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The effect of force on thermodynamics and kinetics: unfolding single RNA molecules

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Abstract

We have used laser tweezers to unfold single RNA molecules at room temperature and in physiological-type solvents. The forces necessary to unfold the RNAs are over the range 10–20 pN, forces that can be generated by cellular enzymes. The Gibbs free energy for the unfolding of TAR (transactivation-responsive) RNA from HIV was found to be increased after the addition of argininamide; the TAR hairpin was stabilized. The rate of unfolding was decreased and the rate of folding was increased by argininamide.

Keywords

argininamide; laser tweezer; single RNA molecule; transactivation-responsive RNA (TAR RNA)

Abbreviation used

TAR, 1 transactivation-responsive

Introduction

Laser tweezers and atomic force microscopes have been used to unfold proteins, DNA molecules and RNA molecules (reviewed in [1–3]). Force is applied to a single molecule to stretch it. For double-stranded DNA, the molecule is extended from a ‘random coil’ to its B-form contour length; then, an over-stretching occurs with an 80% increase in length [4]. RNA molecules are unfolded from compact base-paired structures to extended single strands [5,6]. Proteins are unfolded to extended polypeptides [7]. The advantage of force unfolding is that the reaction can be performed at any temperature and in any solvent. Neither denaturants nor high temperatures are needed. Furthermore, since the force is applied only to the molecule of interest, other molecules in the solution (including ligands) are not affected.

A unimolecular reaction in which the product has a different extension compared with the reactant can be influenced by force; force favours the species with the longer extension. The equilibrium constant for the reaction depends exponentially on the force and the difference in extension. Similarly, the rate constant for the reaction depends exponentially on the force and the distance from the reactant to the transition state [8].

$$\begin{aligned} K &= K^{\circ} e^{F\Delta X/k_B T} \\ k &= k^{\circ} e^{FX^{\ddagger}/k_B T} \end{aligned} \quad (1)$$

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Here, K is the equilibrium constant at force F , K° the equilibrium constant at zero force, k the rate constant at force F and k° the rate constant at zero force. T is the temperature and k_B is Boltzmann's constant. ΔX is the difference in extension between the product and reactant and $X^\ddagger$ is the distance to the transition state.

The transition-state diagram traditionally seen in elementary textbooks is obviously an approximation and simplification for the actual kinetic mechanism. The multidimensional free-energy landscape and the average behaviour of many molecules are represented as a single 'reaction co-ordinate' versus free energy. In force unfolding of single molecules, the transition-state diagram is more understandable. The reaction co-ordinate is the end-to-end distance of the molecule, and the distance to the transition state can be obtained using eqn (1). Intermediates and their transition states can be located along the extension co-ordinate. The fact that the ends of the molecule are tethered at known distances as the reaction progresses limits the possible conformations available for intermediates and transition states. The relative free energies of the native and unfolded states depend on the force applied. At zero force, we assume that the native state is stable relative to the unfolded state. By definition, at $F_{1/2}$, the folded and unfolded states have equal free energies.

We have used laser tweezers to manipulate beads attached by handles to single RNA molecules containing 50–400 nt. Forces over the range 10–30 pN at room temperature in physiological buffers usually suffice to unfold the RNA. These forces are within the range of forces generated by cellular enzymes that operate on nucleic acids, such as polymerases and helicases [3]. By applying force, we have measured rates and equilibria for unfolding/refolding RNA without using denaturants or high temperatures. The effects of ligands have also been determined.

TAR (transactivation-responsive) RNA

The transactivation response element in HIV is a stem-loop RNA hairpin, TAR RNA, which increases the transcriptional efficiency of the viral RNA and is necessary for HIV replication [9]. Its secondary structure is shown in Figure 1(a); the three-base bulge is a specific binding site for arginine and peptides containing arginine [10,11]. We have used the TAR hairpin to learn about the effects of adding a specifically bound ligand (argininamide) on the thermodynamics and kinetics of RNA unfolding.

The construct for applying force to unfold TAR RNA was prepared by the same methods as used in [5,6]. An approx. 1 kb-long RNA with the TAR RNA sequence in the middle was annealed to two 0.5 kb pieces of DNA to form DNA•RNA handles flanking the TAR RNA. One handle contains biotin to attach to a streptavidin-coated bead; the other handle contains digoxigenin to attach to an anti-digoxigenin-coated bead. The beads are held between a micropipette on a piezoelectric actuator and a laser light trap. The distance between the beads and the force on the bead in the trap are measured [12]. Changes in distance of approx. 1 nm and changes in force of approx. 0.1 pN can be detected.

Figure 1(b) shows a force versus extension curve at 20°C for TAR RNA in a buffer consisting of 100 mM KCl, 10 mM Hepes (pH 8.0) and 1 mM EDTA. The micropipette is moved at constant velocity to produce a loading rate of approx. $1.5 \text{ pN} \cdot \text{s}^{-1}$. During unfolding, at forces up to 14 pN, the curve corresponds to straightening of the DNA•RNA handles. Then, an abrupt increase in extension occurs as the RNA hairpin unfolds. Since we measure force by the motion of the bead from the centre of the trap, the slope of the 'rip' is equal to the force constant ($0.1 \text{ pN} \cdot \text{nm}^{-1}$) of the trap. The noise also has the same slope. After the rip, the curve corresponds to straightening the handles and the single-stranded RNA. On lowering the force, the RNA starts refolding near 14 pN, but the folding is not complete until the force is decreased by 2 or 3 pN more. We believe that, as the extension decreases, base-pairs transiently form and break until sufficient native structure has formed to allow the final jump to the native species near

12 pN. The unfolding/refolding process is obviously not reversible; it is kinetically controlled. This has two effects: (i) each force–extension curve will be different because of the stochastic nature of kinetics. (ii) The curves will depend on the loading rate: the higher the loading rate, the larger the hysteresis. In principle, at a low enough loading rate, the process becomes reversible. In practice, drift in the force and distance measurements do not allow us to monitor the reversible reaction and, thus, directly obtain ΔG , the Gibbs free-energy change.

$$\Delta G = \text{reversible work} = \text{force} \times \text{distance}$$

We can measure the ‘irreversible’ work of unfolding the TAR RNA for each trajectory, but to obtain the free-energy change of the reaction requires a different procedure. A large number of irreversible trajectories are measured; the Boltzmann-weighted average of the exponentials of irreversible work provides the Gibbs free energy [13].

$$e^{-\Delta G/kT} = \left\langle e^{-w_{\text{irrev}}/kT} \right\rangle \quad (2)$$

Force-jump kinetics

Here, we describe a kinetic method to establish the reversible reaction force and thereby obtain the reversible work and the free energy. In force-jump kinetics, the force is quickly raised or lowered, then held constant until the molecule reacts [7]. For TAR RNA, we see a single-step increase or decrease in extension and record the time when this occurs. Figure 2 shows an example of two successive experiments. The upper panel shows the time dependence of the force and the lower panel shows the corresponding change in extension. First, the force is rapidly raised and then held constant (Figure 2, upper panel) until the extension suddenly increases (Figure 2, lower panel); then, the force is increased to ensure that the molecule has unfolded completely. Secondly, the force is lowered and then held constant until the extension suddenly decreases; then, the force is decreased to allow the molecule to refold. The experiment is repeated at least 100 times and the lifetimes of the unchanged molecule at each force are recorded. For a two-state reaction, the distribution of times fits a single exponential that provides the first-order rate constant k for the process. The first-order rate constant k can also be obtained as the reciprocal of the average of the measured lifetimes.

$$k = 1 / \langle t_i \rangle \quad (3)$$

We measured rate constants at 0.3 pN intervals over the force range 12.7–14.2 pN for unfolding TAR and 10.9–12.4 pN for refolding TAR. These forces correspond to rate constants in the range 0.05–2 s^{−1} and half-lives from 0.35 to 14 s. The range of measurable half-lives is limited by the time required to increase the force for short half-lives and by drift in the instrument for long lives. The logarithms of the rate constants should be linear in force (eqn 1) if the distance to the transition state is independent of force. A plot of $\ln k$ against force is linear for unfolding ($\ln k = 1.99F - 28.06$; $R^2 = 0.99$) and refolding ($\ln k = -1.90F + 21.56$; $R^2 = 0.93$). These lines intersect at $F_{1/2} = 12.8$ pN, where the rate constants for folding and unfolding are equal ($k = 0.069$ s^{−1}); the equilibrium constant $K = 1$.

From the slopes of the $\ln k$ versus force lines, we obtain the distance to the transition state from the native (hairpin) species ($X^\ddagger_{\text{unfolding}} = 8.2$ nm) and from the unfolded (single-strand) species ($X^\ddagger_{\text{folding}} = 7.8$ nm). For a two-state process, the sum of these transition-state distances (16 nm) should equal the change in length of the molecule on proceeding from the native to the unfolded state near $F_{1/2}$. The measured change in length is 18 ± 2 nm. Since the transition state is close to halfway between the native and unfolded species, the RNA is termed compliant. Compliant transitions are seen for secondary-structure unfolding [5]. Transitions involving

tertiary-structure unfolding are brittle; the transition state is close to the native structure ($X_{\text{unfolding}}^{\ddagger} \leq 2 \text{ nm}$) [6,14].

Free-energy change

The work measured at $F_{1/2}$ equals the equilibrium free-energy change for the transition from native hairpin to unfolded single strand: $\Delta G = F_{1/2} \times X = 12.8 \times 18 \text{ pN} \cdot \text{nm} = 138 \pm 15 \text{ kJ} \cdot \text{mol}^{-1}$. We can consider the free-energy change to have two contributions: the breaking of all the base-pairs and the straightening–stretching of the resulting RNA single strand caused by the applied force. At $F_{1/2}$, there is thus an additional free energy – the stretching free energy – compared with the free-energy change for the transition at zero force. The stretching free energy is calculated from a worm-like chain model for single-stranded RNA with persistence length equal to 1 nm and contour length equal to 0.59 nm per nucleotide [5]. The stretching free energy for the 52-nt TAR RNA at $F_{1/2}$ is $45 \text{ kJ} \cdot \text{mol}^{-1}$; thus about one-third of the change for the transition is due to stretching the single strand. The calculated free-energy change for the unfolding of TAR RNA in 100 mM salt at 20°C and zero force is $93 \text{ kJ} \cdot \text{mol}^{-1}$.

We cannot actually unfold the RNA at room temperature and zero force in 100 mM KCl and therefore, we cannot measure the free energy of the transition directly. Instead, we estimate the free energy of the folded form as the sum of base-pair nearest-neighbour interactions for helices plus the contribution from loops and bulges [15]. The parameters are for a solvent of 1 M salt, but an adjustment for ionic strength can be made. The calculated free-energy change (Mfold server <http://www.bioinfo.rpi.edu/~zukerm/>) for unfolding TAR at zero force at 20°C in 0.1 M salt equals $117 \text{ kJ} \cdot \text{mol}^{-1}$. We do not understand the large discrepancy between the force unfolding and the nearest-neighbour calculation. We have previously obtained reasonable agreement between the two procedures [5]. To get agreement between the calculated ΔG° and the mechanical unfolding value, the force at the midpoint of the transition would have to be over 15 pN. This is not consistent with our measurements.

Argininamide

Arginine and argininamide (with a +2 net charge) bind specifically at the UCU bulge of TAR [10,11]. Adding 10 mM argininamide to TAR RNA in 100 mM Na^+ or K^+ has a large effect on the unfolding/refolding. Figure 3 shows the data for TAR RNA in the presence and absence of argininamide. The kinetics shows two-state behaviour, with exponential distributions of lifetimes, in the presence or absence of argininamide. The unfolding rate constants for TAR RNA are decreased by nearly two orders of magnitude in the presence of argininamide. The refolding rate constants are increased by nearly two orders of magnitude in the presence of argininamide. The net effect on the equilibrium is to stabilize the folded form, as expected. The transition force $F_{1/2}$ is increased to 15.8 pN in 10 mM argininamide; the free energy of the unfolding at this force is $171 \text{ kJ} \cdot \text{mol}^{-1}$. The resulting free energy at zero force at 20°C is $117 \text{ kJ} \cdot \text{mol}^{-1}$. Addition of 10 mM argininamide, thus, stabilizes the folded hairpin by $24 \text{ kJ} \cdot \text{mol}^{-1}$ at room temperature and zero force.

Conclusions

The unfolding of an RNA (or protein) can be studied at room temperature and in non-denaturing solvents by application of force. This process is analogous to the unfolding that occurs when an RNA is translated by a ribosome or unfolded by a helicase. The work required to unfold the RNA (measured as the product of the force and the change in end-to-end distance) is equal to the Gibbs free-energy change when the RNA is unfolded reversibly. Rates of unfolding and refolding are measured by changing the force rapidly to a value in the transition range and determining when the reaction occurs.

For TAR RNA in 100 mM Na⁺ or K⁺ at 20°C, we find that it unfolds reversibly at 12.8 pN, where its equilibrium constant is equal to 1. In the presence of 10 mM argininamide, the equilibrium constant for unfolding/folding becomes 1 at 15.8 pN. The stabilization of the TAR hairpin by 10 mM argininamide at zero force is 24 kJ · mol⁻¹. The kinetics as well as the thermodynamics is affected by argininamide. The folding and unfolding rate constants are equal to 0.069 s⁻¹ for TAR RNA at the midpoint of the transition, $F_{1/2}$. When 10 mM argininamide is added, the unfolding rate constant at the same force is 3.1×10^{-4} s⁻¹ and the folding rate constant is 22 s⁻¹. Argininamide increases the folding rate and decreases the unfolding rate and, thus, stabilizes the hairpin.

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Figure 1. Force unfolding and refolding of TAR RNA

(a) Secondary structure of the TAR hairpin we studied. (b) A force–extension curve for the TAR hairpin in 100 mM KCl, 10 mM Hepes (pH 8.0) and 1 mM EDTA is shown. The loading rate was approx. $3 \text{ pN} \cdot \text{s}^{-1}$; the amount of hysteresis and the distribution of forces at which the rips occur depend on the loading rate.

Figure 2. Force clamp kinetics

Upper panel: the time dependence of two successive force jumps and drops; lower panel: the corresponding changes in extension. Measurement of the lifetime of the unchanged molecule after each jump or drop in force yields the rate constants.

Figure 3. Base-10 logarithms of the rate constants for unfolding and refolding TAR RNA are plotted against force

Unfolding and refolding for TAR RNA in EDTA (pH 8) with 100 mM KCl (circles) or 100 mM NaCl (squares), and TAR RNA in NaCl buffer, but with 10 mM argininamide added. The temperature was 20°C.