Stretching Single Molecules Along Unbinding and Unfolding Pathways with the Scanning Force Microscope

Bruno Samorì*[a]

Abstract: Individual molecules can be stretched with a scanning force microscope and the forces required to rupture bonds and to mechanically drive their structures towards new conformations and states can be measured. The tailoring of the experiments, the possibility of carrying them out in quasi-equilibrium conditions, the relationships between single molecule force measurements, and macroscopic kinetics or thermodynamic data are discussed. Mechanochemical experiments are expanding chemistry into new realms between biology and material science.

Keywords: mechanochemistry · protein folding · scanning probe microscopy · single molecule chemistry

Introduction

One of the most exciting challenges that has been taken up in chemistry in the last twenty years is the exploration of smaller and smaller scales, both in time and in space. At the forefront, on one side is the already mature and well-consolidated femtochemistry, on the other side is the much younger and, in many aspects, still pioneering chemistry of the single molecules. In the last few years in several laboratories a remarkable series of experiments were carried out on stretching single molecules with different tools, such as optical tweezers (OT), bendable microneedles, and the scanning force microscope (SFM), also called atomic force microscope.[1] These investigations were started from the pioneering force versus extension measurements of Bustamante and co-workers made on a single DNA molecule.[2] This work brought an experiment of classical physics into the world of single molecules. The general approach of all these experiments was to measure the force required to mechanically drive the structure of a single molecule towards new conformations and states that are otherwise very hard to reach. This approach was named "force spectroscopy" by several authors.

[a] Prof. B. Samorì
Dipartimento di Biochimica, Università di Bologna
via Irnerio, 48 40126 Bologna (Italy)
Fax: (+39) 051-354387
E-mail: samori@alma.unibo.it

The SFM was developed to image surfaces and single molecules deposited on a flat substrate by raster scanning a very sharp tip over the surface; this is carried out by a flexible cantilever. The force of interaction between the tip and the sample is continuously measured by monitoring the deflection of the cantilever and the force is also kept constant during the scanning.[3] With the SFM tip we can gently touch individual molecules: it is like playing at blind-man's buff with them (Figure 1).

The applications of SFM can go beyond imaging because it is intrinsically a force sensor.[4] The minimum force an AFM can detect is limited by both the noise from the detection system used to monitor the cantilever motion and by thermal motion of the cantilever itself. Under water, for the commercially available 200 μ long cantilever with a spring constant of about 60 mN m^{-1}, the measured force noise amounts to 7.4 pN. For a shorter cantilever the measured force noise can be decreased to 1.3 pN.[5] In manipulations of single molecules the measured force must, therefore, span from the tensile strength of a covalent bond, which is in the range of $2-4.5 \times 10^3$ pN,[6] to around 9 pN, which is needed to unpair a single AT base pair in DNA.[7] It must be emphasized that these nanomanipulation games are played in liquids and, when biological structures are involved, conditions close to those that exist in living organisms can be mantained.

After an introduction to the single-molecule dimension (certainly not very familiar to most chemists), the relationships between the single molecule force spectroscopy data, and the thermodynamic or kinetics data traditionally obtained with ensembles of Avogadro numbers of molecules are discussed. The present technical capabilities are mostly discussed in the next section on the tailoring of the experiments, and an overview of the recent advancements and the most important prospects of the technique have been considered in the section on the equilibrium and non-equilibrium processes and in the Conclusions paragraph.

Tailoring a Force Spectroscopy Experiment to Single Molecules

The commercial SFMs, originally developed for imaging, currently make it possible to approach the tip to a substrate

  0947-6539/00/0623-4249 $ 17.50+.50/0

Figure 1. Imaging and manipulating single molecules with the SFM is like playing at blind-man's buff with them: their shape is reconstructed not in the brain of the blind-man, but in a computer where the deflections of the cantilever are continuously recorded during the raster scanning of the tip on the sample. Reproduction from "la gallina ciega" by F. Goya (Museum EL Prado, Madrid).

upon which polymer molecules has been previously deposited, to load a desired force, and then to retract the tip: this cycle is normally repeated multiple times in sequence. Whenever a molecular bridge is formed between the two moving surfaces at the micro-contact, either because the tip has just picked up one end of a molecule[7], or because a bond was formed,[6, 8] the force acting on this bridge is reported versus the tip displacement, as in Figure 2. These force curves have a saw-tooth shape composed of a rising part followed by a sudden drop. The drop corresponds to breaking of the bridge, and its measure is an estimate of the rupture force. The rising part corresponds to the stretching of the polymeric bridge: the force necessary to stretch the polymer molecule increases with the extent of stretching, because the conformational space that the polymer can sample is increasingly reduced. It is like when one wants to hold a certain volume of gas under a piston; a squeezing force must be loaded against the pressure of the gas. Both cases deal with an entropic elasticity. The stretching of a molecule may in addition induce an increase of its contour length through a conformational transition which extends along the chain. This transition is revealed by a kink or a plateau in the force curve (Figures 2 and 3). In this case the elasticity has acquired an enthalpic component.

Commercial SFMs were modified[9, 10] or true nano-manipulators based on SFM technology were custom built[7, 11] in order to control at will the position and the displacements of the tip along the vertical z axis. This makes it possible to manipulate the molecular bridge repeatedly before reaching its rupture limit, by letting the tip approch and retract, step by step, many times. This capability allows us 1) to engage the tip on the substrate with the so-called "fly-fishing-mode", which minimizes the number of multiple bridges;[12] and 2) to verify

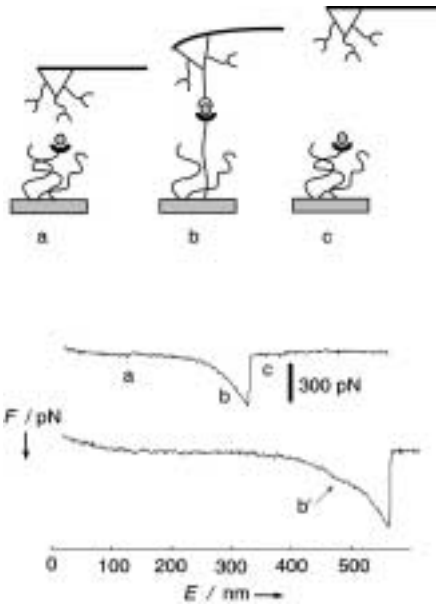

Figure 2. Top: Sketch of a) the approach of an SFM tip to a substrate; b) formation and stretching of a single molecular bridge between them, and c) rupture of the bridge in an approach and retraction cycle. Bottom: Characteristic shape of the force (F) versus extension (E) unbinding traces associated to the rupture the molecular bridge. The force curves have a saw tooth shape composed of a rising part followed by a sudden drop. The drop corresponds to breaking of the bridge, and its measure is an estimate of the rupture force. The rising part corresponds to the stretching of the polymeric bridge. The stretching may induce before the rupture of the bridge a conformational transition which extends along a segment of the chain and is revealed by a kink or a plateau (b′) (reproduced from reference [8]).

the reversibility of the investigated process, as shown by the two examples in Figure 3. The force-extension traces recorded by Fernandez et al.[13] on stretching and subsequently on

0947-6539/00/0623-4250 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 23

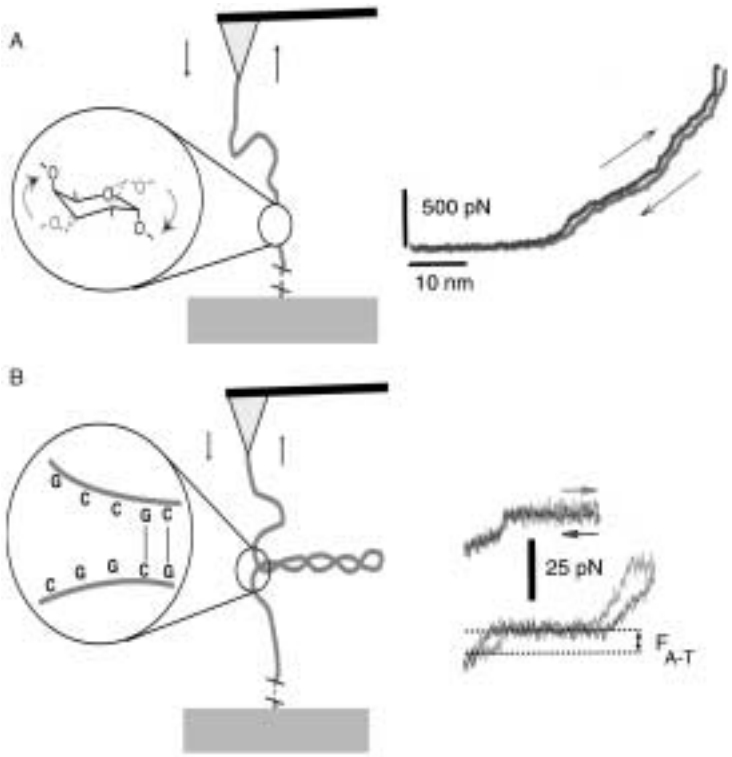

Figure 3. A) The stretching of pectin induces two chair-inversion transitions along the chain that are revealed by the two kinks in the force versus extension curves; the stretching (→) and relaxation (←) traces were superimposable (reproduced from reference [13]). B) A single DNA chain was opened by pulling apart its complementary strands, like the two sides of a zip. Also in this case the stretching and relaxation traces were superimposable (reproduced from reference [7]).

relaxing the same single chain of a native pectin, at a pulling speed of 500 nm s^{-1} are superimposable (Figure 3A). Another reversible behavior was recorded by Gaub et al.[7] while doing experiments of force-induced melting in a very long (48.5 kb) DNA strands. They recognized and assigned a plateau (occurring at 9 pN for a pure AT and at 20 pN for a pure GC sequence) to the zipping–unzipping transition in the double helix. The extension and relaxation curves were superimposable (Figure 3B).

A marked hysterisis was observed instead after a complete pulling cycle when a polymer of T4 lysozyme was unfolded and refolded, with a pulling speed in the same range as the two previous experiments.[10] The unfolding force-extension curve exhibited the characteristic saw-tooth pattern, which had been previously reported for multi-modular proteins composed of tandem arrays of globular domains, like titin (Figures 4 and 5). As first demonstrated by Gaub et al. the equally spaced peaks result from the sequential stepwise unfolding of the individual domains of the polymeric protein.[14] The origin of the hysterisis will be discussed in the section below on the equilibrium and non-equilibrium processes.

Is exactly one single molecule picked up by the tip?: The possibility of having a single molecular bridge between a functionalized tip and the substrate is normally connected to the capability of diluting the chemical functionalities on the surfaces, and, sometimes, also to a slow disentangling of the individual polymer filaments from a polymer brush. Whenever events involving more than one bridge take place, the distribution of the measured forces shows peaks that correspond to multiples of an elementary force.[15] This force, which relates to a single-molecule event, can be statistically evaluated even if only a few events took place at a single-molecule level. On the other hand, if we want to base our study on single-molecule events only and collect and analyze only data coming from them, we must be able to recognize them.

High-resolution SFM imaging can, in some cases, support this aim very effectively, by first locating a single molecule to be manipulated and then imaging the system again after the manipulation. This approach made it possible to estimate the intermolecular forces between individual proteins on a bacterial surface layer and to describe

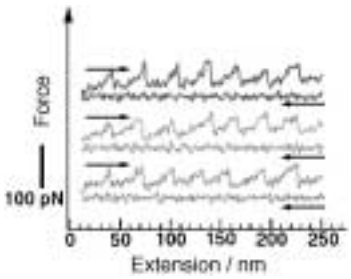

Figure 4. Force curves of the unfolding (→) and the refolding (←) of a polymer of T4 lysozyme (reproduced from reference [10]).

the unfolding pathway during their extraction.[16] Imaging with a single-molecule resolution was combined by Hinterdorfer et al. with force measurements of the molecular recognition of an antigen by an antibody.[17]

In most of the force spectroscopy studies so far reported, the single-molecule level could not be demonstrated by imaging the systems under investigation. This was due either to the inability of most nanomanipulators (in spite of being SFM-based) to image, or to a SFM resolution too poor to make this possible. The force spectroscopy experiments were therefore approached with a peculiar attitude: the main issue was not to reach the full reproducibility of one specific single-molecule event, but to recognize and assign the same event, whenever it took place.

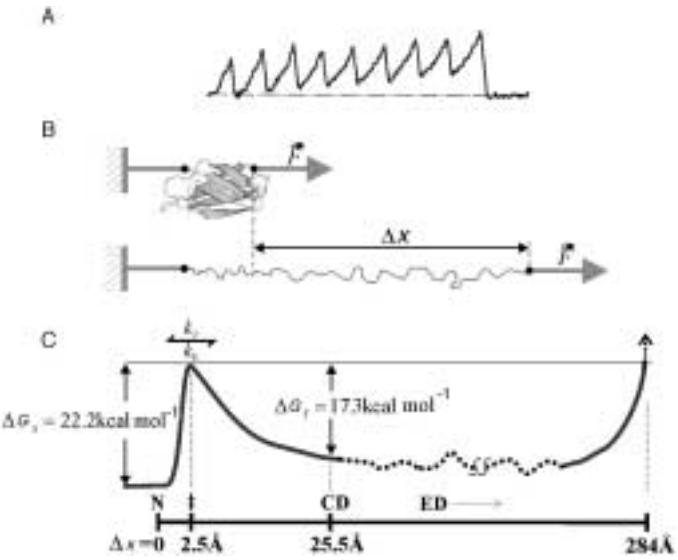

Figure 5. A) Saw-tooth-like pattern of the force curve of the unfolding of a single protein made of tandem repeats of identical immunoglobin modules. B) As the protein is elongated the restoring force increases until one of the domains unfolds; this unravelling suddenly increases the contour length of the protein and allows the force on the cantilever to fall to near zero. Further extension is resisted again by entropic forces until a second domain in the chain unravels and so on a saw-tooth pattern is thus obtained. C) The "energy landscape" of the folding pathway for a single domain was determined by SFM. The changes in free energy (ΔG) are plotted versus the process coordinate (Δx). Three distinct states were identified: native (N, $\Delta x = 0$), condensed denatured (C, $\Delta x = 25.5$ Å), and extended denatured (ED, $25.5 < \Delta x < 284$ Å). The transition state is located 2.5 Å away from the N state and 23 Å away from the C state. (reproduced from reference [36]).

Two approaches are very helpful to recognize when the tip has picked up exactly one single molecule.

1) Normally in a series of experiments the peaks in the force curves occur at different extensions, as also shown in Figure 2: the molecular bridges that are stretched have various contour lengths and therefore different elastic properties. The elastic properties should scale linearly with length in the experiments in which only one chain was picked up.[14, 8]
2) A plateau or a kink, whenever it is present in the force-extension curve, (Figures 2 and 3) can be used to monitor if simultaneous stretching of more than one molecule took place. In fact it is due to a conformational transition which should occur in a single molecule always at the same elementary force.[6, 8, 12, 13]

Can we manipulate only long polymer chains?: The molecular bridges stretched in all the experiments we have mentioned so far were long polymers such as DNA or polysaccharides. With long polymers the stretching experiment can be carried out while keeping the tip and the surface holding the other end of the molecule so far apart from each other, so that the strength of nonspecific interactions between them to a minimum. When smaller molecules are manipulated nonspecific attractive or repulsive interactions can drastically affect the force in the SFM experiments, and also a number of irreproducible features can appear in the force curves and obscure the interactions of interest at short extension (see for instance Figure 5 in ref. [10]). This is the reason why long spacers were introduced in the system to be manipulated.[18] On the other hand with long spacers 1) the load does not rise steadily under constant pulling speed, and the measured values of bond strength are modified;[19] 2) the compliance of the molecular bridge increases, and the force resolution decreases.[20] Non-specific interactions can be decreased also by tailoring the environment[18, 21] and the chemical composition of the two surfaces.[22]

The tailoring of the composition of the bridge should also take into account the possibility of including in the construction a polymeric linker, such as a DNA[23] or a polysaccharide chain,[6, 8, 12, 13] which would introduce a well-defined plateau in the force curve that can be used as an internal strain-gauge to calibrate the force acting on the molecular bridge.

Should we always anchor the chain to the two surfaces by covalent bonds?: Most of the experiments with polysaccarides and proteins were carried out just by adsorbing them onto a clean glass substrate or on gold surface. An individual chain was picked up with a SFM tip by applying a contact force of several nN over seconds. This attachment protocol was shown to be applicable to a broad range of molecules and results in stable attachment under forces of up to a nN.[7]

When the rupture forces measured reach values of the order of one nN, the molecule to be stretched must be covalently bound to the tip and to the substrate. By far the most popular approach to tip and substrate functionalization is to coat them with gold or platinum and then immobilize thiol-functionalized molecules on their metal-coated surfaces. Stripped-template gold surfaces are preferentially used as substrate because of their flatness.[24] Covalent bonding to the tips can alternatively be attained by using organosilane functionalities.

Equilibrium and Non-Equilibrium Processes

The general approach of all the experiments outlined above is to induce ruptures of noncovalent or covalent bonds by applying an external force. If we could wait long enough the force required to rupture one bond would measure zero, because any unbinding barrier can be thermally overcome. An external force, if properly directed, speeds up the kinetics of dissociation by tilting the "energy landscape" of the unbinding path and lowering the activation barrier. The kinetics may be sped up as much as necessary to trigger the unbinding on the timescale of the experiment. Bond strength may be therefore thought of in terms of the force that is most likely to break the bond over a particular timescale.[25–28]

If the applied force is not large enough to eliminate the activation barrier, the surmounting of the barrier is still thermally activated and may take place in quasi-equilibrium conditions. This will occur provided that the pulling velocity is slow enough to let the molecule fluctuate thermally at any extension point, as much as to visit a significant fraction of its accessible conformational space. The re-equilibration of the solvent shell can be normally considered a much faster process that can be accounted for as a background equi-

  0947-6539/00/0623-4252 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 23

librium fluctuation. Molecular dynamics simulations of unbinding processes in quasi-equilibrium conditions have been reported by Schulten et al.[29]

Experiments based on the rupture of the molecular bridge between the tip and the substrate are like digital on-off processes and cannot be reversible. In fact after the bridge was broken the two separated parts relax independently. In contrast, in a stretching experiment based on mechanical manipulations of the bridge before its rupture, equilibrium conditions can be attained, provided that the pulling velocity is slow enough to get a reversible path, like those shown in Figure 3. Can we predict how slow the velocity should be? A thorough theoretical description making it possible to answer this question has not been made available yet. Generally, it has been said that the structural changes must occur much faster than the time the experiment takes,[30] but also the stiffness and the viscoelastic response of the cantilever plays an important role, which is intertwined with that of the pulling velocity.

The thermal fluctuation of the tip position (δz) within the potential well is determined by the stiffness of the cantilever. With a spring constant of 60 pN nm^{-1}, δz is expected to be about 3 Å in air [from Eq. (3) in ref. [29a]], which is of the order of magnitude of the width of the potential well of a covalent bond. This value of fluctuation amplitude is expected to decrease with the solvent and with the tethering of the molecular bridge, unless occasionally reinforcement effects occur.

We may tailor the experiment as to extend the tip in increments followed by a pause at a constant force that allows the fluctuating system to equilibrate. This will take place if the intervals after each extension increment are long compared with the relaxation time of the bond-opening and -reclosing reaction (τ_r). More commonly the manipulation experiments are performed by pulling the tip continuously at a constant speed (v). During its motion the tip allows a δz "breathing" of the system. This breathing may involve opening and reclosing of bonds and/or advancing and relaxing of conformational transitions along the chain. If we want to keep the process on a quasi-equilibrium pathway, the pulling velocity must be slow enough to leave the molecule "breath", at any point sequentially reached, for a time compatible with a thermal equilibration. This ought to take place whenever $\delta z/v$, that is, the time required for the tip to be retracted by a length equal to δz, is longer than τ_r. In analogy with the so-called Deborah number (De) used to compare data of polymer elongation with flows of different viscosity and strain rate, a dimensionless parameter is here proposed and named "stretching parameter" [S in Eq. (1)].

$$S = \tau_r v/\delta z \quad (1)$$

It can be used for comparing data collected with different cantilevers and pulling speeds, and it can be useful for the tailoring of quasi-equilibrium conditions provided that δz has been estimated in the conditions of the stretching experiment. We can try to apply Equation (1) to two experiments previously described by using the in-air δz value. As estimated in reference [31], the S parameter for the experimental conditions of the pectin stretching reported in reference [13] results in a value smaller than one, as required for the quasi-equilibrium condition. In fact the process in those conditions was reported to be reversible.

As theoretically simulated for a dextran chain[30] and experimentally shown in chromatin manipulation by OT,[32] above a critical pulling speed the reversibility is lost: the force starts changing with the pulling velocity and a hysteresis appears between the extension and the relaxation cycle that indicates a transition to a non-equilibrium regime.

After that critical pulling speed the unfolding processes are sped up with respect to equilibrium condition, while the refolding is slowed down by the tensions along the bridge and by the decrease of the rotational freedom of the chain domains. This is the origin of the hysterisis between the extension and the relaxation traces and the dependence of the unfolding force on the pulling speed. In this condition, the molecules are driven by the external force to metastable non-equilibrium conformations which could clearly not occur if the elongation allows the molecules to equilibrate constantly. With no equilibrium the process becomes dissipative, the amount of energy equal to the area inside the hysteresis curve is locally wasted as heat,[9] and because of these irreversible components, the forces at which the processes take place is found to increase with the force loading rate.[27]

The S value estimated in reference [31] for the experimental conditions so far most commonly used in protein unfolding experiments, tells us that quasi-equilibrium conditions could be attained only if the tip velocity is slowed down by three or four orders of magnitude: this is hardly compatible with the stability of the instruments presently available. On the other hand these unfolding processes of multimodular proteins were very recently demonstrated to be kinetic and not equilibrium processes.[33]

In summary, many unfolding and unbinding processes take place in nature in a irreversible way and are accompanied by dissipative work. These processes can be simulated and studied at the single-molecule level by force spectroscopy experiments and the features of their non-equilibrium "energy landscapes" can be explored along the different pathways that we can select by changing the stiffness of the tip cantilever and the pulling rate.[27, 29] If we are interested instead to those processes that take place in quasi-equilibrium conditions and are governed by thermodynamic stability, the capability of recording those events under constant pulling forces can play a crucial role in these studies: this kind of experiment may require in most cases a stability hardly achievable for the nanomanipulators currently available.

Pathways Mechanically Driven in Single Molecules and Pathways Thermally or Chemically Activated in Macroscopic Systems

Unbinding or unfolding forces may be governed by thermodynamic stability (free energy difference between the bound or folded and unbound or denatured states) or by the free energy difference between the bound or folded states and the

transition states. The height of the energy barrier can be estimated from the rate constant of the mechanically driven process extrapolated to zero force. The rate constants are obtained from the force–frequency histogram and from the dependency of the force on the pulling rate, as described in references [18, 30, 34–36]. In parallel, from the same experimental data also a parameter Δx is obtained. It indicates the reaction length over which the force must be applied to reach the transition state. In the case of an unbinding process, this parameter corresponds to the difference in bond length between the bound and the transition state, in the case of an unfolding process, as in Figure 5, it corresponds to the difference in the end-to-end extension between the folded and the transition state.

Across a series of different antibody–antigen complexes Pluncktun et al. very recently found that the unbinding forces correlate with the thermal dissociation rates in solution and not with the activation or equilibrium enthalpies. They also found that Δx correlates with the height of the transition state regardless of the details of the binding site, which most likely reflects the plasticity of the protein.[34] The same indication of a strong similarity between the pathway of the mechanical unbinding and that of the thermal dissociation was also found by Guntherodt et al. in SFM experiments of separation of complementary DNA strands.[37]

Fernandez et al. recently addressed the problem of the relationship between mechanical and chemical unfolding of protein domains.[33, 36] They showed how SFM experiments on single proteins can provide both rate constants extrapolated to zero force and the Δx values, not only for the unfolding but also for the refolding process. On this basis the thermodynamical stability can be also estimated for single molecules. The data they obtained for a series of immunoglobin modules, for their homopolymers and heteropolymers, were compared with those obtained on the same systems by chemical denaturation in solution extrapolated to 0 M denaturant (guanidinium chloride). They found a very close agreement between the SFM and the solution data. Both sets of data suggested that the unfolding force is not governed by their thermodynamic stability, but rather by the unfolding activation energy.

Those similarities between mechanically, thermally, or chemically driven pathways could throw doubt upon the importance of keeping doing experiments on single molecules. What can these experiments add to the traditional bulk ones?

Ensemble-Averaged and Single-Molecule Methods

Single-molecule measurements can be used to record rare events, fluctuating or stockastic behaviors, and can explore properties not detectable with traditional, ensemble-averaged methods. One clear example was very recently provided by Fernandez et al.: by examining the fidelity of mechanical refolding by repeatedly unfolding and refolding an extracellular matrix protein, they captured a "skip" misfold which could significantly affect the function of that protein.[38]

Single-molecule measurements, which overcome the limitation of solution methods of the averaging experimental parameters on the entire ensemble of presumably identical molecules, provide information on the distribution of the observables rather than just their average values. This can give crucial insights, as shown by a constantly increasing number of papers. For instance S. Chu et al. found that identical DNA molecules under identical conditions follow a multitude of paths as they are extended in elongation flows.[39] On imaging by SFM the diffusion of individual *E. coli* RNA polymerase molecules along DNA chains, Bustamante et al. found that several of the imaged molecules followed other mechanisms of facilitated targetting, such as inter-segment transfer and hopping.[40] These mechanisms were demonstrated for the first time by this single-molecule study. One further example is provided by a force spectroscopy experiment on the coordination bond between a histidine tag and Ni-nta complex (nta = nitriloacetate).[8] It revealed that their encounter may lead, with markedly different probabilities, to at least two types of complexes with different stability and with different "energy landscapes" along their force-driven dissociation pathway. All these kinds of information could not be accessed by means of traditional experiments.

The importance of performing single-molecule experiments is evidenced also by recent optical spectroscopy experiments, which provide an unprecedented insight into static and dynamic molecular inhomogeneity previously obscured by ensemble averaging.[41] Distributions and time trajectories of physical observables connected to conformational states, conformational dynamics, and activity of single biological macromolecules can now be measured by fluorescence spectroscopy on single molecules without the impossible need to synchronize all the molecules in the ensemble.[42]

Single-molecule experiments reveal that nature is more heterogeneous than we could so far figure out. For instance, the present picture of the protein folding "landscape" is that it resembles a funnel which guides the protein through many different sequences of traps towards the low-energy folded (native) structure. Why should only one structure be expected at the end of the funnel? Another complex biological process such as enzyme activity will most likely show in the future a multiplicity of kinetics paths that only single-molecule experiments may make possible to characterize.

Conclusion

Mechanochemical experiments are expanding chemistry into new realms between biology and materials. The above-mentioned works on the mechanochemistry of modular proteins have already given important insights on the physical origin of the elasticity of natural adhesives and muscle proteins. On this basis mechanically complex modular proteins, novel tissues, and fibers can be engineered.

A large numbers of molecular motors have been discovered in recent years: they use chemical energy to carry out mechanical processes. Most enzymes act as molecular motors because large conformational changes are involved in their catalytic activity. Their kinetics and thermodynamic proper-

0947-6539/00/0623-4254 $ 17.50+.50/0 *Chem. Eur. J.* **2000**, *6*, No. 23

ties are affected by external forces. Their function and mechanism could be explored on this basis by force spectroscopy experiments.[43]

The construction of nanosized bioelectronic circuits requires methodologies for site-selective positioning and assembly of single molecules. Methodologies of mechanical manipulations of single molecules can crucially support the construction of complex biocatalytic surfaces miniaturized to the nanometer, and, afterwards, also map their functioning at the single-molecule scale. These methodologies are developing very rapidly, and future advances will depend on technical improvements in the time resolution of the measurements and the mechanical and thermal stability of the manipulation tools. Further advances in the theory are still necessary for both the tailoring of the experiments and the interpretation of the data.

Acknowledgement

I thank all my students and collaborators, M. Conti, G. Falini, G. Zuccheri in particular, and also E. Evans (Vancouver) and Gouliang Yang (Philadelphia) for comments on the manuscript, L. Lunazzi (Bologna) for a discussion on the relaxation times used in reference [31]. All my conversations, always so enlightening, with Carlos Bustamante (Berkeley) are gratefully acknowledged. This work was supported by Programmi Biotecnologie legge 95/95 (MURST 5%); MURST PRIN (Progetto Biologia Strutturale 1997–1999, 1999–2001).

[1] B. Samorì, *Angew. Chem.* **1998**, *110*, 2318–2321; *Angew. Chem. Int. Ed.* **1998**, *110*, 2198–2200, and references cited therein.
[2] S. B. Smith, L. Finzi, C. Bustamante, *Science* **1992**, *258*, 1122–1126.
[3] C. Bustamante, D. Keller, *Phys. Today* **1995**, *Dec.*, 32–38.
[4] H. Takano, J. R. Kenseth, S. Wong, J. C. O'Brien, M. D. Porter, *Chem. Rev.* **1999**, *99*, 2485–2490.
[5] M. B. Viani, T. E. Schaffer, A. Chand, M. Rief, H. E. Gaub, P. K. Hansma, *J. Appl. Phys.* **1999**, *86*, 2258–2262.
[6] M. Grandbois, M. Beyer, M. Rief, H. Clausen-Schaumann, H. E. Gaub, *Science* **1999**, *283*, 1727–1730.
[7] M. Rief, H. Clausen-Schamann, H. E. Gaub, *Nat. Struct. Biol.* **1999**, *6*, 346–349.
[8] M. Conti, G. Falini, B. Samorì, *Angew. Chem.* **2000**, *112*, 221–224; *Angew. Chem. Int. Ed*. **2000**, *39*, 215–218.
[9] B. L. Smith, T. E. Schaffer, M. Viani, J. B. Thompson, N. A. Frederick, J. Kindt, A. Belcher, G. D. Stucky, D. E. Morse, P. K. Hansma, *Nature* **1999**, *399*, 761–763.
[10] G. Yang, C. Cecconi, W. A. Baase, I. R. Vetter, W. A. Breyer, J. A. Haack, B. W. Matthews, F. W. Dahlquist, C. Bustamante, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 1–6.
[11] A. F. Oberhauser, P. E. Marszalek, H. P. Erickson, J. M. Fernandez, *Nature* **1998**, *393*, 181–185.
[12] M. Rief, F. Oesterhelt, B. Heymann, H. E. Gaub, *Science* **1997**, *275*, 1295–1297.
[13] P. E. Marszalek, Y.-P. Pang, H. Li, J. El Yazal, A. F. Oberhausen, J. M. Fernandez, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 7894–7898.
[14] M. Rief, M. Gautel, F. Oesterhelt, J. M. Fernandez, H. E. Gaub, *Science* **1997**, *276*, 1109–1112.
[15] a) E. L. Florin, V. T. Moy, H. E. Gaub, *Science* **1994**, *264*, 415–417; b) V. T. Moy, E. L. Florin, H. E. Gaub, *Science* **1994**, *266*, 257–259; c) G. U. Lee, L. A. Chris, R. J. Colton, *Science* **1994**, *266*, 771–773.
[16] a) D. J. Muller, W. Baumeister, A. Engel, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 13170–13174; b) F. Oesterhelt, D. Oesterhelt, M. Pfeiffer, A. Engel, H. E. Gaub, D. J. Muller, *Science* **2000**, *288*, 143–146.
[17] A. Raab, W. Han, D. Badt, S. J. Smith-Gill, S. M. Lindsay, H. Schindler, P. Hinterdorfer, *Nat. Biotechnol.* **1999**, *17*, 902–905.
[18] P. Hinterdorfer, W. Baumgartner, H. J. Gruber, K. Schilcher, H. Schindler, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 3477–3481.
[19] E. Evans, K. Ritchie, *Biophys. J.* **1999**, *76*, 2439–2447.
[20] C. Bustamante, C. Rivetti, D. J. Keller, *Curr. Opin. Struct. Biol*. **1997**, *7*, 709–716.
[21] D. J. Muller, D. Fotiadis, S. Scheuring, S. A. Muller, A. Engel, *Biophys. J.* **1999**, *76*, 1101–1111.
[22] C. Duschl, A. F. Sevin-Landais, H. Vogel, *Biopys. J.* **1996**, *70*, 1985–1995.
[23] A. Noy, D. V. Vezenov, J. F. Kayyem, T. J. Meade, C. M. Lieber, *Chem. Biol*. **1997**, *4*, 519–527.
[24] P. Samorì, J. Diebel, H. Lẅe, J. P. Rabe, *Langmuir* **1999**, *15*, 2592–2594, and references cited therein.
[25] G. I. Bell, *Science* **1978**, *200*, 618–627.
[26] E. Evans, K. Ritchie, *Biophys. J.* **1997**, *72*, 1541–1555.
[27] R. Merkel, P. Nassoy, A. Leung, K. Ritchie, E. Evans, *Nature* **1999**, *397*, 50–53.
[28] K. L. Sebastian, *Phys. Rev. E* **2000**, *62*, 1128–1132.
[29] a) M. Balsera, S. Stepaniants, S. Israilev, Y. Oono, K. Schultzen, *Biophys. J.* **1997**, *73*, 1281–1287; b) S. Izrailev, S. Stepaniants, M. Balsera, Y. Oono, K. Schulten, *Biophys. J.* **1997**, *72*, 1568–1581; c) J. Gullingsrud, R. Braun, K. Schulten, *J. Comput. Phys.* **1999**, *151*, 190–211.
[30] M. Rief, J. M. Fernandez, H. E. Gaub, *Phys. Rev. Lett.* **1998**, *81*, 4764–4767.
[31] For a two-state reaction $\tau_r = 1/(k_1 + k_{-1})$. The first-order rate constant for ring inversion in cyclohexane is 10^4–$10^5\ s^{-1}$ at 300 K. The activation parameters for the same reaction in tetrahydropyrane are very similar to those in cyclohexane (J. B. Lambert, C. E. Mixan, D. H. Johnson, *J. Am. Chem. Soc.* **1973**, *95*, 4634–4639). The substitution of the tetrahydropyrane ring in the pectin chain most likely lowers the corresponding τ_r by at least one order of magnitude. On the other hand with a $\tau_r = 10^{-3}$ s, for $\delta z = 0.3$ nm and $v = 0.5$ nm/ms used in ref. [13], S is still less than 1. In the case of a modular protein the rate constants for the unfolding and refolding were estimated to be 3.3×10^{-4} and $1.2\ s^{-1}$, respectively: 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* **1999**, *96*, 3694–3699. By using the same velocity and cantilever as above, which are the most commonly used, S is about 10^3.
[32] Y. Cui, C. Bustamante, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 127–132.
[33] H. Li, F. Oberhauser, S. B. Fowler, J. Clarke, J. M. Fernandez, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 6527–6531.
[34] F. Schwesinger, R. Ros, T. Strunz, D. Anselmetti, H-J. Guntherodt, A. Honegger, L. Jermutus, L. Tiefenauer, A. Plückthun, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 9972–9977.
[35] P. Hinterdorfer, K. Schilcher, W. Baumgartner, H. J. Gruber, H. Schindler, *Nanobiology* **1998**, *4*, 177–188.
[36] 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* **1999**, *96*, 3694–3699.
[37] T. Strunz, K. Oroszlan, R Scafer, H. J. Guntherodt, *Proc. Natl. Acad. Sci. USA.* **1999**, *96*, 11277–11282.
[38] A. F. Oberhauser, P. E. Marszalek, M. Carrion-Vazquez, J. M. Fernandez, *Nat. Struct. Biol.* **1999**, *6*, 1025–1028.
[39] T. T. Perkin, D. E. Smith, S. Chu, *Science* **1997**, *276*, 2016–2021.
[40] C. Bustamante, M. Guthold, X. Zhu, G. Yang, *J. Biol. Chem.* **1999**, *24*, 16665–16668.
[41] W. E. Moerner, M. Orrit, *Science* **1999**, *283*, 1670–1675.
[42] S. Weiss, *Science* **1999**, *283*, 1676–1683.
[43] D. Keller, C. Bustamante, *Biophys. J.* **2000**, *78*, 541–556.