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Personal report

An appreciation of John D. Ferry and the mechanical properties of single molecules

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1. An appreciation of John D. Ferry

John Ferry pulled me into polymers and viscoelastic properties by offering me a research assistantship in the summer of 1951. His three-page letter was full of words I did not understand such as viscoelastic, but I was impressed that a professor would take the time to write me in such detail. The projects that were described dealt with synthetic polymers or biological polymers: fibrinogen and DNA.

Ferry's students mainly relied on lab-built machines, such as the Schremp apparatus and the Fitzgerald apparatus. We were all taught to keep very careful notebooks, and to record every possible variable that might affect the results, including lot numbers on reagents, etc. I can still remember Ferry's exclamation of 'Good Lord!' the day that none of us had measured the pH of the buffer we had used. It was the same buffer we had prepared many times before, so we assumed it had the same pH. I think that the publication reporting the results had an asterisk on some of the values stating that the pH was only approximate. He wanted to be precise and honest.

John Ferry wanted to be correct, but if there was an error, he wanted to be sure that everyone knew it was not due to sloppiness. All data must be completely described, so the chemicals used and the procedures done, could be reproduced. It seems paradoxical that his desire for accurate data and reproducible results did not prevent him from studying polydisperse synthetic polymers, DNA from calf thymus, and fibrinogen from cow blood. None of these materials was very reproducible. You might guess that a man like Ferry would prefer to work on pure inorganic chemicals. Instead, what I learned from him is that you should work on the frontier where you can't buy the materials or the instruments you need. If you can buy what you need, the field is clearly mature and probably uninteresting. However, because you are a pioneer, you must be particularly sure that your research is carefully done and meticulously described. I doubt if any of Ferry's students can match his combination of originality, honesty, precise communication, and regard for his students and colleagues. But we can all use him as a model for how scientists should behave.

2. Mechanical properties of single polymers

Although as a graduate student I was in a laboratory devoted to dynamic mechanical properties of polymers, I never made any measurements of mechanical properties. The instrument I designed for this purpose didn't work. After leaving Wisconsin I continued research on proteins and nucleic acids, mainly their spectroscopic properties (ultraviolet absorption, optical rotation, circular dichroism, and NMR). But now, nearly 50 years later, I have returned to mechanical properties of polymers.

The experiment is the simplest you can imagine. You grab the ends of a polymer and pull. You measure the distance between the ends and the force that you are applying. The work done on the molecule—the mechanical work—is the integral of force times distance. If the force is increased slowly enough that the molecular response is not limiting; that is, the process is reversible, then the reversible mechanical work is equal to the Gibbs free energy change at constant temperature and pressure. Most of the published work can be considered 'unfolding'—torsion angles are changed as a coil is pulled into an extended chain, hydrogen bonds are broken as proteins and nucleic acids are 'denatured'. For reviews, see Refs. [1,2]. If the force applied is large enough, even covalent bonds can be broken [3].

My goals here are (1) To communicate the excitement of actually measuring the mechanical properties of a single molecule. (2) To encourage the readers to apply the methods to their own problems. I know that John Ferry emphasized that the practical properties of polymers depended on the interactions present in concentrated solutions. However, force can be applied to a single molecule in a concentrated mixture, and force can be applied to cross-linked networks of polymers. The unique advantage of single-molecule measurements is that distributions of properties are

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obtained, rather than averages. A bulk measurement gives an ensemble average over all the molecules in the sample. The distribution may be a broad Gaussian, but it also may be two very distinct, sharp distributions. The average will not distinguish between these two possibilities, or reveal the actual distribution. However, by studying one molecule at a time you can learn the distribution of properties for one molecule, and the distribution of different molecules in a polydisperse sample. Reviews of single-molecules experiments involving force have recently appeared [4,5].

How do you measure the distance between the ends of a polymer, and how do you apply and measure the force? An atomic force microscope and laser tweezers are the usual instruments used; they have complementary characteristics. The atomic force microscope provides 0.1 nm resolution with force resolution in the tens of piconewtons (pN). Laser tweezers approach 1 nm distance and 0.1 pN force resolution. The resolutions for both instruments are continually improving, so there is reasonable expectation of obtaining 0.1 nm and 0.1 pN sensitivity. In the laser tweezers apparatus the ends of the polymer molecule are attached to micron-sized beads; one bead is on a micropipet, the other is held by one or two focused laser beams. The bead in the 'trap' will sit in the center of the focused beams, unless a force is applied. The light produces a harmonic potential for the bead with a force constant dependent on the intensity of the light. The distance the bead moves from the center of the trap is a direct measure of the force. The reason for the factor of 100 difference in the force sensitivity between laser tweezers and atomic force microscopes is their difference in force constants. In a laser tweezer, the movement of the bead by 1 nm corresponds to about 0.1 pN force. In an atomic force microscope the movement of the cantilever by 1 nm corresponds to about 10 pN.

My work has been on RNA: the polyribonucleotide that carries the messages for synthesizing proteins in cells; the genetic material of viruses that include HIV, SARS, and flu; and the class of molecules (RNAi) that has recently been found to be capable of specifically 'knocking-out' any gene. RNAs are synthesized as linear polymers copied from DNA in a process called transcription. In the cell the RNA folds into compact structures stabilized by base pairing, and by site-bound magnesium ions that reduce electrostatic repulsion among the phosphate groups of the nucleotides. The RNA structures have been determined to atomic resolution by NMR and by X-ray diffraction. A less detailed, but nevertheless useful picture of a folded RNA is its secondary structure. This is essentially a catalog of the base pairs formed when RNA folds to produce hairpins with single-stranded loops and bulges separating double-stranded regions. The tertiary structure is defined as the further folding of the secondary structure by interactions among the loops, bulges, and double strands.

During an RNA's life cycle in a cell, it folds and unfolds repeatedly. Forming consecutive base pairs lowers the free energy of the RNA relative to the single strand, but loops and bulges raise the free energy, because of their loss of entropy. In physiological conditions the RNA folds as it is synthesized, but it must then unfold and rearrange its base pairs when it interacts with proteins or other RNAs. An obvious example is messenger RNA, which is unfolded as it is translated by the ribosome to generate proteins. With laser tweezers, we have applied force to unfold RNA molecules, and to measure their free energies and kinetics of unfolding.

Fig. 1a shows a schematic of the experiment [6]. The RNA is extended by approximately 500 nucleotides on each end, and complementary DNA strands are added to make DNA•RNA handles. One DNA strand has a biotin on the end; the other has a digoxigenin group. These groups can be bound tightly by streptavidin beads or antidigoxigenin beads. The handles keep the RNA molecule that is being unfolded away from the beads. The beads are 1–3 μm in diameter, and are the only objects seen in the field of view of the video camera. The procedure is to flow streptavidin beads into the chamber; catch one in the laser trap; and transfer the bead to the micropipet. Next, antidigoxigenin beads, with the RNA attached, are flowed in and a bead is caught by the laser trap. The bead on the micropipet is moved close to make a connection to the biotin on the DNA handle. A successful docking is attained when movement of the micropipet bead causes the laser trap bead to move. At seminars someone always asks "How do you know you only caught one molecule." The answer is that two or more attachments produce very different force-extension curves than those produced by one attachment.

Fig. 1b gives a three-dimensional picture of an RNA molecule we have studied [7]. The RNA is a ribozyme—a catalytic RNA—discovered by Thomas Cech; it was the first ribozyme discovered. The *T. thermophila* L-21 ribozyme contains nearly 400 nucleotides and folds into the compact shape (as determined by X-ray diffraction) shown in Fig. 1b. We chose to study this RNA because so much was already known about it; it was an excellent model to demonstrate what new could be learned from force unfolding. The 3D-structure is an important beginning, but it does not tell you how easy or difficult it is to unfold the RNA and refold it. What are the free energy changes when the RNA goes from its single-strand state, to partially folded intermediates, and finally to its native fully folded form? How rapidly do these changes take place? Of course, we want to answer these questions for conditions similar to those found in biological cells. This is where force unfolding has an important advantage; the RNA can be unfolded at physiological temperatures and solvents. Without force, either high temperatures or denaturing solvents must be used. We think that force has another advantage. RNAs in cells are unfolded by proteins, such as helicases, polymerases, and the ribosome, which convert chemical energy into motion, force and mechanical energy. Our attached beads and the laser trap may mimic the action of these cellular proteins. The beads and cells both use local

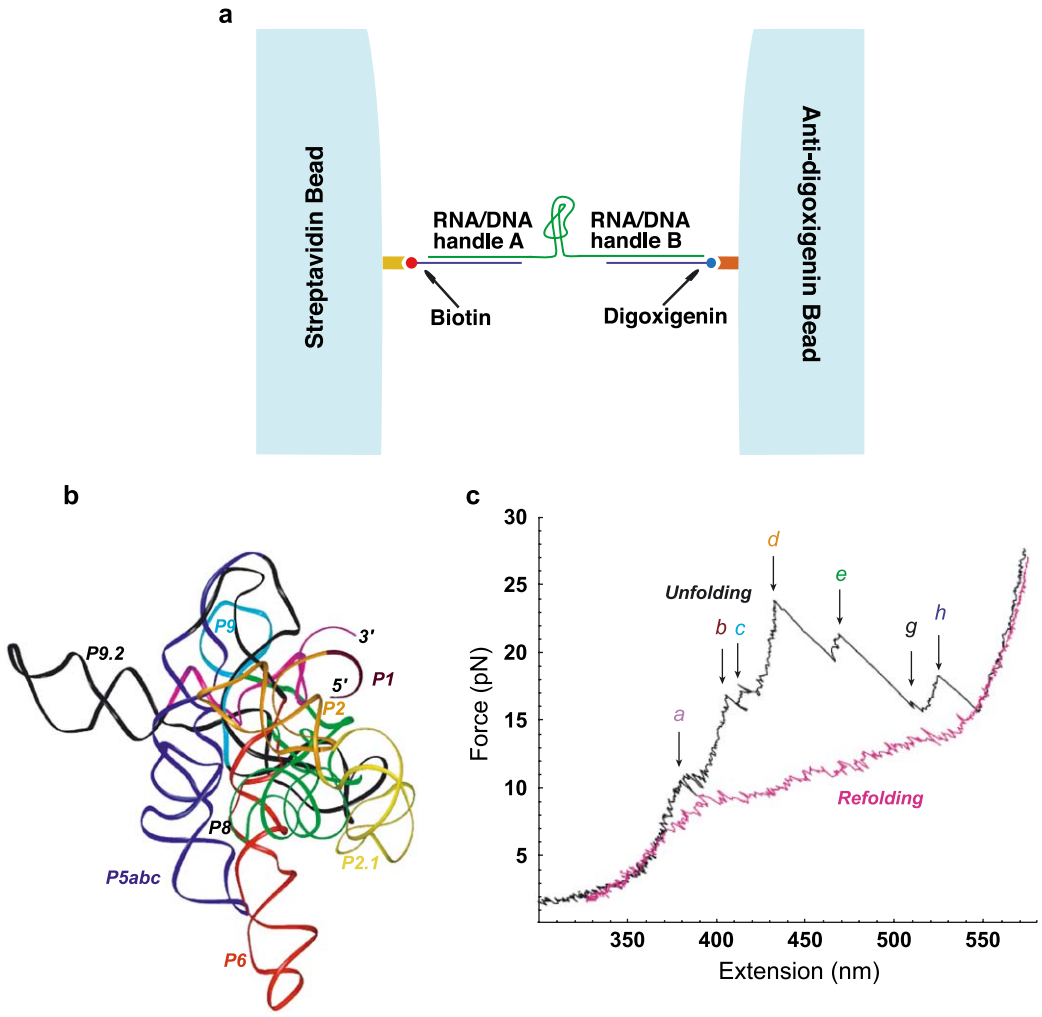

Fig. 1. (a) A schematic of an RNA molecule connected by RNA•DNA hybrid handles to micron-sized polystyrene beads. The handles are held to the beads by a biotin–streptavidin bond on one side and a digoxigenin–antidigoxigenin antibody on the other side. One bead is in a laser trap; the other is held by a micropipet. (b) A ribbon model of the *Tetrahymena thermophila* ribozyme L-21. (c) A force vs. extension curve for unfolding the RNA (black) and refolding it (red). The first transition, labeled *a*, is unfolding of helix P9.2; the last transition, labeled *h*, is unfolding of P5abc. Part c was adapted from Ref. [7]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

perturbations, instead of the global perturbations of temperature and solvent.

Fig. 1c shows the experimental result of unfolding the ribozyme by applying force at room temperature, pH 7, 250 mM NaCl, 10 mM Mg^{2+}. The distance between the beads in nanometers (nm) is plotted vs. the force applied in piconewtons (pN). Each time the RNA is unfolded the curve is somewhat different. The unfolding is a stochastic process; as kinetics is involved, repeating the process gives a different result. The main difference is in the number of transitions seen, and there is a distribution of forces and lengths for each transition. Below 10 pN the curve is the result of straightening the DNA•RNA handles. The curve can be fit by a worm-like-chain model [8] with a persistence length of 10 nm and a contour length of 0.34 nm per base pair. At about 12 nm the first 'rip' occurs (labeled *a* in Fig. 1c); it is assigned to the unfolding of helix P9.2 (see Fig. 1b). Seven rips are seen in the force–extension curve shown, but successive pullings of the RNA reveal eight rips. After the last rip (*h*) assigned to P5abc, the curve fits a worm-like-chain model with properties that are the sum of the handles and the single-strand RNA formed (persistence length 1 nm, contour length 0.59 nm per nucleotide). In the worm-like-chain model we assume that the work of straightening the polymer is due to the loss of entropy going from a coil with many conformations to an extended polymer with fewer conformations. The single-strand RNA is much more flexible than the double-stranded handles (a persistence length one-tenth that of the handles), so its entropy loss is greater and its force–extension curve is steeper.

Assignment of the rips in a pulling curve to molecular unfolding events is based on several criteria. (1) The length of the rip depends on how many base pairs are broken to form single strands. The length thus identifies possible helices. (2) An individual domain of the RNA with only one rip usually shows a rip similar in length and at approx-

imately the same force as in larger pieces, or in the complete RNA. Therefore, unfolding subunits of the RNA can indicate assignments for the complete RNA. (3) To corroborate the assignments, a mutation is made that prevents a certain interaction, or a complementary oligonucleotide is added that competes for an intramolecular interaction. The mutation or competing oligonucleotide is designed to remove a specific rip. If the prediction is fulfilled, we are convinced the assignments are correct.

We assigned each rip to the unfolding of a structural element in the RNA. This revealed the order of unfolding of the RNA, and identified the intermediate, partially unfolded species. It is important to emphasize that the order of unfolding depends on where the force is applied. Cyclizing the RNA and cutting it to produce two new ends allows the force to be applied in different places. This leads to a different order of unfolding. For example, we can make rip *h* occur first instead of last.

The kinetic barriers that produce the intermediates are strongly dependent on Mg^{2+} concentration. In the absence of Mg^{2+}, the barriers disappear; the unfolding curve without Mg^{2+} is very similar to the refolding curve with Mg^{2+} shown in Fig. 1c. We think that the kinetic barriers are produced by tertiary interactions involving Mg^{2+}. The height of the barrier—the force required to overcome it—depends on the number and types of interactions that cause the barrier.

Free energies of unfolding and kinetic rate constants for folding and unfolding are being measured. As the force increases, the free energy of the longer species in a reaction is favored—the unfolded form. Increasing force increases the rate constant for unfolding, and decreases the rate constant for refolding. The force F dependence of the rate constants and equilibrium constants are exponential.

$$k = k(F = 0)\mathrm{e}^{FX^{\ddagger}/kT}$$

The difference is that the rate constant depends on the distance to the transition state $X^{\ddagger}$, whereas the equilibrium constant depends on the end-to-end distance. The rate constant at zero force, $k(F{=}0)$, cannot simply be obtained by extrapolation. The mechanism of unfolding will in general be different for the local perturbation of a directed force and for the global perturbation of temperature or solvent. However, we think that the measured rates and free energies under force are more relevant to biological processes than the thermally measured parameters. The range of forces seen in Fig. 1c for the unfolding of the RNA (10–25 pN) are within the range of forces exerted by cellular enzymes that operate on nucleic acids [9].

Applying force to a single molecule is new; it is different every time you repeat the experiment; it requires equipment built in the laboratory. I think John Ferry would have loved it.

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