BIOPHYSIQUE À L'ÉCHELLE DE LA MOLÉCULE UNIQUE
SINGLE MOLECULE BIOPHYSICS

Foreword

In the past 10 years, biophysics has been revolutionized by the development of methods to visualize and manipulate single bio-molecules. These techniques have been applied both to the study of the physical properties of bio-molecules (elasticity of DNA [1–6], mechanical denaturation of proteins [7–9], etc.) and to the study of molecular motors, enzymes that use chemical energy to perform mechanical work (myosin, kinesin, RNA-polymerase, topoisomerases, etc.). One of the issues to be addressed was the ability of molecular motors to produce a force stroke. The first clear single molecule measurements of the force developed by a single motor (myosin and kinesin) were published in 1993–94 by the groups of S. Block [10], J. Spudich [11] and T. Yanagida [12]. An other fascinating example is the study by the group of K. Kinosita [13] of the adenosine triphosphate (ATP) driven rotation of the γ sub-unit of F_1-ATPase, the usual generator of ATP in the cell which doubles as a rotary motor when ATP is supplied. These experimental approaches are now used to study a growing number of enzymes. In contrast to usual test-tube assays which yield ensemble averaged measurements, single molecule experiments provide information on distribution and time trajectories of observables that do not require the synchronization of the whole ensemble. Enzymatic variables, such as the rate, processivity (the number of cycles made consecutively) and step-size can be directly measured and differences in activity due to the local environment (e.g. sequence) can be quantified. Whereas most ensemble measurements can be deduced from single molecule data, the converse is not true: some single molecule experiments (see DNA unzipping below) are simply impossible to do in ensemble experiment. Another case in point is the intriguing observation by the group of X.S. Xie [14] of molecular individuality and memory in the activity of an enzyme. In this work single molecule fluorescence methods described in the following were used to moninor the natural change in fluorescence of the enzyme's active site during a catalytic cycle. In this special issue we have tried to give a flavor of this rapidly developing field from various perspectives. Since molecular motors were specifically addressed previously [15], we have focus the review more on the the techniques and their various applications.

1. Force at the molecular level

To manipulate single molecules one should be aware of the forces acting at the molecular level and choose a manipulation technique adapted to the relevant force range. The Brownian fluctuations (due to shocks with solvent molecule) are responsible for the smallest measurable forces at the molecular level. For example, the average force acting on a 1 μm size bead every second is equal to about 10^{-14} N $= 10$ fN, which is comparable to its weight. Above this level, one encounters forces typical of molecular motors which transform the chemical energy of ATP into mechanical work. As ATP hydrolysis yields $\sim 20k_{\mathrm{B}}T$ ($k_{\mathrm{B}}T = 4 \cdot 10^{-21}$ J) and molecular dimensions are on the order of 10 nanometers, the characteristic force associated with such motors is of the order of 5 pN (1 pN $= 10^{-12}$ N). Next on the scale come cohesion forces associated with cooperative hydrogen bonding, hydrophobic interactions, etc. These are partly responsible for the stability of bio-molecules, for example the stability of the native state of proteins. They are of order 100 pN, the typical force required to break a non-covalent bond [16,17] and denature a protein [7–9,18,19]. Finally, the strongest forces at the molecular level, of order 1000 pN, are those required to break a covalent bond [20] (of energy O(1 eV) and scale O(1 Å)).

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2. Techniques of single molecule micromanipulation

There are by now many ways to manipulate single molecules: using optical or magnetic tweezers, atomic force (AFM) cantilevers, glass micro-fibers and bio-membrane force probes. In all these techniques, a DNA molecule, protein or other bio-polymer is bound to a surface at one end and to a force sensor at the other (Fig. 1). The force sensor is usually a trapped micron-sized bead or a cantilever, whose displacement can be measured to determine the force. This sensor behaves like a noisy damped oscillator: for a given a temporal resolution, the noise in the force measurement depends solely on the dissipation due to the viscous drag on the sensor.

Different force, displacement and time scales are afforded by these techniques. AFM cantilevers are capable of measuring angstrom-scale, millisecond events and forces larger than 10 pN [26–28]. Glass
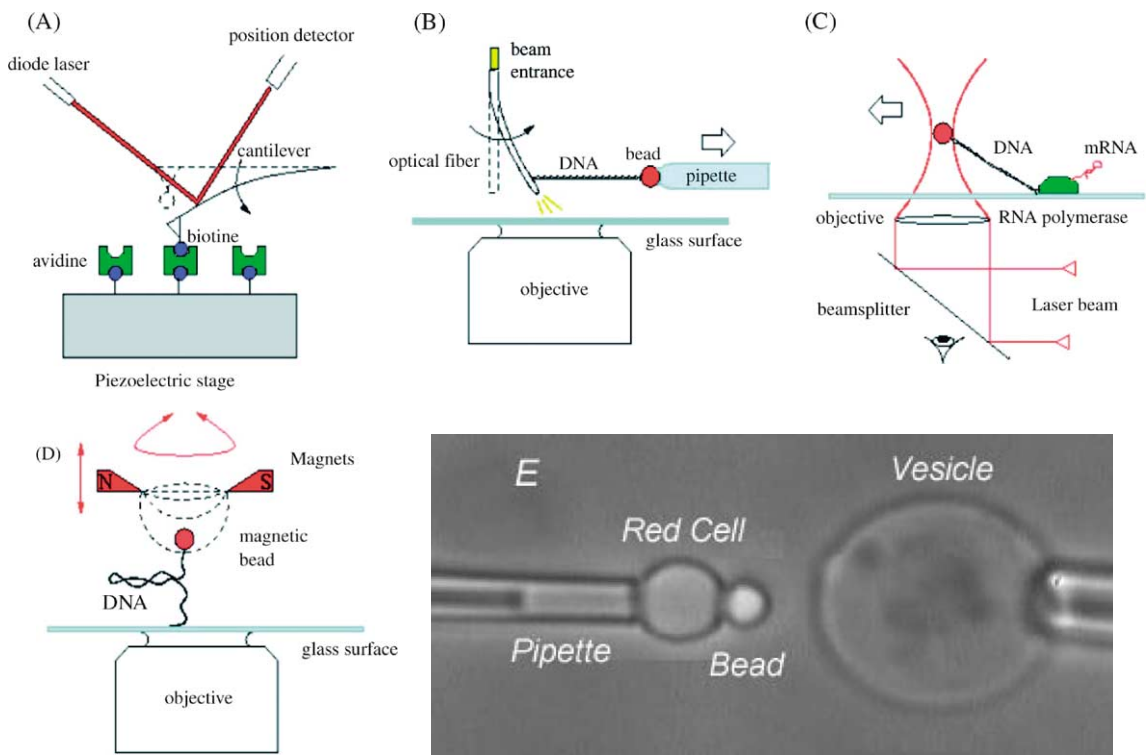

Figure 1. Example of forces transducers. (A) An AFM cantilever is often used as a force transducer during intermolecular force measurements [16] and protein unfolding experiments [7]. Its deflection upon pulling is detected by the displacement of a laser beam reflected from the cantilever. (B) In some of the experiments involving DNA pulling [21,22] and unzipping [23], the force transducer is an optical fiber, whose deflection is detected optically (by measuring the displacement of either the fiber directly on a microscope stage or of a light beam emitted from its pulled end). (C) A force transducer often used to characterize molecular motors [11,24,25] is optical tweezers, which consist of a single, strongly focused laser beam holding a bead at its focal point. The displacement of the bead in the trap is observed with a microscope and, together with the trap stiffness, is used to assess the trapping force. (D) Small magnets can be used to pull and twist a DNA molecule tethering a super-paramagnetic bead to a surface. As explained in the text, in this case the force can be deduced from the amplitude of the Brownian fluctuations. (E) A small vesicle held in a micropipette by a controlled suction can be used as a force probe. A small bead stuck to this vesicle carries a ligand whose interaction with receptors on a nearby cell can be deduced from the deformation of the membrane under tension. Since the stiffness of the sensor is set by the pressure difference across the membrane, a particularly large force range is accessible by this method. (This illustration was kindly provided by F. Pincet and E. Perez.)

micro-fibers that are larger than cantilevers do not achieve such high spatial and temporal resolution, but can be used to measure piconewton forces [21,29,30]. Optical (magnetic) tweezers allow the measurement of pico(femto)-newton forces and nanometer displacements on a sub-second timescale (see [31,32] and [2, 33,34]). Magnetic tweezers and micro-fibers further allow twisting of the molecule by rotating the magnets or the fibers where the bead is attached [33,35–38]. In each technique, it is possible either to keep the force constant and measures a displacement, or to fix the extension and to measure a force. Although some techniques are natural extension clamps (cantilevers, optical tweezers) and others force clamps (magnetic tweezers), an appropriate feedback loop can convert a force clamp into an extension clamp and vice versa. All these techniques rely upon a sensitive detection of the sensor position: often the position of the handle attached to the bio-molecule is measured with an accuracy of a few nanometers. This is feasible but not always simple and we shall see that extracting signal from noise will generally require some work.

Single molecule visualization can be performed using fluorescence, the emission of a Stokes-shifted signal upon excitation with an incident light. Non-fluorescent biomolecules can be observed by grafting fluorophore moities in a non-perturbative way. Unfortunately all fluorophores emit a finite number of photons before being destroyed. This number varies from one fluorophore to the next but usually range from 10^3 to 10^6. All the art of single molecule visualization resides in acquiring a maximum of information from a relatively small number of detected photons. This implies well designed light collection and sensitive detectors.

3. Theoretical models

The first paper by S. Cocco et al. [39] reviews the statistical-mechanical theories of single-molecule micromanipulation experiments on nucleic acids. It presents a rare instance in biophysics of a remarkable accord between experiments and sophisticated physical models. In particular, the agreement between the measurement of the elastic properties of double-stranded DNA and the elastic theories of an ideal (i.e. non-interacting) polymer under tensional and torsional stress (Worm-like and Rod-like chain models) has provided the best estimates of the bending and torsional modulus of DNA. This solid theoretical foundation is being used extensively to study more complex situations involving microscopic interactions such as the elasticity of single-stranded DNA where self-avoidance and the formation of tertiary (hairpin) structures play an essential role, the study of structural transitions in DNA under stress, DNA unzipping and the interaction of DNA with proteins. Building on the known energetics of interactions between base-pairs in DNA and RNA, these theoretical approaches make detailed predictions on the pathway of DNA unzipping and RNA folding and unfolding that are presently tested in single molecule experiments.

4. DNA unzipping

One such experiment, the mechanical unzipping of DNA is presented in the second paper by U. Bockelmann et al. [40]. The DNA construct and manipulation set-up described in that paper are among the most sophisticated systems built for single molecule studies. They permit the reproducible study of the unzipping of more than 10 000 base-pairs long DNA molecules with a resolution of a few base-pairs. There is an excellent agreement between the measured force versus extension (stick–slip) signal and the predictions derived from a model taking into considerations the energetic of base-pairing and the elasticity of the DNA chains and the displacement sensor. Essentially the molecule is storing elastic energy during the stick (force increasing) phase which it releases as a burst of unpairing (melting) in the slip phase. The experimental resolution is completely accounted for by the effective stiffness of the system which decreases as unzipping proceeds, i.e. as the length of the ssDNA strands increases. This suggests that the technique might be used to detect single base-pair mutations or sequence DNA, if the stiffness of the measurement chain could be increased by a factor 10.

5. Observing single enzymatic cycles

The third paper by T. Strick et al. presents an example of the study of DNA/protein interactions by single molecule manipulation techniques [41]. The enzymes studied in that work are topoisomerases, an essential protein that is involved in DNA disentanglement (decatanation, unknotting, etc.). Magnets are used to pull on and rotate a small super-paramagnetic bead anchored to a surface by a single DNA. The molecule can thus be twisted and stretched. The removal of supercoil by a single topoisomerase can be monitored in real time by following the resulting increase in the DNA's extension [42]. The measured extension signal is typical of experiments studying the interaction of proteins (helicases, DNA and RNA polymerases [43,44], transcription factors [45], exonucleases) with DNA, via a modification of its length. The enzyme's rate is easily deduced from the rate of change of the molecule's extension, its processivity by the total change in extension between bursts of enzymatic activity. An other feature common to many DNA-based motors is the coupling of their mechanical activity to a chemical energy source, typically ATP. This coupling may allow one to slow down the enzyme sufficiently to study its individual cycles through the quantized changes (steps) in the DNA's extension. In the case of type II topoisomerases, such a study provided the first direct evidence of the relaxation of supercoils two turns at a time. Finally, single molecule manipulation assays provide one with a new parameter to control the enzymatic activity: the stretching force. It can be used to alter the enzyme's rate [42,44,46] and learn more about its mechanism, as exemplified in this paper.

6. The art of single molecule visualization

Single molecule manipulation experiments are only one facet of the recent revolution in biophysics. The other side has to do with the development of fluorescent dyes and sophisticated techniques to observe and study conformational changes in single bio-molecules. This is the subject of the last two papers in this issue. X. Michalet and S. Weiss review [47] some of the recent advances in detector sensitivity and in instrument design that have enabled researchers to probe single molecule with light and study their spectroscopic properties and temporal evolution. To observe a single fluorescent molecule, great care must be exercised to reduce the background and noise levels. The former can be reduced by total internal reflection microscopy, the use of a confocal design or a near-field scanning optical probe. The signal to noise ratio can be improved by using fluorophores of high quantum efficiency, efficient collecting optics and the low dark current detectors presently available. However, a recurrent problem of all fluorophores is the existence of dark states that limit the number of photons that can be extracted from a single molecule. A corollary of that limitation is that single dye molecules can be observed with a given spatial resolution only for a limited time (usually less than a second). During that time spectral information (either fluorescence lifetime, polarization or energy transfer) can be used to characterize changes in molecular conformation or the molecule environment.

7. Visualizing in the cell

In a biological in vivo context the recent discovery of fluorescent proteins (GFP and DsRed) has opened a new vista in the study of processes at the single cell (if not yet single molecule) level. This is the subject of the last paper by L. Cognet et al. [48]. By mutating the naturally occuring Green Fluorescent Protein, research has generated a number of GFP mutants covering a large part of the visible spectrum. Fusions of these fluorescent proteins to cellular proteins of interest permit the observation of their dynamics and localization during the cell cycle. The major problems which presently hinder the observation of a single fluorescent protein in a live cell are the high level of background auto-fluorescence present in a cellular environment and the limited number of photons (a few thousands) that can be extracted from these proteins before they go into dark states. For these reasons experiments with individual fluorescent proteins are a challenge that will need further research and technological improvement before they are routinely used in biological laboratories.

Single molecule in biophysics is developing rapidly and a full coverage of this subject is beyond the scope of the articles of this issue. The reader can consult several review articles and books: a special *Science* issue was devoted in 1999 to this subject [49]; J. Howard has published a book relating experiments done on molecular motors [50]; reviews covering stretching of polymers, molecular motors, etc., may be found in [51–56]. Recent publications reporting new fascinating results such as the contraction produced by light on a stretched polymer [57], or the study of the motor which package the DNA molecule inside the bacteriophage [58] will complete the picture that we have sketched here.

D. Bensimon
V. Croquette
Laboratoire de physique statistique et Département de biologie
École normale supérieure, UMR 8550 CNRS,
associé aux universités Paris VI et Paris VII,
24, rue Lhomond, 75231 Paris cedex 05, France

Acronyms and definitions

AFM (Atomic Force Microscopy): special microscope derived from the Scanning Tunneling Microscope. A sharp tip is scanned above the surface of interest while the applied force is servoed to remain constant.

ATP (Adenosine Tri-Phosphate): one of the nucleic acids (adenosine) involved in the RNA. It is in the high energy three phosphate form. It's hydrolysis fuels nearly all enzymatic activity.

F_1-ATPase (the enzyme sub-unit F_1-ATPase): sub-unit F1 of the enzyme in charge of producing ATP from its lower energy components: Adenosine Di Phosphate and Phosphate [59].

kinesin (the kinesin molecular motor): a motor protein which moves along microtubules. This protein is very processive it typically transports small vesicles over long distances in the cell (in the neurones for instance. For further information see http://www.blocks.fhcrc.org/~kinesin/index.html).

microtubules (microtubule filament protein): a globular protein made of two sub-units which assembles in tubular form creating a very rigid filament involved in many cellular processes.

myosin (the myosin molecular motor): a motor protein family associated with the actin filament. Myosin II is a major component found in muscle and responsible for contraction (more information may be found at: http://www.mrc-lmb.cam.ac.uk/myosin/myosin.html).

BFP (biomembrane force probes): a force sensor introduced by E. Evans [60] based on the attachment of either a red blood cell or a phospholipid vesicle held at the tip of a micropipette under a slight depression. The stiffness of this sensor can be evaluated directly from the depression and the measure of the diameters of the pipette-tip and the vesicle.

biotin (biotin molecule): a small molecule also called Vitamin H. It is the paradigm of the key in the key-lock (ligand–receptor) model of molecular binding.

steptavidin (steptavidin molecule): a protein having four cavities presenting a tremendous affinity with biotin. It is the paradigm of the lock in the key-lock (ligand–receptor) model of molecular binding.

FJC (Free Jointed Chain): the simplest polymer model which describes the molecule as a series of independent monomers.

WLC (Worm Like Chain): a polymer model which describes the molecule as a flexible tube (or worm).

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