Non-specific binding of Na\(^+\) and Mg\(^{2+}\) to RNA
determined by force spectroscopy methods

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ABSTRACT

RNA duplex stability depends strongly on ionic conditions, and inside cells RNAs are exposed to both monovalent and multivalent ions. Despite recent advances, we do not have general methods to quantitatively account for the effects of monovalent and multivalent ions on RNA stability, and the thermodynamic parameters for secondary structure prediction have only been derived at 1M [Na\(^+\)]. Here, by mechanically unfolding and folding a 20 bp RNA hairpin using optical tweezers, we study the RNA thermodynamics and kinetics at different monovalent and mixed monovalent/Mg\(^{2+}\) salt conditions. We measure the unfolding and folding rupture forces and apply Kramers theory to extract accurate information about the hairpin free energy landscape under tension at a wide range of ionic conditions. We obtain non-specific corrections for the free energy of formation of the RNA hairpin and measure how the distance of the transition state to the folded state changes with force and ionic strength. We experimentally validate the Tightly Bound Ion model and obtain values for the persistence length of ssRNA. Finally, we test the approximate rule by which the non-specific binding affinity of divalent cations at a given concentration is equivalent to that of monovalent cations taken at 100-fold concentration for small molecular constructs.

INTRODUCTION

RNA hairpins are elementary structures found in many macromolecular assemblies. It is generally accepted that a deeper understanding of their dynamics is a critical step toward the elucidation of many biological processes, like the regulation of gene expression (1–5); the catalytic activity in many reactions (6,7); the ligand-binding specificity (8); or the RNA folding problem (9,10). DNA and RNA hairpins are also appealing model systems for their simplicity as they are amenable to exhaustive studies using a more physically oriented approach, where theoretical models can be rigorously tested using simulations and experiments (11,12).

Many different and complementary biophysical methods have been used to study these structures (13–21). For example, using time-resolved nuclear magnetic resonance (NMR) spectroscopy and thermal denaturation experiments, kinetics and thermodynamics of bistable RNA molecules were studied (22). Recently, a photolabile caged RNA was designed to stabilize one ground-state conformation and study the folding kinetics by NMR and CD spectroscopy under different conditions, including Mg\(^{2+}\) (23). Laser temperature-jump experiments have also been used to characterize the folding kinetics of small RNA hairpins at the ns and \(\mu\)s timescales (24–29).

Using coarse-grained Go-like models, it was predicted that hairpins unfold in an all-or-none process in mechanical experiments (30), in agreement with experimental results (31–33). Within the cell, many dynamical processes involving transient melting events of DNA and RNA double strands are driven by the application of localized forces by molecular motors. Therefore, single-molecule experiments are ideal to understand the thermodynamics and kinetics of macromolecules inside cells (34,35). As pointed out by Hyeon \textit{et al.} (30), force-denaturation using single-molecule experiments are intrinsically different from thermally induced denaturation: in bulk experiments where the unfolded state is accessed by raising the temperature or lowering the concentration of ions, the unfolded state is a high-entropy state while in mechanical pulling experiments the unfolding process is a transition...
from a low-entropy state to another low-entropy state. Regions of the free energy landscape normally inaccessible by conventional methods are probed using mechanical experiments. Consequently, pathways and rates of thermally induced and mechanical unfolding processes are expected to be different.

In a previous work (36), we pulled an RNA hairpin using optical tweezers (37,38) to study the base-pairing thermodynamics, kinetics and mechanical properties at a fixed monovalent condition. A kinetic analysis was introduced to determine the location of the force-dependent kinetic barrier, the attempt rate, and the free energy of formation of the molecule. Here, we performed a systematic study by mechanically pulling the same RNA hairpin at different monovalent cation concentrations and also at mixed ionic conditions containing different concentrations of Mg\(^{2+}\) cations. This is important because RNAs also have limited information about RNA helix stability in mixed monovalent/multivalent ionic conditions (44,45). In fact, the thermodynamic parameters for secondary structural elements of RNAs have only been derived in the temperature of the fixed standard salt condition of 1M [Na\(^+\)] (15–21). Here, we derived numbers such as the persistence length describing the elastic response of ssRNA and also the free energy of formation of an RNA hairpin at different monovalent and mixed monovalent/Mg\(^{2+}\) conditions. Our results are compatible with predictions obtained using the Tightly Bound Ion (TBI) model for mixed ion solutions, which treats monovalent ions as ionic background and multivalent ions as responsible from ion–ion correlation effects, and which takes into account only non-sequence-specific electrostatic effects of ions on RNA (43–45). Our findings demonstrate the validity of the approximate rule by which the non-specific binding affinity of multivalent cations is equal to that of monovalent cations taken around 100-fold concentration for small molecular constructs (46,47).

MATERIALS AND METHODS

Molecular synthesis

The RNA molecule was prepared as previously described (48). Oligonucleotides CD4F (5′-AATTCACACG CGAG CCATAA TCTCATTG GAAAGATGAG ATTA TGGCTCGC ACACA-3′) and CD4R (5′-AGCTTTGTGT GCGAGCCATA ATCTCATC TGGTTTCCAGAT GAGGCTCG CACACA-3′) were annealed and cloned into the pBR322 DNA plasmid (GenBank J01749) digested with EcoRI (position 4360) and HindIII (position 30). The annealed oligonucleotides contain the sequence that codes for a modified version of CD4-42F class I hairpin that targets the mRNA of the CD4 receptor of the human immunodeficiency virus (49). Oligonucleotides T7 Forward (5′-TATAGCAG CTA CTATTAGG GACTGGTA GTACTCA ACCAA GTC-3′) and T7 Reverse (5′-TA GGAAGC AGGCCAG T AGTAGG-3′) were used as primers to amplify by PCR a product of 1201 bp from the recombinant clone containing the CD4 insert. This amplicon contains the T7 RNA Polymerase promoter at one end, and was used as a template to synthesize an RNA containing the RNA hairpin (20 bp stem sequence and tetraloop GAAA) and the RNA components of handles A (527 bp) and B (599 bp). The DNA components of handles A and B were obtained by PCR from the pBR322 vector (positions 3836-1 for handle A and positions 31-629 for handle B). Handle A was 3′ biotinylated while handle B was tagged with a 5′ digoxigenin. Hybridization reactions were performed in a formamide-based buffer (50) with a step-cool temperature program: denaturation at 85°C for 10 min, followed by 1.5 h incubation at 62°C, 1.5 h incubation at 52°C and finished with a cooling to 10°C within 10 min.

Measurement protocol

All experiments were performed using a dual-beam force measuring optical trap (37,38) at 25±1°C in buffers containing 100 mM Tris–HCl (pH 8.1), 1 mM EDTA and NaCl concentrations of 0, 100, 500 and 1000 mM, or in buffers containing 100 mM Tris–HCl (pH 8.1) and MgCl\(_2\) concentrations of 0.01, 0.1, 0.5, 1, 4 and 10 mM. The monovalent cation concentration [Mon\(^+\)] includes the contributions from [Na\(^+\)] ions and dissociated [Tris\(^-\)] ions. At 25°C and pH 8.1, about half of the Tris molecules are protonated, therefore 100 mM Tris buffer adds 50 mM to the total monovalent ion concentration (51). Anti-digoxigenin polyclonal antibody-coated polystyrene microspheres (AD beads) of 3.0–3.4 μm (Spherotech, Libertyville, IL, USA) were incubated at room temperature with the molecular construct for 20 min. The second attachment was achieved inside the microfluidics chamber using a single optically trapped AD bead previously incubated with the RNA hairpin and a streptavidin-coated polystyrene microsphere (SA bead) of 2.0–2.9 μm (G. Kisker GbR, Products for Biotechnologie) positioned at the tip of a micropipette by suction (Figure 1A and B). Tethered molecules were repeatedly pulled at two constant loading rates of 1.8 pN/s or 12.5 pN/s by moving up and down the optical trap along the vertical axis between fixed force limits and the resulting force-distance curves (FDCs) were recorded (Figure 2A). A pulling cycle consists of an unfolding process and a folding process. In the unfolding process, the tethered molecule is stretched from the minimum value of force, typically in the range of 5–10 pN, where it is always at its native folded state, up to the maximum value of force, typically in the range of 25–30 pN, where the molecule is always unfolded. In the folding process the molecule is released from the higher force limit (unfolded state) up to the lower force limit (native folded state) (52). A minimum of two molecules (different bead pairs) were tested at each ionic condition, and a minimum of 100 cycles were recorded in each case (detailed statistics are given in Section S1 of the Supplementary Data).

Hairpin model

Under applied force it is feasible to reduce the configurational space of an RNA hairpin containing N base pairs (bps) to a minimum set of N+1 partially unzipped RNA
structures (36,53,54). Each configuration in this set contains \(n\) adjacent opened bps in the beginning of the fork followed by \(N-n\) closed bps, with \(0 \leq n \leq N\). The folded state (F) is defined as the configuration in which \(n = 0\) (all bps are formed), and the unfolded state (U) is the hairpin configuration in which \(n = N\) (all bps are dissociated). Based on a simple calculation (see Section S2 of the Supplementary Data) we conclude that fraying (55) plays a rather minor role (if any) on the folding/unfolding kinetics of the sequence under study (Figure 1A) and we do not include it in our analysis. The stability of each configuration \(n\) with respect to the F conformation is given by
\[
\Delta G_n(f) = \Delta G_n(0) + \Delta G_{\text{ssRNA}}^n(f) + \Delta G_{\text{d}}^0(f).
\]
In Equation 1 \(\Delta G_n(0)\) is the free energy difference at zero force between a hairpin in the partially unzipped configuration \(n\) and a hairpin in the completely closed configuration; \(\Delta G_{\text{ssRNA}}^n(f)\) is equal to the reversible work needed to stretch the ssRNA strands of the hairpin in configuration \(n\) (2 \(n\) opened bases) from a random coiled state to a force-dependent end-to-end distance \(x_n(f)\); and \(\Delta G_{\text{d}}^0(f)\) is the contribution related to hairpin stem orientation (56,57). An estimation of \(\Delta G_n(0)\) at 1M [Mon+] can be obtained by using the nearest-neighbor (NN) energy parameters widely employed to predict the stability of RNA secondary structures (15,19). It is given by the sum of the stacking contributions of the duplex region, containing \(N-n\) bps. The elastic term \(\Delta G_{\text{ssRNA}}^n(f)\) is given by (56):
\[
\Delta G_{\text{ssRNA}}^n(f) = -\int_0^f x_n(f') df'.
\]
The molecular extension of ssRNA, \(x_n(f)\), can be estimated using polymer theory (see following section). Finally, the last term in Equation 1, \(\Delta G_{\text{d}}^0(f)\), is equal to the free energy of orientation of a monomer of length \(d_0\) along the force axis (57):
\[
\Delta G_{\text{d}}^0(f) = k_B T \log \left[ \frac{k_B T}{f d_0} \sinh \left( \frac{f d_0}{k_B T} \right) \right]
\]
where \(f\) is the applied force, \(k_B\) is the Boltzmann constant, \(T\) is the bath temperature and \(d_0\) is the diameter of a double stranded chain, taken equal to 2 nm.

**Elastic models of ssRNA**

To model the elastic response of ssRNA we employed both the interpolation formula for the inextensible Worm Like Chain (WLC) model and the Freely Jointed Chain (FJC) model, which give the equilibrium end-to-end distance \(x\) of a polymer of contour length \(l_n\) stretched at a given force \(f\). These models have been mainly tested for long polymers. However, several studies indicate that they are generally applicable when the contour length is

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**Figure 1.** Experimental setup and RNA sequence. (A) A single RNA hairpin is attached to two polystyrene microspheres through RNA/DNA heteroduplexes used as handles. The anti-digoxigenin antibody-coated microsphere is optically trapped while the streptavidin-coated microsphere is positioned at the tip of a micropipette by air suction. (B) RNA hairpin sequence.

**Figure 2.** Pulling experiments. (A) A few pulling cycles for the RNA hairpin showing the unfolding (red) and folding (blue) trajectories. (B) Experimental distribution for the unfolding first rupture forces at 1.8 pN/s (red filled squares) and 12.5 pN/s (blue filled circles), and for the folding first rupture forces at 1.8 pN/s (red empty squares) and 12.5 pN/s (blue empty circles).
larger than the persistence length. The inextensible WLC is given by:

\[ f = \frac{k_BT}{P} \left[ \frac{1}{4(1 - x/l_n)^2} - \frac{1}{4} + \frac{x}{l_n} \right] \]  

(4)

where \( k_B \) is the Boltzmann constant, \( T \) is the bath temperature and \( P \) is the persistence length (58,59). The FJC model is given by

\[ x = l_n \left[ \coth \left( \frac{fb}{k_BT} \right) - \frac{k_BT}{fb} \right] \]  

(5)

where \( b \) is the Kuhn length.

There are other models, such as the Thick Chain, that are more general than the WLC or the FJC and that have been used to fit the elastic response of biopolymers. Despite of their greater complexity, we do not expect a qualitative improvement of our results by using them.

Kinetic analysis

We applied Kramers rate theory (60) to study the kinetics of the transition between states F and U. The framework for understanding the effect of an external force on rupture rates was first introduced in (61) and extended to the case where the loading force increases with time (62,63). The assumption that the transition state does not move under an applied force \( f \) can be relieved by considering that the effective barrier that must be crossed by a Brownian particle is force dependent, \( B_{\text{eff}}(f) \). The unfolding and folding rates can be obtained as the first passage rates over the effective barrier,

\[ k_U(f) = k_0 \exp \left( \frac{-B_{\text{eff}}(f)}{k_BT} \right), \]  

(6a)

\[ k_F(f) = k_0 \exp \left( \frac{B_{\text{eff}}(f) - \Delta G_N(f)}{k_BT} \right). \]  

(6b)

In Equation 6, F was selected as the reference state and \( \Delta G_N(f) \) has been defined in Equation 1. \( k_0 \) is the attempt rate for activated kinetics. The effective barrier \( B_{\text{eff}}(f) \) can be obtained analytically from Kramers rate theory (KT) (64,65) (detailed derivation provided in the Section S3 of Supplementary Data) as

\[ B_{\text{eff}}^{KT}(f) = k_BT \log \left[ \sum_{n=0}^{N} h(n) \exp \left( \frac{\Delta G_n(f)}{k_BT} \right) \right] \]  

(7)

with \( h(n) = \sum_{n'=0}^{\infty} \exp \left( \frac{-\Delta G_{n'}(f)}{k_BT} \right) \). Importantly, the location of the barrier along the reaction coordinate can be obtained from the first derivatives of \( B_{\text{eff}}(f) \) with respect to force,

\[ x_{\text{eff}}^F(f) = -\frac{dB_{\text{eff}}(f)}{df}, \]  

(8a)

\[ x_{\text{eff}}^U(f) = \frac{dB_{\text{eff}}(f) - \Delta G_N(f)}{df} \]  

(8b)

where \( x_{\text{eff}}^F(f) \) and \( x_{\text{eff}}^U(f) \) are the distances from the effective barrier to the F and U states, respectively. The force-dependent fragility parameter \( \mu(f) \) (36),

\[ \mu(f) = \frac{x_{\text{eff}}^F(f) - x_{\text{eff}}^U(f)}{x_{\text{eff}}^F(f) + x_{\text{eff}}^U(f)} \]  

(9)

lies in the range \([-1:1]\) and is a measurement of the compliance of a molecule under the effect of tension. Compliant structures deform considerably before the transition event and are characterized by positive values of \( \mu(f) \), i.e. \( x_{\text{eff}}^F(f) > x_{\text{eff}}^U(f) \). In contrast, brittle structures are defined by negative values of \( \mu(f) \), \( x_{\text{eff}}^F(f) < x_{\text{eff}}^U(f) \). A given sequence can display different fragilities at different force regimes, due to changes in the location of the transition state (TS) with force.

From the measured transition rates (see following section) we can get estimators for the effective barrier \( B_{\text{eff}}^{U/F}(f) \) for unfolding and folding using the expressions in Equation 6:

\[ \frac{B_{\text{eff}}^{U}(f)}{k_BT} = -\log k_U(f) + \log k_0, \]  

(10a)

\[ \frac{B_{\text{eff}}^{F}(f)}{k_BT} = -\log k_F(f) + \log k_0 + \frac{\Delta G_N(f)}{k_BT}. \]  

(10b)

By comparing the experimental estimators of the kinetic barrier \( B_{\text{eff}}^{U/F}(f) \) with the effective barrier \( B_{\text{eff}}^{U/F}(f) \) as predicted by Kramers rate theory (Equation 7) we can extract the free energy of formation of the hairpin \( \Delta G_N(0) \), the attempt rate \( k_0 \) and the parameters that characterize the elastic response of the ssRNA (36). While \( k_0 \) always can be determined by doing this comparison, there is a trade-off between the contributions of the elastic response of the ssRNA and the free energy of formation of the hairpin. Although this is not strictly true (the stretching contribution term is force dependent whereas the free energy of formation term is not) it holds to a very good degree. Therefore, if only the free energy of formation of the hairpin is known a priori, then we can extract the elastic properties of the ssRNA by matching Equation 10a and b with Equation 7. On the contrary, if we only know the elastic properties of the ssRNA, then we can extract the free energy of formation of the hairpin (see Section S4 of the Supplementary Data).

Data analysis

The molecular transitions during unfolding and folding can be identified as force rips in a FDC (56). In order to extract the unfolding and folding rates (Equations 6a and b) from experiments we have collected the first rupture forces associated with the unfolding and folding parts of each pulling cycle (Figure 2A and B). By plotting the number of trajectories in which the molecule remained at the initial configuration (F state during the stretching part and U state in the releasing part of the cycle) as a function of force \( N(f) \), divided by the total number of trajectories \( N_0 \), we obtained experimental estimators for survival probabilities \( P_{U/F}(f) = N(f)/N_0 \) of the U and F states. Moreover, we obtained an experimental estimator
for the probability densities $\rho_{U/F}(f)$ of unfolding and folding first rupture forces by doing normalized histograms of both data sets ($P_{U/F}(f) = \Delta N/\Delta f \times N_0$), where $\Delta N$ is the number of events in the range between $f$ and $f + \Delta f$). The survival probabilities are related to $P_{U/F}(f)$ by the following equations,

$$
P_U(f) = 1 - \int_{f_{\text{max}}}^{f} \rho_U(f') df',
$$

$$
P_F(f) = 1 - \int_{f}^{f_{\text{min}}} \rho_F(f') df'.
$$

If we assume a two-state transition, the time evolution of the survival probabilities is described by the following master equations (66):

$$
\frac{dP_U(f(t))}{dt} = -k_U(f(t))P_U(f(t)),
$$

$$
\frac{dP_F(f(t))}{dt} = -k_F(f(t))P_F(f(t)).
$$

With this assumption and the experimental estimators for survival probabilities and densities, it is possible to extract the transition rates $k_{U/F}(f)$ from rupture force measurements using $k_{U/F}(f) = -\rho_{U/F}(f)/P_{U/F}(f)$, with $r$ being the pulling speed (36,56,66).

Salt corrections

It is interesting to experimentally measure the effect of salt on the free energy of formation of nucleic acid hairpins. However, UV absorbance experiments cannot be carried out for this particular sequence because its melting temperature is too high to obtain reliable results (see Section S5 of the Supplementary Data). Therefore, as mentioned in the ‘Hairpin model’ section, the estimation of the free energy of formation of the RNA hairpin at 1 M [Mon$^+$] is obtained using the NN energy parameters proposed by refs. (15,19). To introduce the effect of monovalent salt concentration [Mon$^+$] we assume a sequence-independent correction $g_1([\text{Mon}^+])$ for the free energy of formation of one base pair. As the free energy is measured relative to the F state we get for the free energy correction of a hairpin with $n$ unzipped bps:

$$
\Delta G_n^{[\text{Mon}^+]}(0) = \Delta G_n^{\text{1M}}(0) - ng_1([\text{Mon}^+])
$$

where $\Delta G_n^{\text{1M}}(0)$ corresponds to the free energy of formation of the n-th configuration at 1000 mM [Mon$^+$] at zero force. In the case of mixed monovalent/Mg$^{2+}$ conditions we add a second sequence-independent correction term $g_2([\text{Mon}^+])$ that captures the effect of Mg$^{2+}$ ions on the hairpin free energy of formation:

$$
\Delta G_n^{[\text{Mon}^+],[\text{Mg}^{2+}]}(0) = \Delta G_n^{\text{Mg}^{2+}}(0) - ng_1([\text{Mon}^+])
$$

$$
- ng_2([\text{Mg}^{2+}]).
$$

In what follows, unless stated otherwise, all monovalent and divalent salt concentrations [Mon$^+$], [Mg$^{2+}$] are expressed in mM units.

RESULTS

Effect of force on thermodynamics and kinetics of an RNA hairpin

We pulled the RNA hairpin at loading rates of 1.8 pN/s and 12.5 pN/s in buffers containing different ionic conditions (see ‘Measurement protocol’ section). From the unfolding and folding FDC we measured the first rupture forces along many cycles. The resulting probability distributions at each pulling speed and ionic condition can be found in the Section S5 of Supplementary Data. As previously observed (36), this hairpin displays a two-state behavior, with force jumps signaling the transition between F and U states (Figure 2A) and with no evidence of fraying or intermediate states (see Section S2 of the Supplementary Data).

The order of magnitude of the resulting rupture forces and hysteresis effects are compatible with previous force-melting experiments carried out for other simple nucleic acid structures, like P5ab and TAR RNA hairpins (31–33) or short DNA hairpins (55). Moreover, results at 1 M [Na$^+$] were in significant agreement with solution predictions (15,73). As expected, hysteresis effects strongly depend on the loading rate, being lower at 1.8 pN/s and higher at 12.5 pN/s. This can be seen in the experimental distributions of unfolding and folding first rupture forces (Figure 2B), which are closer for pulling cycles performed at 1.8 pN/s.

Experiments at different monovalent cation concentrations

We performed pulling experiments at four different NaCl concentrations (see ‘Measurement protocol’ section). We find that the RNA duplex stability increases at higher [Mon$^+$] concentrations. For instance, rupture force distributions are displaced to higher forces (Figure 3A) as we increase the concentration of NaCl. The greater duplex stability at higher salt concentrations can also be observed as an increase in the mean rupture force with the logarithm of the salt concentration (Figure 3B). The standard deviation of rupture unfolding (folding) forces, that are known to be proportional to $k_BT/\kappa_{\text{eff}}(f)$ (56), remain almost constant along the salt range explored. That might denote that the position of the TS mediating the unfolding and the folding transitions does not depend on [Mon$^+$], despite the fact that both transitions occur at higher forces. In Figure 3C, we see that the unfolding (folding) kinetic rates decrease (increase) with the salt concentration, which again shows the stabilizing effect of salt on the RNA hairpin.

Experiments at 1M NaCl

From the current set of NN energy parameters for RNA secondary structures obtained at 1000 mM NaCl concentration we can predict the free energy of formation $\Delta G_n(0)$ for the RNA hairpin at this particular condition using the mfold server (18,21,67,68). We get $\Delta G_n^{\text{fold}}(0) = 63.0 \ k_BT$ (although our experiments were performed at 1050 mM of monovalent salt, we do not expect significant differences by comparing with the
prediction at 1000 mM). By applying the kinetic method introduced in ‘the Kinetic analysis’ section we can evaluate the kinetic barrier associated to the unfolding reaction \( B^{\text{eff}}(f) \) and \( B^{\text{U/F}}(f) \) for a given elastic model for the ssRNA and find the one for which the theoretical prediction by Kramers rate theory (Equation 7) best matches the experimental results (Equation 10). The procedure is shown in Figure 4A and explained in the Section S4 of the Supplementary Data. We found the best fit to our data using the inextensible WLC model with persistence length \( P = 0.75 \pm 0.05 \) nm and interphosphate distance \( a = 0.665 \) nm/base. The free energy of formation obtained is equal to \( \Delta G_N(0) = 65.3 \pm 0.3 \) k\( B_T \), in reasonable agreement with the aforementioned value for \( \Delta G^{\text{Mfold}}_N(0) \).

Selection of elastic parameters: experiments with monovalent salts

In order to know \( \Delta G_N(0) \) at monovalent ionic conditions different from 1050 mM we need to know the effect of salt on the elastic contribution of ssRNA strands. The elastic behavior of single-stranded RNA (poly-U) has been studied in single-molecule stretching and fluorescence experiments carried out at various \([\text{Na}^+]\) concentrations (69–71). Despite the extremely different contour length of the molecules under consideration [we are dealing with 44 bases-long chain in contrast to a polynucleotide of 1500–4000 bases in ref. (70)], we take the values for the persistence length \( P \) proposed in previous stretching experiments and add our value obtained at 1050 mM. We assume that the elastic properties of ssRNA strands are independent of sequence, which can lead to a small error in the values obtained for the elastic properties of ssRNA strands in the case of sequence-dependent behavior. In fact, a sequence-dependent elastic behavior for ssDNA strands was previously considered as a possible explanation for the specific salt corrections found for the NN energy values obtained from unzipping experiments (37). In the inset of Figure 5 we plot the persistence lengths versus the monovalent salt concentration. To fit the data, we employ the following dependence of \( P \) on the Debye screening length \( \lambda_D \),

\[
P \sim \lambda_D^{-\nu} \propto \frac{1}{[\text{Mon}^+]^{\nu \tau}}.
\]  

No unique scaling law can be derived, as the value of \( \nu \) can be 1, 2 or <1, depending on the polymer properties (72).

![Figure 3. Kinetic analysis of experiments at varying [Mon\(^+\)].](image)
free parameter, which results in a value of $\nu = 0.60 \pm 0.06$. We then interpolate both fits, in order to infer the values of $P$ in our experimental conditions of $[\text{Mon}^+]$ concentration (50, 150, 550 and 1050 mM), as shown in Table 1.

### Table 1. Parameters obtained from experiments at different $[\text{Mon}^+]$

| $[\text{Mon}^+]$ (mM) | $P$ (nm) | $\log k_0$ (1/s) | $\Delta G_N(0)$ ($k_B T$) |
|-----------------------|----------|------------------|--------------------------|
| 1050                  | 0.75 ± 0.05 | 10.9 ± 0.4      | 65.1 ± 0.3               |
| 550                   | 0.82 ± 0.02 | 10.5 ± 0.4      | 64.0 ± 0.4               |
| 150                   | 1.01 ± 0.01 | 11.3 ± 0.5      | 59.4 ± 0.4               |
| 50                    | 1.27 ± 0.03 | 12.4 ± 0.4      | 54.0 ± 0.4               |

ssRNA persistence length $P$, log $k_0$, and free energy of formation ($\Delta G_N(0)$) for the RNA hairpin at different monovalent ion concentrations.

**Monovalent salt correction to the free energies of formation of the RNA hairpin**

Having obtained the elastic parameters that allow us to appropriately describe the elastic response of ssRNA strands at different $[\text{Mon}^+]$ concentrations, we still need to characterize the effect of salt on the energies of formation of the RNA hairpin at each intermediate
configuration \( n \). It is generally assumed a sequence-independent correction to the free energies of formation of nucleic acids duplexes (73–75). However, we have previously shown that a sequence-dependent salt correction to the NN energy parameters of DNA improves the free energy prediction of both unzipping and melting experiments (37). Related to this, it has been found that cation concentration affects RNA stability in a sequence-dependent manner (76). In the absence of RNA sequence-specific parameters available, we adopted a sequence-independent salt correction (Equation 13) given by \( g_1([\text{Mon}^+]) = m \log([\text{Mon}^+] / 1000) \), where [Mon\(^+\)] is expressed in mM units. As we will see, there are experimental and theoretical evidences that support the logarithmic effect of monovalent ions to the stability of nucleic acid hairpins.

Using this correction, the variation of \( \Delta G_n(0) \) with monovalent salt concentration depends strictly on the value of the constant \( m \). In order to derive \( m \) from our data, we compared the estimators of \( B_{\text{eff}}(f) \) obtained experimentally (\( B_{\text{eff}}^{(I)}(f) \) and \( B_{\text{eff}}^{(P)}(f) \) in Equation 10) with the theoretical prediction (\( B_{\text{eff}}^{\text{KT}}(f) \) in Equation 7) at different values of \( m \). In Figure 4A–D, we see the correspondence between theory and experiments at each monovalent ion concentration. For all salt concentrations, we found the best agreement at \( m = 0.10 \pm 0.01 \text{ kcal/mol} \). This value agrees with the sequence-independent salt correction reported for DNA duplex oligomers in melting experiments (37). Figure 3D summarizes all the results. At a given force we see that the height of the kinetic barrier increases with salt concentration, which again indicates that salt increases kinetically the stability of the RNA structure.

In Figure 5 we show the dependence of the measured \( \Delta G_n(0) \) of the RNA hairpin on the monovalent ion concentration. As expected from earlier observations on DNA (39,78,79) and from the application of counterion condensation theory to interpret polyelectrolyte effects on equilibrium involving highly charged, locally rod-like polyelectrolytes (40,80–82), we observe an approximately linear dependence of RNA duplex stability on the logarithm of monovalent salt concentration, \( m = 0.104 \pm 0.010 \text{ kcal/mol} \) (37). Figure 3D summarizes all the results. At a given force we see that the height of the kinetic barrier increases with salt concentration, which again indicates that salt increases kinetically the stability of the RNA structure.

In Figure 5 we show the dependence of the measured \( \Delta G_n(0) \) of the RNA hairpin on the monovalent ion concentration. As expected from earlier observations on DNA (39,78,79) and from the application of counterion condensation theory to interpret polyelectrolyte effects on equilibrium involving highly charged, locally rod-like polyelectrolytes (40,80–82), we observe an approximately linear dependence of RNA duplex stability on the logarithm of monovalent salt concentration. Interestingly, our data can also be well described by the empirical expressions derived in refs. (43–45), where the TBI model is used to predict the hairpin free energies at different ionic conditions (see Section S7 of Supplementary Data).

By deriving the effective barrier as a function of force we can measure the distance of the TS to the F and U states, \( x_{\text{eff}}^f(f) \) and \( x_{\text{eff}}^u(f) \) (Equation 8a and b), and the fragility \( \mu(f) \) of the molecule as a function of the applied force (Equation 9). Figure 6 shows the two extreme cases with 50 and 1050 mM [Mon\(^+\)] (continuous and dashed lines, respectively). In Figure 6A we observe that the location of the TS changes as a function of force. The same trend is observed for the fragility in panel B, where the experimentally measured points, the predicted force-dependent fragility (black curves) and the expected values of the fragility for all possible locations \( n \) of the TS along the stem. Continuous black lines are the theoretical prediction using Kramers rate theory for data at 50 mM [Mon\(^+\)], and dashed gray line corresponds to the WLC prediction when \( n = 19 \) or \( n = 6 \) bps are unzipped at 50 mM [Mon\(^+\)]. As seen, at an intermediate value of forces \( n = 6 \) coincides with the TS for both ionic conditions. (B) Dependence of fragility \( \mu(f) \) with force. Gray lines indicate the value of the fragility for different locations \( n \) of the TS along the stem. Continuous black lines are the theoretical prediction using Kramers rate theory for data at 50 mM [Mon\(^+\)], and dashed black lines for data at 1050 mM [Mon\(^+\)]. Blue and green points are the experimental evaluation of \( x_{\text{eff}}^f(f) \) and \( \mu(f) \) for folding and unfolding data collected at 50 mM [Mon\(^+\)]. Red and purple points are the experimental evaluation for folding and unfolding at 1050 mM [Mon\(^+\)].

![Figure 6](image_url)
Experiments in mixed monovalent/Mg$^{2+}$ conditions

We have also performed pulling experiments in mixed monovalent/Mg$^{2+}$ buffers, containing a fixed concentration of Tris$^+$ ions (50 mM) and varying concentrations of Mg$^{2+}$ (see ‘Measurement Protocol’ section). The rupture force distributions for all mixed monovalent/Mg$^{2+}$ conditions can be found in Section S6 of the Supplementary Data. We found two regimes in the behavior of the average rupture forces for unfolding and folding processes along the range of [Mg$^{2+}$] experimentally explored. Below 0.1 mM [Mg$^{2+}$], there is no significant difference between control (no Mg$^{2+}$ added) and magnesium-containing conditions (Figure 7A and B). However, at higher magnesium concentrations, we found a linear dependence of average rupture forces with the logarithm of [Mg$^{2+}$] (Figure 7A and 7B). Interestingly, Owczarzy et al. (51) have made a similar observation in DNA melting experiments done in mixed monovalent/Mg$^{2+}$ conditions. They found that the ratio $R = \sqrt{[\text{Mg}^{2+}] / [\text{Mon}^+] }$ (both salts in molar units) is a convenient parameter to determine whether divalent or monovalent ions are dominant on duplex stability. If $R$ is less than 0.22, then monovalent ions are dominant and the presence of Mg$^{2+}$ can be ignored. In our experiments, $R = 0.0632$ and $R = 0.2$ for the 0.01 mM and 0.1 mM [Mg$^{2+}$] conditions, respectively. As in the case of pure monovalent ion conditions, the standard deviation of rupture forces remains almost constant and we also observed a linear dependence of log $k_U(f)$ and log $k_F(f)$ on the applied force for the different [Mg$^{2+}$] tested (Figure 7C).

In order to obtain the free energy of formation $\Delta G_N(0)$ of the RNA hairpin at different magnesium concentrations, we employed the empirical expression derived in refs. (44,45) by applying the TBI model to predict the RNA helix stability in mixed monovalent/Mg$^{2+}$ ionic conditions (see Figure 9, inset). Using this mixed salt correction, it is possible to obtain the sequence-independent correction of one base pair $g_2([\text{Mg}^{2+}])$ at any mixed salt condition using Equation 14:

$$g_2([\text{Mg}^{2+}]) = \frac{1}{N} (\Delta G_{\text{N}}^{\text{Fold}}(0) - \Delta G_{\text{N}}^{\text{BI}}(0) - Nm \log([\text{Mon}^+/1000]))$$

(16)

From this expression, we can extract the value of $\Delta G_{\text{N}}^{\text{Mon}}([\text{Mg}^{2+}](0)$ in Equation 14 for any intermediate state $n$. By varying the mixed salt-dependent values for...
the persistence length \( P \) of the ssRNA for each [Mg\(^{2+}\)], we can now determine the value of \( P \) that results in better agreement between the predicted effective barrier \( B_{\text{eff}}(f) \) and our experimental estimations (Figure 8 and Section S4 of Supplementary Data). All the results are summarized in Figure 7D, where we can see that the stability of the hairpin increases with magnesium concentration. The dependence of \( P \) on [Mg\(^{2+}\)] is shown in Figure 9.

Table 2 summarizes the results obtained for the persistence length \( P \) and the attempt frequency \( k_0 \). The position of the TS varied with [Mg\(^{2+}\)] in a way similar to what we found for [Mon\(^{+}\)]. In a specified force the TS mediating the unfolding and folding transitions is shifted toward the U state as the [Mg\(^{2+}\)] is raised (Figure 10A), in agreement with the Hammond’s postulate (83). The force-dependence of the position of the TS with respect to the F state \( x_{\text{eff}}(f) \) and the hairpin fragility \( \mu(f) \) are similar in both monovalent and mixed ionic conditions (Figures 6 and 10). At low forces the TS is located near the loop, whereas at intermediate forces it is located in the stem region \( n = 6 \pm 1 \) (Figure 10A and B).

**DISCUSSION**

The effect of monovalent ion concentration on DNA stability has been extensively studied and there is a variety of empirical salt corrections available in the literature (84,85). There is no general agreement about the accuracy and limitations of use of salt corrections in terms of sequence length and range of salt concentrations (84). Recently, we have reported 10 NN salt correction parameters for prediction of DNA duplex stability derived from single-molecule experiments (37). However, there is no equivalent study on RNA duplexes and the
Table 2. Parameters obtained for experiments at different [Mg2+]  

| [Mg2+] (mM) | P (nm) | log k_0 (1/s) | ΔG^TBI^B(0) (k_BT) | g_2 (kcal/mol) |
|-------------|--------|---------------|-------------------|----------------|
| 0.00        | 1.27±0.03 | 12.40±0.40 | 55.58             | 0.000±0.005    |
| 0.01        | 1.50±0.15 | 12.05±0.30 | 55.50             | 0.007±0.005    |
| 0.10        | 1.25±0.10 | 11.45±0.30 | 55.55             | 0.005±0.005    |
| 0.50        | 0.90±0.15 | 11.50±0.30 | 57.06             | 0.039±0.005    |
| 1.00        | 0.80±0.10 | 11.40±0.50 | 58.63             | 0.0858±0.005   |
| 4.00        | 0.75±0.10 | 11.15±0.50 | 62.60             | 0.2033±0.005   |
| 10.0        | 0.75±0.10 | 10.40±0.50 | 64.77             | 0.2678±0.005   |

Persistence length for ssRNA P, log k_0, theoretical predictions for the free energies of formation based on the TBI model, ΔG^TBI^B(0), and sequence-independent correction g_2([Mg2+]) for the RNA hairpin at different magnesium concentrations. A fixed concentration of 50 mM [Mon+] was used in all ionic conditions.

Experimental data available for the salt effects on RNA duplex stability are limited to short sequences that display a two-state behavior (76). Different polyelectrolyte theories try to characterize the interaction between counterions and nucleic acids to study ionic effects to the molecular stability. The most accepted are mean field theories such as the Poison–Boltzmann and the counterion condensation theories (80,81). Recently, the TBI model has been proposed (43). It incorporates correlation and fluctuation effects for bound ions, and has been extended to treat RNA helices under mixed monovalent/divalent salt conditions (44,45). It was shown that the TBI improves the prediction of the stabilities of RNA duplexes smaller than 15 bp (44,86).

Here, we performed a detailed characterization of the effect of monovalent and mixed monovalent/magnesium concentrations on the stability of a RNA hairpin containing a stem of 20 bp by mechanically unfolding and folding the molecule using optical tweezers. The results we have obtained can be very well described by the empirical formulas derived from the TBI model for folding and unfolding data collected at 0.01 mM [Mg2+]. Red and purple points are the experimental evaluation for folding and unfolding at 0.01 mM [Mg2+], and dashed gray line corresponds to the WLC prediction when n = 19 or n = 6 bps are unzipped at 0.01 mM [Mg2+]. Dashed black lines are the empirical predictions for folding and unfolding data collected at 0.01 mM [Mg2+]. Red and purple points are the experimental evaluation for folding and unfolding at 10 mM [Mg2+].

![Figure 10](image-url)

Figure 10. Barrier location and mechanical fragility at 0.01 mM and 10 mM [Mg2+]. (A) Force-dependence of the barrier position measured with respect to the F state, x_F^<w>(f), at 0.01 mM [Mg2+]. Continuous gray line is the WLC prediction when n = 19 or n = 6 bps are unzipped at 0.01 mM [Mg2+], and dashed gray line corresponds to the WLC prediction when n = 19 or n = 6 bps are unzipped at 10 mM [Mg2+]. At an intermediate range of force the TS coincides with n = 6 for both ionic conditions. (B) Dependence of fragility μ(f) at 0.01 mM and 10 mM [Mg2+]. Blue and green lines are the empirical predictions using the TBI model for data at 0.01 mM [Mg2+], and dashed black lines correspond to the WLC prediction when n = 19 or n = 6 bps are unzipped at 10 mM [Mg2+]. Blue and green points are the theoretical predictions for folding and unfolding data collected at 0.01 mM [Mg2+]. Red and purple points are the experimental evaluation for folding and unfolding at 10 mM [Mg2+].

use the numbers derived from single-molecule stretching experiments for the elastic behavior of ssRNA molecules (as explained in the ‘Experiments at different monovalent cation concentrations’ section) to appropriately account for the energetic contribution of stretching the ssRNA strands during mechanical unfolding.

By applying the Kramers rate theory and correctly accounting for the elastic contribution of ssRNA stretching we were able to obtain the free energy of formation of the RNA hairpin from pulling experiments done in non-equilibrium conditions. This allowed us to obtain a large number of trajectories at different ionic conditions in a feasible timescale. In this way, we obtained the effective barrier of the unfolding reaction as a function of force for different ionic conditions.

The ability of Mg2+ ions to stabilize RNA structures at much lower concentrations than monovalent ions was recognized almost 40 years ago (89). In fact, by plotting the values of the free energies of formation versus salt concentration (expressed in mM units) we can collapse
data for both types of salt into a single master curve by multiplying \([\text{Mg}^{2+}]\) by a factor 100 (Figure 11A). This effect has been previously observed (24,46,47,90,91) and can be explained using the counterion condensation theory that accounts for strong correlations between counterions and polyelectrolytes (46,81,82,92). A similarity (but not a data collapse) is obtained for the persistence length values of ssRNA (Figure 11B). It can be interpreted as the screening effect of the counterions and polyelectrolytes (46,81,82,92). A conformational switch at the 3’ end of a plant virus RNA regulates viral replication. 

Figure 11. Comparison between \([\text{Mon}^+]\) and \([\text{Mg}^{2+}]\) results. (A) Free energy of formation of the RNA hairpin at different salt conditions. Magnesium concentrations have been multiplied by 100 along the horizontal axis. (B) Persistence length values for the ssRNA hairpin at different salt conditions. Magnesium concentrations have been multiplied by 100 along the horizontal axis.

As a further step, it would be very interesting to extend this study by mechanically unfolding an RNA hairpin containing a \(\text{Mg}^{2+}\)-specific binding site using our experimental setup. Eventually, the energetic contributions of different specific binding sites could be dissected and incorporated into current models of RNA structure prediction.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–8, Supplementary Methods and Supplementary References [1–15].

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