

Probing the kinetic and thermodynamic consequences of the tetraloop/tetraloop receptor monovalent ion-binding site in P4–P6 RNA by smFRET

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Abstract

Structured RNA molecules play roles in central biological processes and understanding the basic forces and features that govern RNA folding kinetics and thermodynamics can help elucidate principles that underlie biological function. Here we investigate one such feature, the specific interaction of monovalent cations with a structured RNA, the P4–P6 domain of the *Tetrahymena* ribozyme. We employ single molecule FRET (smFRET) approaches as these allow determination of folding equilibrium and rate constants over a wide range of stabilities and thus allow direct comparisons without the need for extrapolation. These experiments provide additional evidence for specific binding of monovalent cations, Na⁺ and K⁺, to the RNA tetraloop–tetraloop receptor (TL–TLR) tertiary motif. These ions facilitate both folding and unfolding, consistent with an ability to help order the TLR for binding and further stabilize the tertiary contact subsequent to attainment of the folding transition state.

Introduction

RNA molecules play roles beyond acting as simple intermediaries in information flow from DNA to proteins and numerous RNAs adopt folded structures in order to function [1]. These RNAs, in conjunction with associated proteins, function in epigenetics and genome maintenance [2,3], pre-mRNA splicing [4], RNA processing [5], gene expression regulation [6], protein synthesis [7], and protein transport [8]. It is important to study these complex macromolecular machines directly and also to uncover the physical features and forces that mediate their folding through systematic and controlled investigation of simpler, model RNA systems (e.g., [9–11]).

All RNAs, as polyelectrolytes, are subject to electrostatic interactions that disfavour folding and typically favour RNA–protein interactions [12,13]. Thus, these processes cannot be understood without deciphering electrostatics. Electrostatic interaction energies are modulated by a surrounding ion atmosphere and higher concentrations of ions and higher ion valences generally better screen charge and enhance RNA folding [11,13–17]. Ions can also directly bind to RNA molecules, and several metal ion binding sites on RNA have been identified through structural and functional studies (e.g., [18–25]). Distinguishing effects of ions in the atmosphere from those that are site bound is a major but

necessary challenge in dissecting electrostatic interactions and effects (e.g., [21,24,26–29]).

Most attention on specific metal-ion binding has focused on divalent metal ions and, in particular, Mg²⁺, as it represents the predominant cellular divalent metal ion. In principle, if monovalent ions only occupied the ion atmosphere and did not engage in specific interactions with RNA, they could be used simply to modulate charge screening through the ion atmosphere. However, there is compelling evidence for greater complexity of monovalent cation effects in RNA folding [30].

Monovalent cations preferentially occupy the ion atmosphere of a simple DNA helix inversely as a function of size [11] and studies on the relaxation of short DNA helices attached by a short, flexible, linker revealed the same trend [31]. However, Draper and colleagues [30] observed monovalent ion preferences for the folding of several RNAs that were distinct from size order obtained in the helix systems and different from one another. One of the RNAs exhibiting such a distinct behaviour, so-called ‘tecto-RNA’ [32], involves the association of two RNA pieces via two tetraloop–tetraloop receptor (TL–TLR) tertiary motifs. TL–TLR tertiary motifs are found in many structured RNAs [33] and prior studies with the P4–P6 RNA had provided evidence for a specific K⁺-binding site within its TL–TLR (Figure 1C; [18,34]).

P4–P6 is an independently folding RNA derived from the *Tetrahymena* group I intron, with two sets of coaxially stacked helices, a sharp bend mediated by the J5/5a junction and two tertiary interactions that connect the helical stacks, a

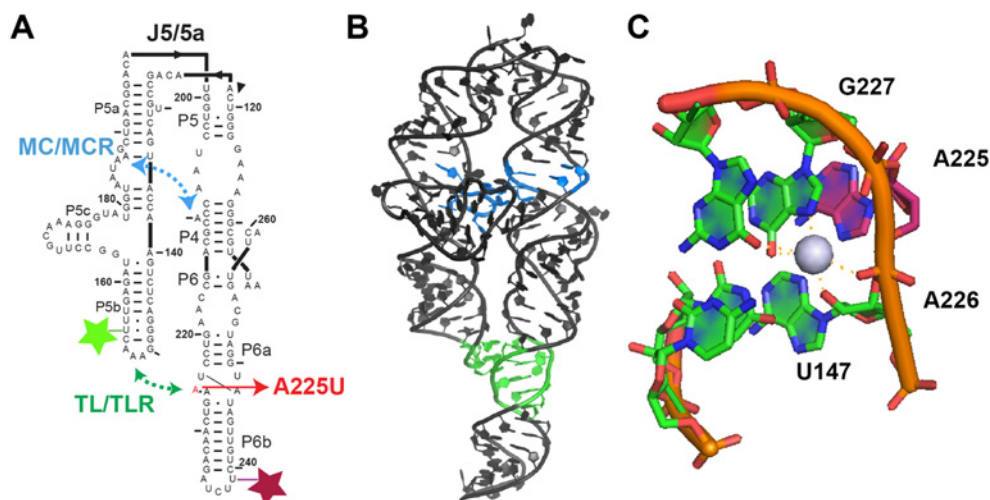
Key words: ion atmosphere, ribonucleic acid (RNA) folding, single-molecule fluorescence resonance energy transfer (FRET), tertiary contact motif.

Abbreviations: MC, metal core; MCR, metal core receptor; smFRET, single molecule FRET; TL, tetraloop; TLR, tetraloop receptor.

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Figure 1 | P4–P6 RNA and its crystallographic K⁺ ion-binding site

(A) Secondary structure of the P4–P6 domain. Tertiary contacts are coloured, the TL–TLR (green) and the MC–MCR (blue). Dye placements for smFRET are shown, Cy3 (light green) and Cy5 (maroon). A mutation used to disrupt the monovalent ion-binding site, A225U, is highlighted in red. Fluorescently-labelled P4–P6 molecules used in smFRET experiments were generated using splinted ligation of synthetic and transcribed RNA pieces, as described in [48,49]. (B) P4–P6 tertiary structure rendering based on the PDB: 1GID [35]. Tertiary contacts coloured as in (A). (C) Monovalent ion-binding site in the TLR of the *Azoarcus* group I intron, rendering from structure (PDB: 1T42, [42]). The same residues are found in the TLR in P4–P6 RNA and numbering from P4–P6 is used in the figure. The mutated residue, A225U, is coloured in red.



metal core–metal core receptor (MC–MCR) and a TL–TLR (Figures 1A and 1B) [35–38]. We have analysed the effect of monovalent cation identity on P4–P6 folding to compare it to prior studies with RNAs containing TL–TLR motifs. We used single molecule FRET (smFRET) to measure folding kinetics and thermodynamics for wild-type and mutant P4–P6 over a wide dynamic range.

Equilibrium effects of monovalent ions on P4–P6 folding

Figure 2(A) shows the equilibrium folding of P4–P6 RNA in a series of monovalent salts each present at 1.8 M for wild-type P4–P6 and A225U mutant P4–P6 (Figure 1A). P4–P6 RNA folds in ~ 1 mM Mg²⁺, but requires higher concentrations of monovalent cation to fold, because monovalent cations are much less effective in charge screening of polyelectrolytes than higher valency cations and because the stabilizing MC–MCR tertiary interaction only forms in the presence of some divalent cations [14,19,26]. For each RNA, under each condition we show the cumulative FRET distribution for all of the molecules, with individual and combined fits to a two-Gaussian model (Figure 2A). The folding and unfolding rate constants are shown in Figure 2(B) for each condition, with the values obtained for each molecule plotted and demonstrating good agreement across the molecular populations studied. Indeed, one of the reasons we chose P4–P6 for in-depth and focused biophysical studies is the

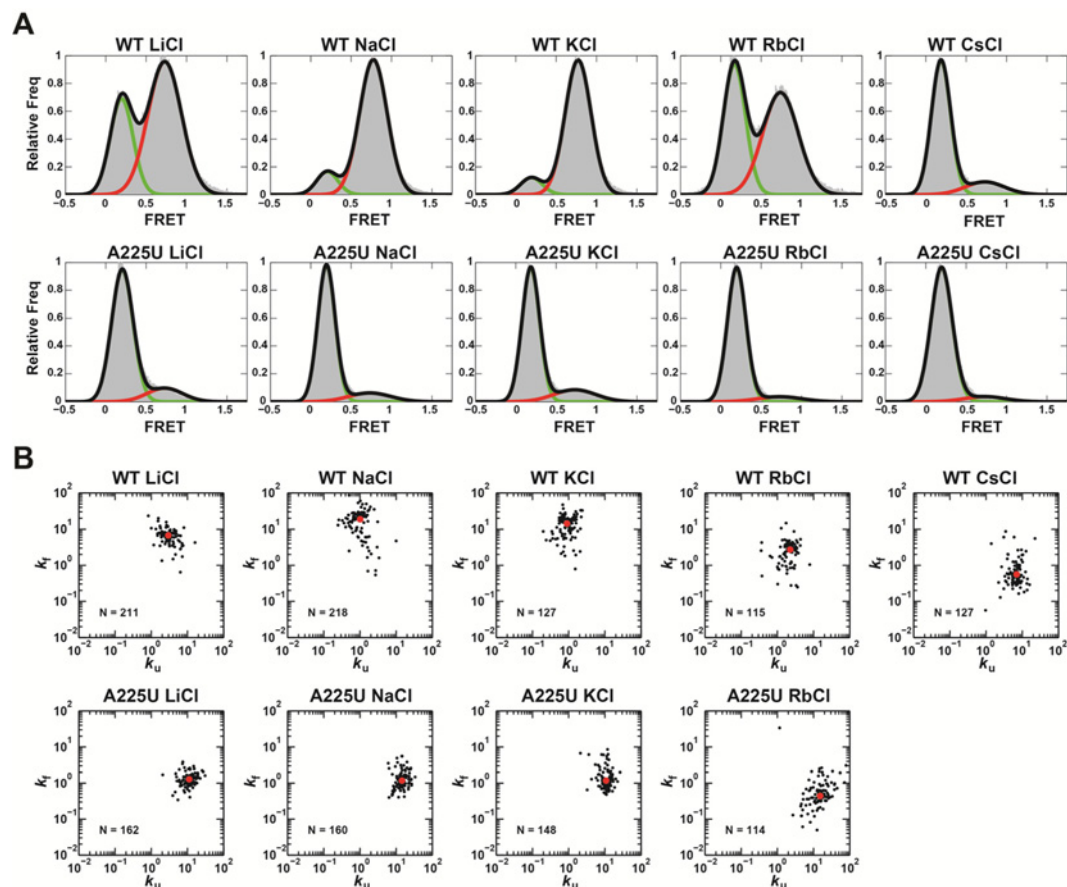
ability to obtain pure and homogeneous populations of this RNA [39].

As expected for a model that describes the data well, there is good agreement between the equilibrium constants obtained from the ratio of rate constants and those obtained directly from the fraction of time spent in high and low FRET states (Table 1). smFRET exhibits an extended dynamic range relative to bulk measurements [40] and we could obtain high precision measurements over equilibrium constants ranging from 0.03 to 20, nearly three orders of magnitude. Nevertheless, we were unable to reliably detect any folded mutant P4–P6 in Cs⁺ and thus can only report a limit for this equilibrium constant based on a fit to the overall FRET distribution and we could not determine values for its folding and unfolding rate constants.

The folding equilibrium for P4–P6 is plotted as a function of hydrated cation radius in Figure 3(A). As expected based on simple ion size considerations, the order of folding stability for wild-type P4–P6 is as follows: Na⁺ \sim K⁺ > Rb⁺ > Cs⁺. However, Li⁺ would be expected to give greater folding stability than Na⁺ or K⁺, but instead gives an ~ 7 -fold less favourable folding equilibrium (Figure 3A, black; Table 1). This deviation suggests that at least one cation engages in specific and stabilizing interactions with unfolded or folded P4–P6. To further probe the origin of this deviation, we investigated the folding of the A225U mutant of P4–P6. There was prior evidence that mutations in the TLR disrupts the monovalent cation-binding site (Figures 1A and 1C; [18,30]). A225U forms an ‘A-platform’ [41] with A226 that sits directly below the monovalent cation-binding site; this

Figure 2 | P4–P6 folding thermodynamics and kinetics in different monovalent ion solutions

(A) Distribution of FRET intensity for all traces at each condition (grey). FRET intensity is defined as the intensity of each molecule in the acceptor channel divided by the intensity found in both the donor and the acceptor channels. The apparent FRET values range from < 0 to > 1 as a result of varying background and noise contributions. The FRET intensity distribution was fit with a two-Gaussian model (overlay in black), representing the fraction of total time the population of molecules spend in the high FRET state (red line) compared with the low FRET state (green line). (B) Scatter plots of the folding compared with unfolding rate constants for each molecule selected under each condition. The mean values for each rate constant are shown in red. Rate constants were determined by analysing each FRET trace with the SMART (Single Molecule Analysis Research Tool) analysis data package, which utilizes a hidden Markov modelling-based algorithm that fits single-molecule trace data to kinetic models [47]. For P4–P6 folding, a simple two-state model was used. smFRET imaging, trace selection and processing was done as described previously [49,50], using a custom total internal reflection (TIRF) setup with image acquisition by Andor iXon Ultra camera and the Nikon Elements software. All smFRET data were taken with a 20 ms exposure time, an average single-to-noise ratio of 2.0 (defined in [47]), in 1.8 M monovalent-chloride salt, 100 mM Na–MOPS, pH 7.0, 0.1 mM EDTA solutions at 25°C, with an oxygen scavenging system of 2 mg/ml glucose, 1.8 mM Trolox, 100 units/ml glucose oxidase and 1000 units/ml catalase.



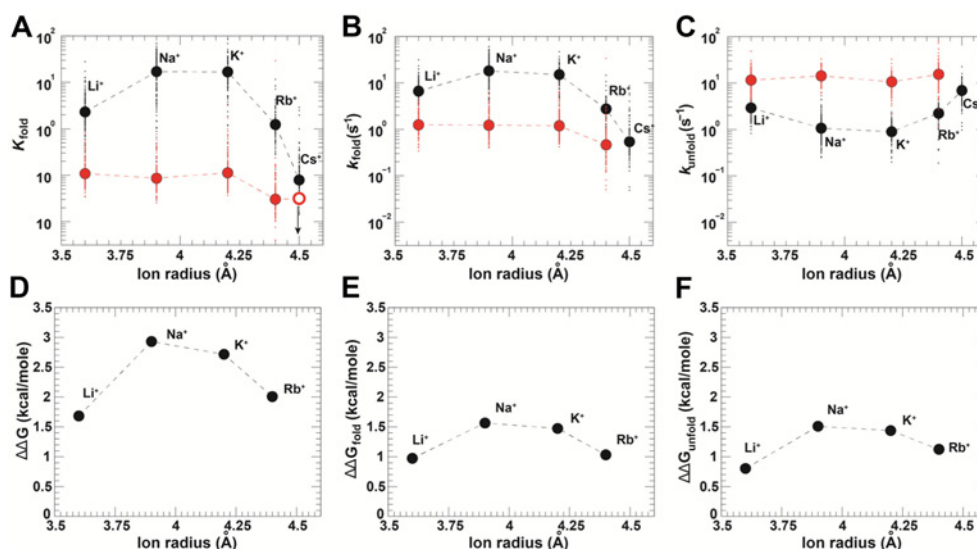
A-platform in the TLR makes multiple interactions with the monovalent cation based on the crystal structure of the *Azoarcus* group I intron [42] (Figure 1C) and thus the mutation A225U might be predicted to disrupt the association with the crystallographically-defined monovalent ion.

The observation that the preferential folding in Na^+ and K^+ over Li^+ is eliminated for A225U P4–P6 (Figure 3A, red) suggests that this folding preference arose from ion binding to this crystallographically-defined monovalent ion-binding site. Folding of the mutant is favoured in Li^+ , Na^+ and

K^+ relative to the larger Rb^+ or Cs^+ ions, consistent with expectations for a simple dependence on ion size. However, Li^+ did not give more favourable folding than Na^+ or K^+ , as would have been predicted based solely on ion size and we do not know why such an effect is not seen. It is possible that the crystallographic site is not fully ablated or that a different weaker site, still with some specificity for Na^+ and K^+ , remains. A225U mutant P4–P6 folds less well in each monovalent cation, with $\Delta\Delta G$ values ranging from ~ 3 kcal/mol (1 kcal \equiv 4184 J) in Na^+ or K^+ to ~ 1.5 kcal/mol

Figure 3 | P4–P6 folding kinetics and thermodynamics compared with hydrated ion radius

(A) Folding equilibria of wild-type (black) and mutant (red) P4–P6 compared with hydrated monovalent ion radius [11,51]. The folding equilibrium is defined as the ratio of the folding rate constant to unfolding rate constant, except for the data for A225U in CsCl, which is a limit obtained from the FRET distribution (Table 1) and is designated with an open circle and arrow. (B and C) Folding (B) and unfolding (C) rate constants of wild-type (WT) and mutant P4–P6 compared with ion size. The rate constants obtained for each molecule are plotted in small circles and the mean value of the population is plotted in large circles. (D) Effect on folding equilibrium of the A225U mutation compared with monovalent ion size, defined as $\Delta\Delta G = \Delta G_{A225U} - \Delta G_{WT}$. (E) Effect on the folding and (F) unfolding rate constants of the A225U mutation compared with ion size, defined as:

$$\Delta\Delta G_{\text{fold}}^{\ddagger} = -RT \ln \left(\frac{k_{\text{fold},A225U}}{k_{\text{fold},WT}} \right); \Delta\Delta G_{\text{unfold}}^{\ddagger} = -RT \ln \left(\frac{k_{\text{unfold},WT}}{k_{\text{unfold},A225U}} \right).$$


in Li⁺ or Rb⁺ (Figure 3D). These data indicate that there are deleterious effects of the mutation that remain in Li⁺ or Rb⁺, suggesting that the mutation disrupts interactions in addition to those involving monovalent ion binding.

The ion trends for wild-type and TLR-mutant P4–P6 closely match those observed for wild-type and a TLR mutant tecto-RNA [30]. As tecto-RNA also associates by TL–TLR interactions but has these interactions embedded within a different RNA context, these similarities suggest that the major contribution to the monovalent ion-specific effects lie within the TL–TLR and are not influenced by non-TL–TLR components of either tecto-RNA or P4–P6 RNA.

A simplified TL–TLR RNA, with these tertiary components tethered together via a flexible ssRNA linker, gave the same folding equilibrium and folding and unfolding rate constants in Na⁺ and K⁺ [43], consistent with the results in the more complex tecto- and P4–P6 constructs, but other monovalent cations were not investigated in the simplified TL–TLR system.

Rate effects of monovalent ions on P4–P6 folding

The folding and unfolding rate constants from the data in Figure 2(B) are compared, as a function of cation size, in Figure 3(B) and 3(C) respectively. For the mutant, there

is very little difference in these rate constants with folding in Rb⁺ being ~30% slower and unfolding possibly being slightly faster (red circles). These results stand in contrast with the dependence of the folding and unfolding rate constants for wild-type P4–P6 (black circles), with folding being ~2-fold faster and unfolding ~2–3-fold slower in Na⁺ and K⁺ relative to Li⁺ and Rb⁺ (Table 1). These results provide further support for the presence of a specific monovalent cation-binding site in wild-type P4–P6 and ablation of the site by the mutation.

The effects of the ions on the folding compared with unfolding rates can be considered in terms of phi analysis [44]. Phi analysis, borrowed from so-called linear free energy relationships in physical organic chemistry, compares equilibrium effects to effects on forward and reverse rate constants. Phi analysis has typically been applied to mutational effects, but here we consider the cation identity as our perturbant and other perturbations have also been analysed using similar frameworks [45].

The simplest scenarios for phi analysis are when the full effect of the perturbation on the equilibrium is manifested in either the folding or the unfolding rate constant. For example, if the full effect is on the folding rate constant, it is suggested that the interaction that is affected by the perturbation is not formed in the unfolded ground state but is (fully) formed sometime prior to the rate-limiting transition state. If the full

Table 1 | P4-P6 folding parameters obtained from smFRET

P4-P6 variants	Ion	Ion size* (Å)	$k_{\text{fold}}^{\ddagger}$ (s^{-1})	$k_{\text{unfold}}^{\ddagger}$ (s^{-1})	$K_{\text{fold, kin}}^{\ddagger}$	$K_{\text{fold, FRETdist}}^{\ddagger}$	ΔG^{\S} (kcal/mol)	SNR Cy3	SNR Cy5	Number of molecules	Trace lifetime (s)
WT	Li ⁺	3.6	6.3 ± 0.43	3.1 ± 0.22	2.1 ± 0.24	2.2	-0.42 ± 0.50	2.3	2.1	211	11
	Na ⁺	3.9	14.5 ± 1.7	1.0 ± 0.08	14.0 ± 2.2	7.6	-1.5 ± 0.67	2.8	2.2	218	17
	K ⁺	4.2	12.2 ± 1.5	0.90 ± 0.08	13.5 ± 1.8	9.4	-1.5 ± 0.45	3.2	2.5	127	18
	Rb ⁺	4.4	2.3 ± 0.30	1.9 ± 0.21	1.1 ± 0.17	1.3	-0.07 ± 0.48	2.3	2.4	115	22
	Cs ⁺	4.5	0.59 ± 0.09	6.3 ± 0.54	0.093 ± 0.018	0.20	1.4 ± 0.53	2.6	2.8	127	32
A225U	Li ⁺	3.6	1.3 ± 0.10	11.3 ± 0.82	0.11 ± 0.011	0.17	1.3 ± 0.34	2.2	2.2	162	14
	Na ⁺	3.9	1.3 ± 0.12	14.2 ± 0.75	0.091 ± 0.010	0.16	1.4 ± 0.37	3.0	2.7	160	18
	K ⁺	4.2	1.3 ± 0.15	10.2 ± 0.64	0.13 ± 0.018	0.22	1.2 ± 0.46	2.9	2.7	148	18
	Rb ⁺	4.4	0.51 ± 0.09	14.1 ± 1.76	0.036 ± 0.009	0.09	1.9 ± 0.56	2.4	2.7	114	21
	Cs ⁺	4.5	N.D.	N.D.	N.D.	<0.03	N.D.	N.D.	N.D.	153	27

*Ion size defined as the radius of the hydrated ions [51] and approximated as in [11] as the distance from the ion to the water oxygen atom in the first hydration shell plus 1.4 Å for the water layer.

†Error estimated through bootstrapping of measurements to determine 95% confidence intervals of the mean value.

‡ $K_{\text{fold, FRETdist}}$ determined through a fit of the total FRET distribution of a population of molecules to two-Gaussians (Figure 2A) and taken as the ratio of the total time spent in the high FRET state relative to the low FRET state. These values are considered less accurate at high and low equilibrium values than the values obtained from the kinetic modelling, as the kinetic fits directly take into account the experimental noise.

§ $\Delta G = -RT \ln(K_{\text{fold, kin}})$. Reported error is the standard deviation of the ΔG values for the population of molecules.

||Value considered a limit as the small amount of apparent high FRET signal could arise from noise. N.D., not determined; SNR, average signal-to-noise ratio, defined in [47]. All reported data have a SNR > 1.5.

effect is on the unfolding rate constant, it is suggested that the interaction that is affected by the perturbation is formed in the folded state and broken in the unfolding process prior to the rate-limiting transition state; in the folding direction this would mean that the interaction is made after the rate-limiting transition state.

For the monovalent cation effects on P4-P6 folding, we have the more complex scenario in which the cation identity affects both folding and unfolding rate constants and to similar extents (Figures 3E and 3F), i.e., folding is faster in Na⁺ and K⁺ than Li⁺ by 2-3-fold and unfolding is slower by a similar extent. Although there are many possible interpretations for such complex behaviour, the simplest model would be that folding occurs with the monovalent cation associated prior to the rate-limiting transition state, helping to align or organize the TLR and rendering a higher fraction of TL-TLR encounters productive and subsequent to this transition state additional TL-TLR interactions are made that involve TLR components that are affected by the bound monovalent cation.

Implications

The ability of smFRET to measure rate and equilibrium constants over a wide range of stabilities and conditions allows comparisons to be made that would otherwise not be possible through bulk techniques. In this case we have determined the effects of a series of monovalent cations on folding of the P4-P6 RNA and a mutant of this RNA under identical ion concentration conditions. The measured equilibrium constants span a range of 400-fold and the rate constants, a range of 40-fold (Table 1).

An issue that often arises in smFRET studies is heterogeneity between different individual molecules, which can be an intrinsic property of the molecule or an artifact from damage or other covalent heterogeneity [39,46]. Our plots comparing the kinetic behaviour of all of the molecules allow ready visual inspection of heterogeneity and more rigorous tools to assess heterogeneity are available as needed [47]. The data of Figure 2(B) are reasonably tightly clustered and comparisons between wild-type and mutant P4-P6 and cation identity reveal clear differences (Table 1 and Figure 3).

Our results confirm and extend our knowledge of ion-specific behaviour of RNAs containing TL-TLR tertiary motifs. Whereas the larger Rb⁺ and Cs⁺ ions give less folding, as expected for their poorer screening ability [11,31], Na⁺ and K⁺ are both more effective in stimulating folding than the smaller Li⁺ ion, consistent with binding of these monovalent cations [18,34,42].

Finally, the observation of increased folding rates and decreased unfolding rates in Na⁺ and K⁺ and only with wild-type and not A225U mutant P4-P6, is consistent with a model in which Na⁺ or K⁺ binding to the TLR favours more structures within its structural ensemble that can productively bind to the TL. After the folding transition state, additional stabilizing interactions would be made according to this model that would decrease the rate of unfolding.

Detailed models such as this underscore the need to follow individual reaction steps during the folding process and to probe RNA conformational ensembles. Future work will allow the development of more generalizable and predictive models for RNA folding and will facilitate the dissection and manipulation of the complex behaviour of large and structured biological RNAs.

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