[10] P.G. Wolynes, Biophys. J. 100 (2011) 5.
[11] D. Eisenberg, Proc. Natl. Acad. Sci. USA 100 (2003) 11207–11210.
[12] C.J. Epstein, R.F. Goldberger, C.B. Anfinsen, Cold Spring Harbor Symp. Quant. Biol. 28 (1963) 439–449.
[13] C.B. Anfinsen, Science 181 (1973) 223–230.
[14] L. Pauling, R.B. Corey, J. Am. Chem. Soc. 72 (1950) 5349.
[15] L. Pauling, R.B. Corey, Proc. Natl. Acad. Sci. USA 37 (1951) 251–256.
[16] L. Pauling, R.B. Corey, Proc. Natl. Acad. Sci. USA 37 (1951) 241–250.
[17] L. Pauling, R.B. Corey, H.R. Branson, Proc. Natl. Acad. Sci. USA 37 (1951) 205–211.
[18] J.C. Kendrew, G. Bodo, H.M. Dintzis, R.G. Parrish, H. Wyckoff, D.C. Phillips, Nature 181 (1958) 662–666.
[19] M.F. Perutz, M.G. Rossmann, A.F. Cullis, H. Muirhead, G. Will, A.C.T. North, Nature 185 (1960) 416–422.
[20] C.C.F. Blake, D.F. Koenig, G.A. Mair, A.C.T. North, D.C. Phillips, V.R. Sarma, Nature 206 (1965) 757–761.
[21] C. Levinthal, J. Chim. Phys. Phys. Chim. Biol. 65 (1968) 44–45.
[22] P.E. Leopold, M. Montal, J.N. Onuchic, Proc. Natl. Acad. Sci. USA 89 (1992) 8721–8725.
[23] K.A. Dill, S. Bromberg, K.Z. Yue, K.M. Fiebig, D.P. Yee, P.D. Thomas, H.S. Chan, Protein Sci. 4 (1995) 561–602.
[24] D. Shortle, Y. Wang, J.R. Gillespie, J.O. Wrabl, Protein Sci. 5 (1996) 991–1000.
[25] D. Shortle, Nat. Struct. Biol. 6 (1999) 203–205.
[26] K.A. Dill, Biochemistry 29 (1990) 7133–7155.
[27] V. Daggett, A.J. Li, L.S. Itzhaki, D.E. Otzen, A.R. Fersht, J. Mol. Biol. 257 (1996) 430–440.
[28] A.R. Fersht, V. Daggett, Cell 108 (2002) 573–582.
[29] P.S. Kim, R.L. Baldwin, Annu. Rev. Biochem. 51 (1982) 459–489.
[30] O.B. Ptitsyn, J. Protein Chem. 6 (1987) 273–293.
[31] M. Karplus, D.L. Weaver, Biopolymers 18 (1979) 1421–1437.
[32] D.A. Dolgikh, R.I. Gilmanshin, E.V. Brazhnikov, V.E. Bychkova, G.V. Semisotnov, S.Y. Venyaminov, O.B. Ptitsyn, FEBS Lett. 136 (1981) 311–315.
[33] L.S. Itzhaki, D.E. Otzen, A.R. Fersht, J. Mol. Biol. 254 (1995) 260–288.
[34] V. Daggett, A.R. Fersht, Trends Biochem. Sci. 28 (2003) 18–25.
[35] A.C. Ferreon, A.A. Deniz, Biochim. Biophys. Acta (1814) 1021–1029.
[36] P. Weinkam, F.E. Romesberg, P.G. Wolynes, Biochemistry 48 (2009) 2394–2402.
[37] L.J. Lapidus, Curr. Opin. Struct. Biol. 23 (in press), doi: 10.1016/j.sbi.2012.10.003.
[38] R. Barattin, N. Voyer, Chem. Commun. (Camb) (2008) 1513–1532.
[39] JPK Instruments, JPK NanoWizard Manual, Germany, Berlin, 2009. pp. 7–131.
[40] S. Santos, V. Barcons, H.K. Christenson, J. Font, N.H. Thomson, PLoS One 6 (2011) e23821.
[41] D.P. Allison, N.P. Mortensen, C.J. Sullivan, M.J. Doktycz, Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol. 2 (2010) 618–634.
[42] P. Eaton, P. West, Atomic force microscopy, Oxford University Press, Oxford, 2010.
[43] P. Parot, Y.F. Dufrene, P. Hinterdorfer, C. Le Grimellec, D. Navajas, J.L. Pellequer, S. Scheuring, J. Mol. Recognit. 20 (2007) 418–431.
[44] N.C. Santos, M.A.R.B. Castanho, Biophys. Chem. 107 (2004) 133–149.
[45] F.A. Carvalho, S. Connell, G. Miltenberger-Miltenyi, S.V. Pereira, A. Tavares, R.A.S. Ariëns, N.C. Santos, ACS Nano 4 (2010) 4609–4620.
[46] I.C. Martins, F. Gomes-Neto, A.F. Faustino, F.A. Carvalho, F.A. Carneiro, P.T. Bozza, R. Mohana-Borges, M.A.R.B. Castanho, F.C. Almeida, N.C. Santos, A.T. Da Poian, Biochem. J. 444 (2012) 405–415.
[47] F.A. Carvalho, F.A. Carneiro, I.C. Martins, I. Assunção-Miranda, A.F. Faustino, R.M. Pereira, P.T. Bozza, M.A.R.B. Castanho, R. Mohana-Borges, A.T. Da Poian, N.C. Santos, J. Virol. 86 (2012) 2096–2108.
[48] F.A. Carvalho, S. Connell, G. Miltenberger-Miltenyi, S.V. Pereira, A. Tavares, R.A.S. Ariëns, N.C. Santos, Biophys. J. 100 (2011) 479.
[49] F.A. Carvalho, N.C. Santos, IUBMB Life 64 (2012) 465–472.
[50] F.A. Carvalho, S. de Oliveira, T. Freitas, S. Gonçalves, N.C. Santos, PloS One 6 (2011) e18167.
[51] J. Zlatanova, S.M. Lindsay, S.H. Leuba, Prog. Biophys. Mol. Biol. 74 (2000) 37–61.
[52] W.F. Heinz, J.H. Hoh, Trends Biotechnol. 17 (1999) 143–150.
[53] J.N. Israelachvili, P.M. McGuiggan, Science 241 (1988) 795–800.
[54] A. Vinckier, G. Semenza, FEBS Lett. 430 (1998) 12–16.
[55] H. Clausen-Schaumann, M. Seitz, R. Krautbauer, H.E. Gaub, Curr. Opin. Chem. Biol. 4 (2000) 524–530.
[56] C. Bustamante, J.F. Marko, E.D. Siggia, S. Smith, Science 265 (1994) 1599–1600.
[57] M. Rief, F. Oesterhelt, B. Heymann, H.E. Gaub, Science 275 (1997) 1295–1297.
[58] M.E. Fauver, D.L. Dunaway, D.H. Lilienfeld, H.G. Craighead, G.H. Pollack, IEEE Trans. Biomed. Eng. 45 (1998) 891–898.
[59] J.L. Hutter, J. Bechhoefer, Rev. Sci. Instrum. 64 (1993) 1868–1873.
[60] A. Ebner, P. Hinterdorfer, H.J. Gruber, Ultramicroscopy 107 (2007) 922–927.
[61] C.D. Frisbie, L.F. Rozsnyai, A. Noy, M.S. Wrighton, C.M. Lieber, Science 265 (1994) 2071–2074.
[62] D.T. Cheung, M.E. Nimni, Connect. Tissue Res. 10 (1982) 187–199.
[63] I. Migneault, C. Dartiguenave, M.J. Bertrand, K.C. Waldron, Biotechniques 37 (790–796) (2004) 798–802.
[64] M. Ouyang, J.L. Huang, C.M. Lieber, Annu. Rev. Phys. Chem. 53 (2002) 201–220.
[65] U. Dammer, O. Popescu, P. Wagner, D. Anselmetti, H.J. Guntherodt, G.N. Misevic, Science 267 (1995) 1173–1175.
[66] R. Barattin, N. Voyer, Methods Mol. Biol. 736 (2011) 457–483.
[67] P. Carl, H. Schillers, Pflugers Arch. 457 (2008) 551–559.
[68] L. Sirghi, J. Ponti, F. Broggi, F. Rossi, Eur. Biophys. J. 37 (2008) 935–945.
[69] I. Casuso, F. Rico, S. Scheuring, J. Mol. Recognit. 24 (2011) 406–413.
[70] C.K. Lee, Y.M. Wang, L.S. Huang, S. Lin, Micron 38 (2007) 446–461.
[71] R. Merkel, P. Nassoy, A. Leung, K. Ritchie, E. Evans, Nature 397 (1999) 50–53.
[72] N. Almqvist, R. Bhatia, G. Primbs, N. Desai, S. Banerjee, R. Lal, Biophys. J. 86 (2004) 1753–1762.
[73] V. Dupres, F.D. Menozzi, C. Locht, B.H. Clare, N.L. Abbott, S. Cuenot, C. Bompard, D. Raze, Y.F. Dufrene, Nat. Methods 2 (2005) 515–520.
[74] Y.F. Dufrene, P. Hinterdorfer, Pflugers Arch. 456 (2008) 237–245.
[75] M. Rief, H. Grubmuller, ChemPhysChem 3 (2002) 255–261.
[76] W.A. Linke, A. Grutzner, Pflugers Arch. 456 (2008) 101–115.
[77] M. Sotomayor, K. Schulten, Science 316 (2007) 1144–1148.
[78] F. Valle, G. Zuccheri, A. Bergia, L. Ayres, A.E. Rowan, R.J. Nolte, B. Samori, Angew. Chem. Int. Ed. Engl. 47 (2008) 2431–2434.
[79] M. Carrion-Vazquez, A.F. Oberhauser, T.E. Fisher, P.E. Marszalek, H. Li, J.M. Fernandez, Prog. Biophys. Mol. Biol. 74 (2000) 63–91.
[80] A. Sarkar, S. Caamano, J.M. Fernandez, J. Biol. Chem. 280 (2005) 6261–6264.
[81] G.I. Bell, Science 200 (1978) 618–627.
[82] E. Evans, K. Ritchie, Biophys. J. 72 (1997) 1541–1555.
[83] E. Evans, K. Ritchie, R. Merkel, Biophys. J. 68 (1995) 2580–2587.
[84] M. Rief, M. Gautel, F. Oesterhelt, J.M. Fernandez, H.E. Gaub, Science 276 (1997) 1109–1112.
[85] M. Carrion-Vazquez, A.F. Oberhauser, S.B. Fowler, P.E. Marszalek, S.E. Broedel, J. Clarke, J.M. Fernandez, Proc. Natl. Acad. Sci. USA 96 (1999) 3694–3699.
[86] H.B. Li, J.M. Fernandez, J. Mol. Biol. 334 (2003) 75–86.
[87] H.B. Li, W.A. Linke, A.F. Oberhauser, M. Carrion-Vazquez, J.G. Kerkviliet, H. Lu, P.E. Marszalek, J.M. Fernandez, Nature 418 (2002) 998–1002.
[88] H.B. Li, A.F. Oberhauser, S.D. Redick, M. Carrion-Vazquez, H.P. Erickson, J.M. Fernandez, Proc. Natl. Acad. Sci. USA 98 (2001) 10682–10686.
[89] H.B. Li, A.F. Oberhauser, S.B. Fowler, J. Clarke, J.M. Fernandez, Proc. Natl. Acad. Sci. USA 97 (2000) 6527–6531.
[90] R.B. Best, B. Li, A. Steward, V. Daggett, J. Clarke, Biophys. J. 81 (2001) 2344–2356.
[91] P.M. Williams, S.B. Fowler, R.B. Best, J.L. Toca-Herrera, K.A. Scott, A. Steward, J. Clarke, Nature 422 (2003) 446–449.
[92] S. Garcia-Manyes, J. Brujic, C.L. Badilla, J.M. Fernandez, Biophys. J. 93 (2007) 2436–2446.
[93] D. Sharma, G. Feng, D. Khor, G.Z. Genchev, H. Lu, H. Li, Biophys. J. 95 (2008) 3935–3942.
[94] J.L. Neira, E. Vazquez, A.R. Fersht, Biochemistry 267 (2000) 2859–2870.
[95] K.B. Wong, J. Clarke, C.J. Bond, J.L. Neira, S.M.V. Freund, A.R. Fersht, V. Daggett, J. Mol. Biol. 296 (2000) 1257–1282.
[96] A. Jollymore, H. Li, J. Mol. Biol. 402 (2010) 610–617.
[97] A.E. Brown, R.I. Litvinov, D.E. Discher, J.W. Weisel, Biophys. J. 92 (2007) L39–L41.
[98] M. Schlierf, H. Li, J.M. Fernandez, Proc. Natl. Acad. Sci. USA 101 (2004) 7299–7304.
[99] I. Schwaiger, C. Sattler, D.R. Hostetter, M. Rief, Nat. Mater. 1 (2002) 232–235.
[100] M. Rief, R.S. Rock, A.D. Mehta, M.S. Mooseker, R.E. Cheney, J.A. Spudich, Proc. Natl. Acad. Sci. USA 97 (2000) 9482–9486.
[101] K. Visscher, M.J. Schnitzer, S.M. Block, Nature 400 (1999) 184–189.
[102] M. Wang, Y. Cao, H. Li, Polymer 47 (2006) 2548–2554.
[103] F.N. Barrera, L.A. Alcaraz, E. Hurtado-Gomez, J.L. Neira, Curr. Protein Pept. Sci. 9 (2008) 626–637.
[104] A. Kedrov, H. Janovjak, K.T. Sapra, D.J. Muller, Annu. Rev. Biophys. Biomol. Struct. 36 (2007) 233–260.
[105] D.J. Muller, D. Fotiadis, S. Scheuring, S.A. Muller, A. Engel, Biophys. J. 76 (1999) 1101–1111.
[106] D. Oesterhelt, W. Stoeckenius, Methods Enzymol. 31 (1974) 667–678.

[107] A. Kedrov, H. Janovjak, C. Ziegler, W. Kuhlbrandt, D.J. Muller, J. Mol. Biol. 355 (2006) 2–8.
[108] F. Benedetti, C. Micheletti, G. Bussi, S.K. Sekatskii, G. Dietler, Biophys. J. 101 (2011) 1504–1512.
[109] F.A. Carvalho, F.A. Carneiro, I.C. Martins, I. Assunção-Miranda, A.F. Faustino, M.A.R.B. Castanho, R. Mohana-Borges, A.T. Da Poian, N.C. Santos, FEBS J. 279 (2012) 10–11.
[110] I.C. Martins, F.A. Carneiro, R. Mohana-Borges, A. Faustino, R.M. Pereira, F.A. Carvalho, M.A.R.B. Castanho, F. Almeida, N.C. Santos, A.T. Da Poian, Biophys. J. 100 (2011) 403–404.
[111] H. Maeda, Langmuir 13 (1997) 4150–4161.
[112] M.H. Trinh, M. Odorico, L. Bellanger, M. Jacquemond, P. Parot, J.L. Pellequer, J. Mol. Recognit. 24 (2011) 503–510.
[113] M. Baclayon, G.K. Shoemaker, C. Uetrecht, S.E. Crawford, M.K. Estes, B.V.V. Prasad, A.J.R. Heck, G.J.L. Wuite, W.H. Roos, Nano Lett. 11 (2011) 4865–4869.
[114] B.V.V. Prasad, M.E. Hardy, T. Dokland, J. Bella, M.G. Rossmann, M.K. Estes, Science 286 (1999) 287–290.
[115] M. Grandbois, M. Beyer, M. Rief, H. Clausen-Schaumann, H.E. Gaub, Science 283 (1999) 1727–1730.
[116] O. Mayans, J. Wuerges, S. Canela, M. Gautel, M. Wilmanns, Structure 9 (2001) 331–340.
[117] A.P. Wiita, R.K. Ainavarapu, H.H. Huang, J.M. Fernandez, Proc. Natl. Acad. Sci. USA 103 (2006) 7222–7227.
[118] C. Verbelen, D. Raze, F. Dewitte, C. Locht, Y.F. Dufrene, J. Bacteriol. 189 (2007) 8801–8806.
[119] P. Vanhee, J. Reumers, F. Stricher, L. Baeten, L. Serrano, J. Schymkowitz, F. Rousseau, Nucleic Acids Res. 38 (2010) D545–D551.
[120] S. Maurer-Stroh, M. Debulpaep, N. Kuemmerer, M.L. de la Paz, I.C. Martins, J. Reumers, K.L. Morris, A. Copland, L. Serpell, L. Serrano, J.W.H. Schymkowitz, F. Rousseau, Nat. Methods 7 (2010) 237–242.
[121] L. Baeten, J. Reumers, V. Tur, F. Stricher, T. Lenaerts, L. Serrano, F. Rousseau, J. Schymkowitz, PLoS Comput. Biol. 4 (2008).
[122] J.W.H. Schymkowitz, F. Rousseau, I.C. Martins, J. Ferkinghoff-Borg, F. Stricher, L. Serrano, Proc. Natl. Acad. Sci. USA 102 (2005) 10147–10152.
[123] A.M. Fernandez-Escamilla, F. Rousseau, J. Schymkowitz, L. Serrano, Nat. Biotechnol. 22 (2004) 1302–1306.
[124] O.F. Lange, D. Baker, Proteins 80 (2012) 884–895.
[125] R.Y.R. Wang, Y. Han, K. Krassovsky, W. Sheffler, M. Tyka, D. Baker, PloS One 6 (2011) e22060.
[126] F. Khatib, S. Cooper, M.D. Tyka, K.F. Xu, I. Makedon, Z. Popovic, D. Baker, F. Players, Proc. Natl. Acad. Sci. USA 108 (2011) 18949–18953.
[127] D. Baker, Nature 405 (2000) 39–42.
[128] Y. Zhang, Proteins 69 (2007) 108–117.
[129] A. Roy, A. Kucukural, Y. Zhang, Nat. Protoc. 5 (2010) 725–738.
[130] S.J. De Vries, M. van Dijk, A.M.J.J. Bonvin, Nat. Protoc. 5 (2010) 883–897.
[131] C. Dominguez, R. Boelens, A.M.J.J. Bonvin, J. Am. Chem. Soc. 125 (2003) 1731–1737.