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# Counterion Charge Density Determines the Position and Plasticity of RNA Folding Transition States

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The self-assembly of RNA structure depends on the interactions of counterions with the RNA and with each other. Comparison of various polyamines showed that the tertiary structure of the *Tetrahymena* ribozyme is more stable when the counterions are small and highly charged. By monitoring the folding kinetics of the ribozyme as a function of polyamine concentration, we now find that the charge density of the counterions determines the positions of the folding transition states. The transition state ensemble (TSE) between U and N moves away from the native state as the counterion valence and charge density increase, as predicted by the Hammond postulate. The TSE is broader and less structured when the RNA is refolded in polyamines rather than $Mg^{2+}$. That the charge density of the counterions determines the plasticity of the TSE demonstrates the importance of interactions among condensed counterions for the self-assembly of RNA structures. We propose that the major barrier to RNA folding is dominated by entropy changes when counterion charge density is low and enthalpy differences when it is high.



*Keywords:* group I intron; RNA folding; polyamines; Hammond postulate; counterion condensation

## Introduction

Folding of RNAs into their biologically active structure requires cations to shield the electrostatic repulsion of the negatively charged phosphate groups. The association of ions with the RNA is primarily determined by the charge of the counterion, and by the electrostatic field of the folded RNA.[1,2] In general, multivalent metal cations such as $Mg^{2+}$ or cobalt hexammine$^{3+}$ stabilize folded RNA structures more efficiently than monovalent ions, because fewer multivalent ions must be localized around the RNA, and because the multivalent ions are strongly attracted by regions of high negative charge density.[3,4]

Although it is well appreciated that the valence of counterions is important, the size and geometry of the counterions also influence the stability of the folded RNA.[5] This is because counterions that are condensed around the RNA interact with each other, as well as with the RNA. Theoretical models suggest that correlated fluctuations in the positions of the condensed counterions and in the RNA produce attractive forces that stabilize the folded RNA.[4,6,7] Simulations and experiments, however, have shown that large counterions approach the nucleic acid less closely and exclude other ions from its vicinity.[5,8–10] In addition, large counterions interact with each other more strongly due to their excluded volume and are hence more difficult to pack around the RNA. Thus, large counterions neutralize the RNA charge less efficiently than small ions, raising the free energy of the folded structure.

Because the valence and size of counterions influence the stability of the native RNA, we expect their charge density to determine the free energies of transition states and intermediates along the folding pathway.[11,12] Characterizing the transition state ensemble (TSE) is difficult in RNA, because the energy landscape is rugged.[13] We use variations of methods introduced in protein folding[14–16] to describe the counterion-dependent movements of the TSE for folding of the *Tetrahymena* ribozyme.

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Abbreviations used: TSE, transition state ensemble; $spd^{3+}$, spermidine; $spm^{4+}$, spermine; $put^{2+}$, putrescine; $2C^{2+}$, 1,2-ethanediamine; $3C^{2+}$, 1,3-propanediamine; $5C^{2+}$, 1,5-pentanediamine.

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We previously observed that the *Tetrahymena* ribozyme folds ten times faster in spermidine$^{3+}$ than in cobalt hexamine$^{3+}$, suggesting that counterions with a lower charge density produce compact RNA structures that are more dynamic.[11] Here, we compare the folding kinetics of the *Tetrahymena* ribozyme in a series of polyamines of different valence and size. We find that the valence and charge density of the counterions determines the relative position and breadth of the major TSE. The results show that the structure of the counterions, and not just their charge alone, must be considered to understand the mechanism of RNA folding.

We use polyamines to study the role of counterion structure in RNA folding, because their hydration energy is less likely to be perturbed by changes in valence and size than is the case for metal ions.[17] Polyamines are abundant in nature, and interact with chromatin and cellular RNAs.[18] In X-ray crystallographic structures, spermine and other amines primarily interact with the sugar-phosphate backbone of tRNA and DNA,[19,20] either directly or indirectly *via* water molecules. Like metal ions, polyamines are attracted to regions of strong negative potential, and in some cases, favorable interactions with the nucleic acid may overcome the energetic penalty for dehydrating the counterion. Spectroscopic data,[21] and molecular dynamics and Monte Carlo simulations,[22,23] however, show that polyamines predominantly remain flexible and hydrated when associated with DNA.

## Results and Discussion

### Native gel electrophoresis assay for folding kinetics

Previous studies showed that a small fraction (5–10%) of the *Tetrahymena* ribozyme folds directly to the native state N in ~1 s.[24–26] However, the majority of the RNA population reaches the native state over several minutes through an ensemble of intermediates I in which the ribozyme core is mispaired.[27] These slower folding transitions (U → I → N) were monitored by native gel electrophoresis as previously described.[24] In low ionic strength buffer, the unfolded ribozyme U was trapped as a mixture of non-native conformers that migrated slowly in the gel (Figure 1(a)). By contrast, pre-incubation in polyamines produced near-native conformations that were captured as the native ribozyme in native gels containing 3 mM $MgCl_2$.[4,5] Because the running buffer was kept at 4 °C and the samples enter the gel within a few seconds, only intermediates that are close in energy and structure to the native state are detected by this assay. To measure the folding kinetics, the ribozyme was incubated with polyamines for various times, and the observed rate of folding was determined from the fraction of native RNA in the gel (Figure 1(b)).

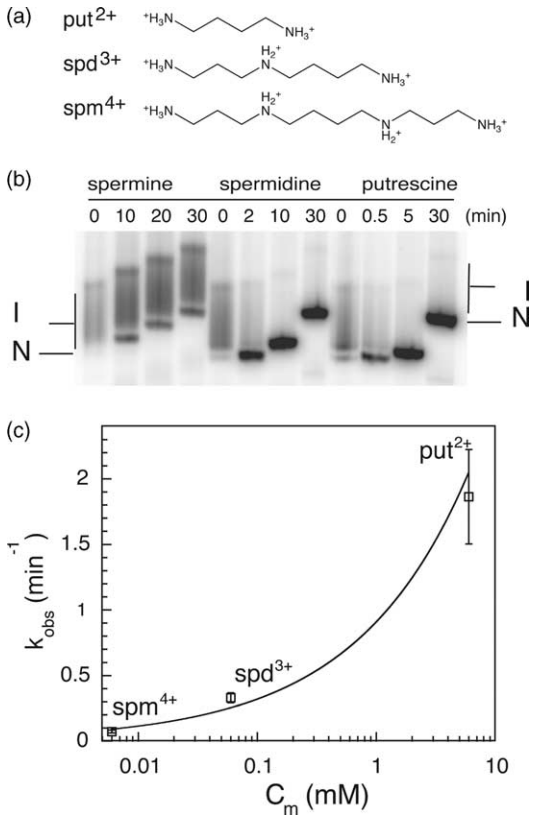


**Figure 1.** Effect of polyamine charge on folding of the *Tetrahymena* ribozyme. (a) Structures of natural polyamines putrescine ($put^{2+}$), spermidine ($spd^{3+}$), spermine ($spm^{4+}$). (b) Uniformly $^{32}P$-labeled L-21 ribozyme was incubated in various concentrations of polyamines at 30 °C for 0–30 min before native gel electrophoresis. I, misfolded RNA; N, native RNA. The concentrations of cations in reactions shown on the gel are: 0.01 mM spermine·$(HCl)_4$ ($spm^{4+}$); 0.3 mM spermidine·$(HCl)_3$-$\sigma\pi d^{3+}$; 100 mM putrescine·$(HCl)_2$ ($put^{2+}$). (c) Observed folding rate ($k_{obs}$) at the midpoint ($C_m$) of the folding equilibrium *versus* $C_m$.

### Folding kinetics and polyamine charge

We previously found that the folded ribozyme becomes more stable as the valence of the polyamine, $Z$, increases.[4,5] Hence, polyamines with higher valence should stabilize the folding intermediates to a greater extent and increase the free energy barrier between I and N. As expected, the ribozyme folded faster in putrescine ($put^{2+}$) than in spermidine ($spd^{3+}$) or spermine ($spm^{4+}$) over a broad range of polyamine concentrations (Figure 1 and Figure S1 in Supplementary Data). For each increment in $Z$, the midpoint of the folding equilibrium ($C_m$) shifted to lower polyamine concentrations by about 100-fold, and the rate of folding decreased two-to tenfold (Figure 1(b)).

### Folding kinetics and polyamine size

We previously found that the smaller polyamines, which have a higher charge density, also