doi:10.1006/jmbi.2001.4660 available online at http://www.idealibrary.com on IDEAL® J. Mol. Biol. (2001) 309, 57–68

JMB

Role of Counterion Condensation in Folding of the *Tetrahymena* Ribozyme II. Counterion-dependence of Folding Kinetics

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Condensed counterions contribute to the stability of compact structures in RNA, largely by reducing electrostatic repulsion among phosphate groups. Varieties of cations induce a collapsed state in the *Tetrahymena* ribozyme that is readily transformed to the catalytically active structure in the presence of Mg^{2+}. Native gel electrophoresis was used to compare the effects of the valence and size of the counterion on the kinetics of this transition. The rate of folding was found to decrease with the charge of the counterion. Transitions in monovalent ions occur 20- to 40-fold faster than transitions induced by multivalent metal ions. These results suggest that multivalent cations yield stable compact structures, which are slower to reorganize to the native conformation than those induced by monovalent ions. The folding kinetics are 12-fold faster in the presence of spermidine^{3+} than $[Co(NH_3)_6]^{3+}$, consistent with less effective stabilization of long-range RNA interactions by polyamines. Under most conditions, the observed folding rate decreases with increasing counterion concentration. In saturating amounts of counterion, folding is accelerated by addition of urea. These observations indicate that reorganization of compact intermediates involves partial unfolding of the RNA. We find that folding of the ribozyme is most efficient in a mixture of monovalent salt and Mg^{2+}. This is attributed to competition among counterions for binding to the RNA. The counterion dependence of the folding kinetics is discussed in terms of the ability of condensed ions to stabilize compact structures in RNA.

Keywords: RNA folding; group I ribozyme; counterion condensation; polyamines

Introduction

Counterions are essential for the stability of RNA tertiary structure.[1–3] While some metal ions bind specifically to well-defined sites on the folded RNA, the majority of cations are attracted non-specifically by the negative electrostatic field of the RNA. These non-specifically bound, or ''condensed'', ions are mobile with respect to the RNA, and are often associated less tightly with the RNA than ions that occupy specific pockets. Nonetheless, they contribute significantly to the stability of the folded structure by reducing the repulsion between phosphate groups. Both thermodynamic and kinetic folding transitions are coupled to binding of monovalent and divalent counterions, e.g.[4–9] Because counterions are needed to mediate the close packing of RNA helices, they play an important role in the folding process itself.

Theoretical considerations and experiments on several RNAs indicate that RNA should rapidly collapse to structures that are more compact in the presence of counterions.[10,11] Condensation of counterions around the RNA reduces the effective phosphate charge by 75-95%.[12] Reduction of the electrostatic repulsion between the phosphates after

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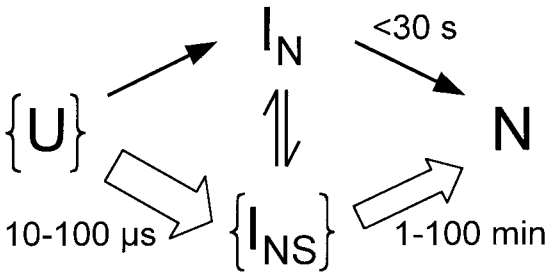

Figure 1. Non-specific collapse and the mechanism of RNA folding. Unfolded RNA (U) is proposed to collapse rapidly to a collection of compact structures (I_{NS}) that undergo further rearrangements to more stable structures.[10] The latter requires about one to ten seconds in 10 mM $MgCl_2$ at 42 °C.[20] Most of the RNA molecules are trapped in metastable intermediates (I) that contain some non-native interactions. These intermediates must partially unfold before reaching the native state (N).[15] A fraction of the RNA population may specifically collapse to a native-like intermediate (I_N) that leads directly to N.

the counterions condense is expected to trigger the formation of less extended structures (Figure 1).

A fraction of the RNA population may fold directly to the native structure (*via* I_N).[13] In practice, the majority of the RNA collapses non-specifically to a disordered state (I_{NS}) that contains a collection of metastable intermediates.[10] Transitions from I_{NS} to the native state involve partial unfolding of the RNA, and typically require minutes or hours.[10,14]

The presence of parallel folding mechanisms has been substantiated by investigations of the Mg^{2+}-dependent folding of the *Tetrahymena* ribozyme. During refolding *in vitro*, the ribozyme is kinetically trapped in intermediates containing some non-native and many native interactions.[15–17] Tertiary interactions in the P4-P6 domain appear in tens of milliseconds to one second, depending on ionic strength, while those in peripheral helices P2/P2.1 and P9.1 appear within ten seconds.[18–20] In contrast, the P3-P7 domain tends to mispair and folds more slowly.[17,20,21] This correlates with the long times (10-100 minutes) required for the ribozyme to gain full catalytic activity.[21] A point mutation that corrects the tendency of P3 to mispair enables 80 % of the RNA to fold rapidly (1 $second^{-1}$).[22] Single-molecule FRET experiments have shown that a small fraction of the wild-type RNA population folds at a rate of 1 $second^{-1}$, although the majority of the RNA folds more slowly through an intermediate state.[23] These experiments directly validate the kinetic partitioning mechanism of RNA folding.[11]

The appearance of tertiary structure in the ribozyme coincides with a rapid (more than one minute) reduction in its average hydrodynamic radius, as measured by small angle X-ray scattering.[24] This is consistent with the idea that collapse to compact structures occurs early in the folding process. The ribozyme from *Bacillus subtilis* RNase P undergoes similar multi-state transitions involving early collapse to a partially disordered state containing kinetically trapped intermediates.[25–27]

We previously found that equilibration of the *Tetrahymena* ribozyme in a variety of cations produces a collection of intermediates (I_N) that are readily transformed to the active structure once Mg^{2+} is added to the folding reaction.[28] Because the transition from I_N to N occurs rapidly at low temperatures, we infer that I_N has significant native character in the ribozyme core. Nearly molar concentrations of Na^+ or K^+ are required to induce this transition, while only micromolar amounts of $[Co(NH_3)_6]^{3+}$ are required. For a given valence, large cations induce I_N less effectively than ions with a smaller radius.[28]

The inverse relationship between the charge of the counterions, and the efficiency with which they stabilize I_N, may be understood in terms of classical counterion condensation theory.[29–31] This theory predicts that multivalent cations condense more strongly around the nucleic acid than monovalent ions, because the entropic cost of localizing fewer ions is reduced. Multivalent counterions neutralize the phosphate charge more completely than monovalent ions, and the reduced net charge favors folded structures. Multivalent ions can also stabilize folded structures by introducing attractive electrostatic interactions that ''bridge'' phosphate groups.[28]

Here, we show that I_N is formed much more slowly in multivalent cations than in monovalent cations, despite the fact that multivalent ions stabilize I_N more efficiently than monovalent ions. The inverse correlation between counterion charge and the folding kinetics can be explained by the premise that multivalent ions produce structures that are more compact and hence more thermodynamically stable than those produced by monovalent salts. Since multivalent cations are more effective in stabilizing compact structures, conformational rearrangements involving partial unfolding of the RNA, such as the transition from I_{NS} to I_N or N, are expected to occur more slowly in the presence of multivalent cations than monovalent salts. This basic model can account for the thermodynamics and kinetics of ion-induced folding of RNA.

Results

Native gel assay for counterion-induced RNA condensation

The conformational state of the ribozyme was monitored using native polyacrylamide gel electrophoresis as illustrated in Figure 2. Unrenatured L-21 ribozyme results in a collection of inactive forms (I) when loaded on native gels containing 3 mM $MgCl_2$ at 4-10 °C.[22] This is consistent with the idea that initial collapse of the RNA is non-specific. After renaturation in 2 mM $MgCl_2$, 85-95 % of the RNA migrates as a single band cor-

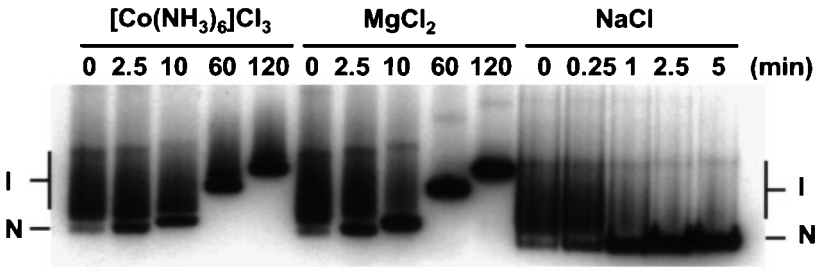

Figure 2. Native gel assay for folding kinetics. L-21 ribozyme was incubated in HE buffer (24.2 mM sodium, 50 mM Hepes, 1 mM EDTA (pH 7.5)) plus additional counterion at 30 °C for the time indicated above the lanes. Samples were immediately loaded on a native gel (10 °C) containing 3 mM $MgCl_2$ as described.[22] The gel was run continuously during the assay, so the samples that were loaded last have traveled a shorter distance. The counterion concentrations used were 1 mM $[Co(NH_3)_6]Cl_3$; 1.25 mM $MgCl_2$; 1 M NaCl.

responding to the active form of the ribozyme (N). Although cations other than Mg^{2+} or Mn^{2+} are not sufficient for full catalytic activity,[32] they induce a conformational state I_N that is transformed to the native structure during the brief time (15-30 seconds) that the RNA is exposed to Mg^{2+} in the wells of the gel.[28]

Previous experiments have shown that little if any refolding occurs in the gel matrix, which is maintained at 10 °C (the running buffer is 2-4 °C).[33,34] Hence, the amount of band N is taken to represent the fraction of ribozyme in the I_N state just before the samples are loaded on the gel. This assay was used to monitor the dependence of the equilibrium folding transition on the concentration of various counterions, including Na^+, K^+, Ba^{2+}, Mg^{2+}, $[Co(NH_3)_6]^{3+}$ and spermidine^{3+}.[28]

Kinetics of counterion-induced transitions

While the non-specific binding of counterions to polynucleotides is diffusion-controlled,[35] reorganization of non-native intermediates of the ribozyme requires several minutes or longer.[15,36] To investigate the kinetics of the counterion-dependent transitions, the ribozyme was incubated for times ranging from 30 seconds to four hours in 50 mM Hepes buffer supplemented with monovalent, divalent or trivalent cations at 30 °C. The fraction of I_N or N was monitored by native PAGE (Figure 2). Little native RNA is observed in the initial time of the experiment (~30 seconds), but nearly all of the RNA was able to fold after prolonged incubation with counterions.

Folding experiments were carried out in a range of counterion concentrations (Figure 3). First-order rate constants were obtained from the increase in

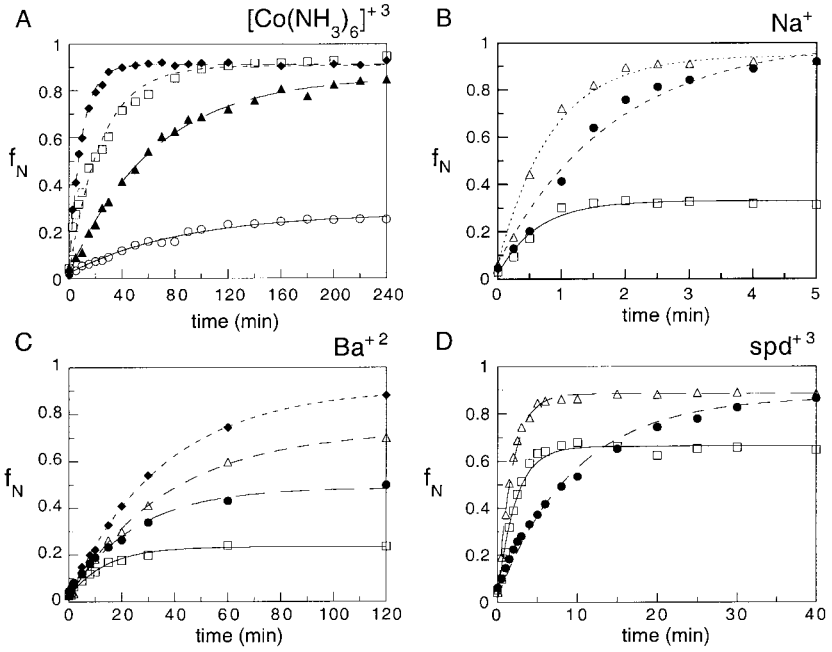

Figure 3. Kinetics of counterion-induced folding. Folding reactions were carried out in HE buffer plus the indicated salt at 30 °C as described in Materials and Methods and illustrated in Figure 2. Aliquots were loaded on a native gel at the times indicated. The data were fit to a first-order rate equation. (a) $[Co(NH_3)_6]^{+3}$: 12 μM (○), 18 μM (▲), 100 μM (◆), or 350 μM (□). (b) NaCl: 0.5 M (□), 1.0 M (△) or 1.5 M (●). (c) $BaCl_2$: 2.2 mM (□), 2.4 mM (●), 2.6 mM (△), or 3.0 mM (◆). (d) Spermidine-HCl: 0.085 mM (□), 0.2 mM (△), or 5 mM (●).

Table 1. Rate constants for cation-induced folding of ribozyme at 30 °C

Cation	C_m (mM)[a]	C_{max} (mM)	$k_{obs,\ max}$ (minute^{-1})	$f_{N,\ max}$
$[Co(NH_3)_6]^{3+}$	0.012	0.03	0.041 ± 0.001	0.90 ± 0.01
Spermidine^{3+}	0.054	0.2	0.55 ± 0.02	0.88 ± 0.01
Mg^{2+}	0.27	1.25	0.067 ± 0.003	0.90 ± 0.02
Ba^{2+}	2.4	3.0	0.028 ± 0.001	0.91 ± 0.01
Na^{+}	460	1000	1.29 ± 0.15	0.95 ± 0.03

Folding reactions were carried out in 50 mM Na-Hepes, 1 mM EDTA plus varying concentrations of counterion. Rate constants reported here were obtained in saturating concentrations of counterions (C_{max}). Amplitudes ($f_{N,max}$) and observed rate constants ($k_{obs,max}$) were determined from non-linear fits to a first-order rate equation as described in Materials and Methods. Errors are from uncertainties in the fits.

[a] The midpoint of equilibrium folding transition. Data taken from Heilman-Miller *et al.*[28]

the fraction of N (or I_N) with time (Table 1). The observed folding rate in 1.25 mM $MgCl_2$ was 0.07 minute^{-1} under these conditions. When the RNA was incubated in other cations, the folding rates ranged from 0.03 minute^{-1} in 3 mM $BaCl_2$ to greater than 1 minute^{-1} in NaCl or KCl (Table 1). Because these observed rates are much slower than diffusion ($\tau \approx 5$ μs), we conclude that reorganization of the RNA is required to form I_N after the initial binding of counterions.

Valence and size control folding rates

In general, we find that the rate of folding decreases as the charge on the metal cation increases (Table 1). Hence, it is likely that the free energy barriers between different conformational states are greater in the presence of multivalent ions than monovalent salts. From the folding rates in Table 1, the difference in the barrier is approximately $\Delta G^{\ddagger}_{Co} - \Delta G^{\ddagger}_{Na} \approx 2$ kcal mol^{-1}.

Spermidine also stimulates folding of the *Tetrahymena* ribozyme; the midpoint of the thermodynamic folding transition is 55 μM. However, the observed folding rate is 12-fold higher in saturating spermidine^{3+} than in saturating concentrations of $[Co(NH_3)_6]^{3+}$ (0.5 minute^{-1} *versus* 0.04 minute^{-1}; Table 1). As discussed below, larger cations such as spermidine are expected to produce more extended RNA structures than smaller metal ion complexes such as $[Co(NH_3)_6]^{3+}$, even when both ions carry the same total charge. This idea explains why reorganization of the ribozyme occurs more readily in spermidine than cobalt hexammine.

If folding is slower in $[Co(NH_3)_6]^{3+}$ because trivalent metal ions stabilize compact RNA structures, then we also expect I_N to be more stable under these conditions. Accordingly, I_N should unfold more slowly in $[Co(NH_3)_6]^{3+}$ than in Na^{+} or spermidine. In preliminary experiments, ribozyme annealed in 100 μM $[Co(NH_3)_6]^{3+}$ unfolded very slowly (0.006 min^{-1}) when the concentration of $[Co(NH_3)_6]^{3+}$ was shifted to 5 μM (data not shown). In contrast, comparable states (I_N) induced by spermidine or Na^{+} unfolded rapidly (1.4 minute^{-1} and ~10 minute^{-1}, respectively) when the counterion was diluted tenfold. These observations also suggest that reorganization of the RNA occurs more slowly in the presence of multivalent metal ions than in polyamines or monovalent salts.

Dependence of folding rates on counterion concentration

Our previous data showed that I_N is stabilized by increasing concentrations of counterions in a cooperative fashion.[28] If the formation of secondary and tertiary structure is rate determining, the observed rate of folding (to I_N) should increase with the counterion concentration. However, if unfolding of metastable intermediates is rate determining, the observed folding rate should decrease with higher counterion concentration. This is because the counterions also stabilize non-native interactions present in I_{NS}. An inverse dependence of the folding rate on $MgCl_2$ concentration was previously observed for the *Tetrahymena* pre-rRNA and ribozyme,[34,37] demonstrating that the folding kinetics correlate with the stability of the intermediates.

The rates of the transitions induced by Na^{+}, Ba^{2+}, $[Co(NH_3)_6]^{3+}$ and spermidine^{3+} were measured as a function of cation concentration (Figure 4). The equilibrium transition in $[Co(NH_3)_6]^{3+}$ has a midpoint of 12 μM, and approaches saturation between 30-100 μM.[28] The observed rate of the transition increases approximately tenfold between 12 to 100 μM $[Co(NH_3)_6]^{3+}$, under conditions in which additional counterion is needed to drive the formation of I_N. Above saturation (100 μM), the folding rate decreases with higher $[Co(NH_3)_6]^{3+}$ concentration, presumably because it becomes increasingly difficult for the non-native conformations present in I_{NS} to unfold.

Similar behavior was observed in spermidine, except that the observed folding rate remains roughly constant over the range of 5 to 500 μM. This concentration range encompasses the equilibrium transition ($C_m = 54$ μM).[28] Above 500 μM, the observed folding rate decreases exponentially as the concentration of spermidine is raised (Figure 4(b)).

For reactions containing Ba^{2+} and Na^{+} (Figure 4(c) and (d)), the folding rates decreased over the entire range of concentrations tested. For these ions, the folding kinetics appears to be domi-

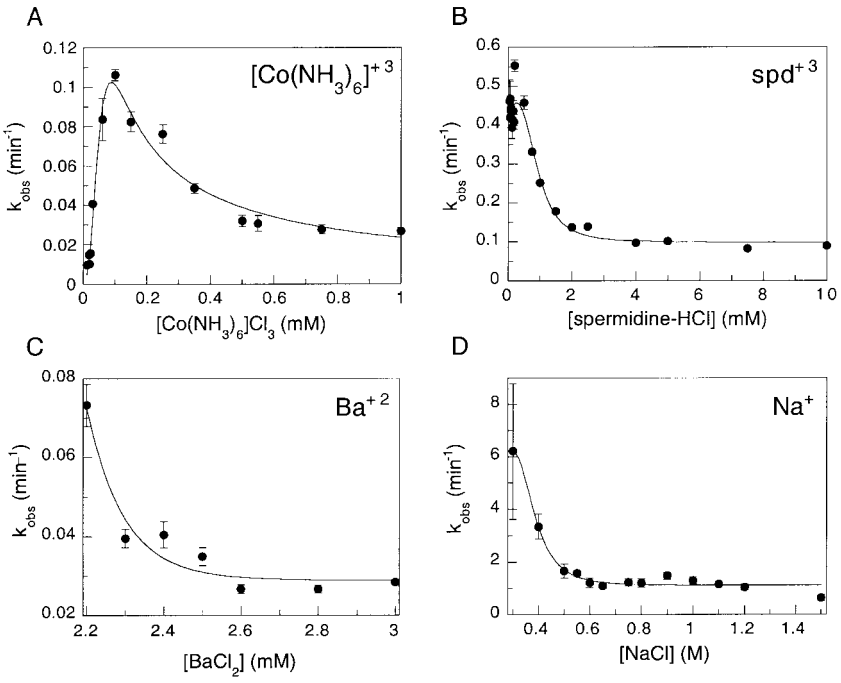

Figure 4. Counterion concentration dependence of folding kinetics. The observed rate constants from experiments similar to those shown in Figure 3 are plotted *versus* the cation concentration. To obtain a semi-empirical description of the system, the data were fit to a cooperative ion binding model to two states of the ribozyme (I_{NS} and I_N) as described in Materials and Methods. Error bars reflect the uncertainties in fitted values of k_{obs}. (a) $[Co(NH_3)_6]^{3+}$; (b) spermidine^{3+}; (c) Ba^{2+}; (d) Na^+.

nated by reorganization of the RNA during the transition from I_{NS} to I_N. Presumably, there exists a concentration range over which the folding rate increases with added Ba^{2+} or Na^+. As this would occur at concentrations well below the midpoint of the folding equilibrium, we are not able to observe this behavior, because the amplitudes of the folding reactions are too small to obtain reliable rates. The behavior of the ribozyme in Ba^{2+} and Na^+ is qualitatively similar to the dependence of the folding rate on $MgCl_2$ concentration.[34,37]

Effect of urea on counterion-induced folding kinetics

Moderate concentrations of urea increase the overall folding rate of the ribozyme in $MgCl_2$ because the misfolded intermediates are destabilized.[15,35,36] To determine whether the formation of I_N is limited by partial unfolding of nonnative interactions, the effect of urea on the folding kinetics in $[Co(NH_3)_6]^{3+}$ was examined. As anticipated, urea thermodynamically destabilizes the folded structure of the ribozyme. The midpoint of the $[Co(NH_3)_6]^{3+}$-induced transition increased from 12 μM in HE buffer to 25 μM in buffer plus 0.8 M urea (Figure 5(a)).

Two scenarios that describe the effect of urea on the folding kinetics may be considered. In model A, the reaction is dominated by transitions from I_{NS} and U to I_N. This will be true if fluctuations between compact intermediates (I_{NS}) and extended structures (U) occur more rapidly than I_N is formed. Under these conditions, $k_{obs} \approx k_u + k_f$, and the observed rate will reach a minimum near the equilibrium midpoint. This ''chevron'' behavior would be most likely to apply to reactions in subsaturating concentrations of counterion. If the stability of I_{NS} is comparable to I_N, as would be the case near the midpoint, then the addition of urea should moderately raise k_{obs} by increasing the unfolding rate. Under conditions in which I_N is more stable than I_{NS}, urea should decelerate folding. In contrast, urea should accelerate formation of I_N if the observed rate is limited by unfolding of stable misfolded intermediates.[38] This is best described by model B, in which folding is dominated by transitions from I_{NS} to I_N.

As shown in Figure 5(b), addition of 0.8 M urea to folding reactions containing 30 and 60 μM $[Co(NH_3)_6]^{3+}$ decreases the rate at which I_N is formed. In 20 μM $[Co(NH_3)_6]^{3+}$, a concentration that is above the midpoint in water ($C_m = 12$ μM) but below the midpoint in urea ($C_m = 25$ μM), addition of urea leads to a faster observed rate but a very low extent of folding. These results conform to the model A described above, and suggest that folding is determined by the equilibrium between I_N and I_{NS} under these conditions. These results are also consistent with the fact that the observed folding rate increases with $[Co(NH_3)_6]^{3+}$ concentration over the range of 20-100 μM (Figure 4(a)). In contrast to previous studies in $MgCl_2$,[15] the changes in k_{obs} due to urea are modest. This is because the free energy of the transition in $[Co(NH_3)_6]^{3+}$ is small (0.7 kcal mol^{-1}), implying that the stabilities of I_{NS} and I_N are similar.

In $[Co(NH_3)_6]^{3+}$ concentrations greater than 100 μM, the folding rate decreases with additional counterion (Figure 4(a)). This suggests that formation of I_N is limited by unfolding of I_{NS}, in saturating concentrations of $[Co(NH_3)_6]^{3+}$ (model B). As anticipated, addition of 0.8 M urea increases

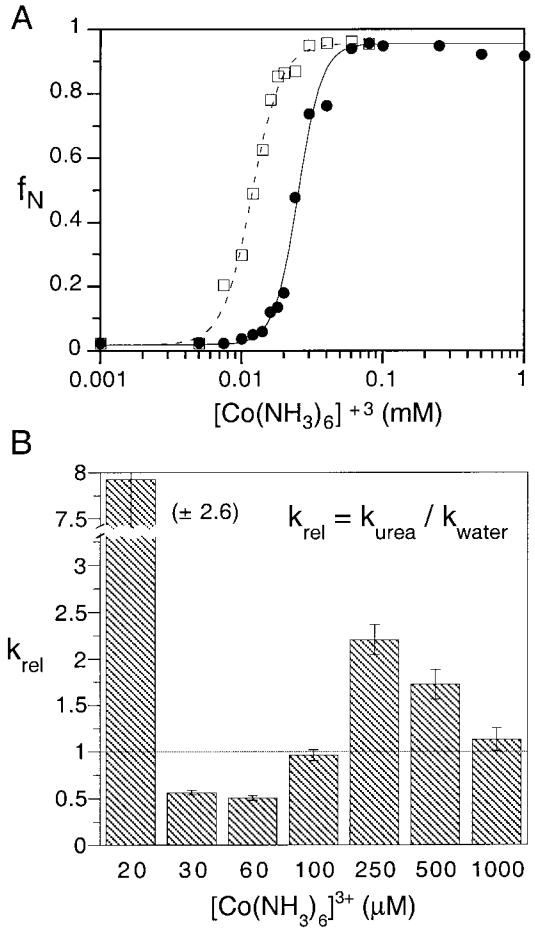

Figure 5. Urea alters folding kinetics in cobalt (III) hexammine. (a) Fraction of native RNA at equilibrium as a function of $[Co(NH_3)_6]^{3+}$ concentration, in HE buffer (□) or buffer plus 0.8 M urea (●). Reactions were incubated four hours before electrophoresis. (b) Folding reactions were carried out in $[Co(NH_3)_6]^{3+}$ as described for Figure 4, in the presence or absence of 0.8 M urea. The ratio of the observed rates, $k_{rel} = k_{obs}(urea)/k_{obs}(water)$, is plotted *versus* $[Co(NH_3)_6]Cl_3$ concentration. Error bars represent the standard deviation of the fitted parameters.

the observed folding rate of reactions containing more than 100 μM $[Co(NH_3)_6]^{3+}$. In 100 μM $[Co(NH_3)_6]^{3+}$, the folding rate is nearly the same in the presence and absence of urea (Figure 5(b)). Under these conditions, we conclude that transitions from I_{NS} to I_N involve at least partial unfolding of non-native interactions. In 1 mM $[Co(NH_3)_6]^{3+}$, 0.8 M urea has only a small effect on the rate of folding, presumably because more denaturant is needed to destabilize I_{NS}.

Monovalent salts increase the rate of Mg^{2+}-dependent folding

To determine whether monovalent salts increase the folding rate of the ribozyme in the presence of Mg^{2+}, folding reactions were carried out at 30 °C in 4 mM $MgCl_2$ plus 0-500 mM NaCl. As shown in Figure 6, the rate of folding increased fourfold in the presence of NaCl. Similar results were obtained when K^+ or NH_4^+ was added to the folding reactions (data not shown). In the absence of NaCl, the amplitude of folding reactions containing more than 3 mM $MgCl_2$ is low (20-30 %). This is probably due to the persistence of kinetically stable conformations at 30 °C, as the ribozyme folds completely when heated to 95 °C and cooled to 30 °C. The addition of NaCl resulted in an increase in the extent of the folding at 30 °C (Figure 6), suggesting that NaCl reduced the fraction of ribozyme that is irreversibly trapped in misfolded structures. These results are consistent with previous work showing that the folding rate of the P4-P6 domain of the ribozyme increases roughly 25-fold as the Na^+ concentration is increased from 10 to 200 mM.[18,19]

The observation that Na^+ increases the folding rate can be understood in terms of a competition between the Mg^{2+} and Na^+ for association with the RNA. As the Mg^{2+} concentration in these experiments (4 mM) is high enough to saturate the folding transition of the ribozyme, binding of 2 Na^+ must be accompanied by the release of Mg^{2+}. The fact that monovalent ions can compete with Mg^{2+} is demonstrated by the fact that 250 mM K^+ or 200 mM NH_4^+ increase the midpoint of the Mg^{2+}-dependent folding to 1.9 and 3.2 mM, respectively,[39] compared to 0.27 mM in HE buffer (Table 1). As Mg ions are displaced by Na^+, the structures represented by I_{NS} are expected to become less compact (relative to Mg^{2+}-induced structures) and have fewer long-range contacts between phosphate groups. This results in faster refolding of the RNA, in agreement with our results.

Discussion

Because RNA is a polyanion, the folding thermodynamics and kinetics are exquisitely sensitive to the nature of the counterions. By systematically comparing the effects of cations with different charge or size, one can begin to understand the principles underlying this process. Although ions other than Mg^{2+} are not sufficient to stabilize the catalytic structure, they promote the formation of conformations (I_N) that are readily transformed to the native structure ($\tau \leqslant 30$ seconds) in the presence of Mg^{2+}. This observation emphasizes the importance of non-site-specific interactions of metal ions with RNA. It also suggests that nucleation events need not involve binding of Mg^{2+} to specific sites. The first paper of this series[28] describes how the effect of valence and size on the efficiency of counterion-induced transitions can be predicted from classical counterion condensation theory. Here, we show that the folding kinetics can be generally understood in terms of the extent to

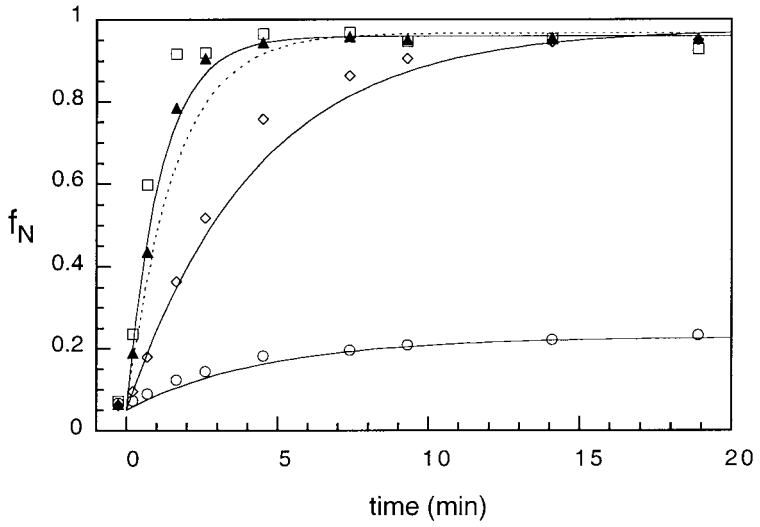

Figure 6. Acceleration of Mg^{2+}-dependent folding by NaCl. Folding reactions were carried out in HE buffer with 4 mM $MgCl_2$ plus varying amounts of NaCl at 30 °C. Fraction of native ribozyme (f_N) *versus* time was fit to a first-order rate equation. (○) No NaCl (0.22(±0.01) minute^{-1}); (◇) 100 mM NaCl (0.23(±0.02) minute^{-1}); (▲) 250 mM NaCl (0.64(±0.07) minute^{-1}); (□) 500 mM NaCl (0.89(±0.12) minute^{-1}).

which counterions stabilize compact conformations of the ribozyme.

Counterion-induced collapse and folding timescales

Theoretical arguments and experimental results on the *Tetrahymena* ribozyme support the model illustrated in Figure 7, in which a rapid counterion-induced collapse is followed by slower reorganization of the RNA.[10,11] With few condensed counterions, the RNA is expected to prefer extended conformations, due to electrostatic repulsion between the phosphate groups. This is borne out by the observation that the R_g value of the ribozyme is considerably larger in low ionic strength buffer than in 10 mM $MgCl_2$.[24] Condensation of counterions around the RNA reduces the net charge as much as 90 %,[28] and permits the chain to explore compact conformations. The collapse from extended to more compact structures is driven by interactions such as base stacking and hydrogen bonding, and by attractive interactions between the bound counterions and the phosphates.[40] The initial collapse is followed by a diffusive search for more stable conformations, in which energetically favorable interactions are optimized. After sufficient time, the majority of RNA reaches the native state (N) or a collection of intermediates that are compatible with the native structure (I_N).

The association of counterions with polynucleotides is expected to occur close to the diffusion controlled limit.[35] The timescale of counterion condensation can be estimated using $\tau_{CC} \approx 6\pi\eta\sigma/RTC^{2/3}$, where C is the counterion concentration, η is the viscosity of the solution, and σ is the coun-

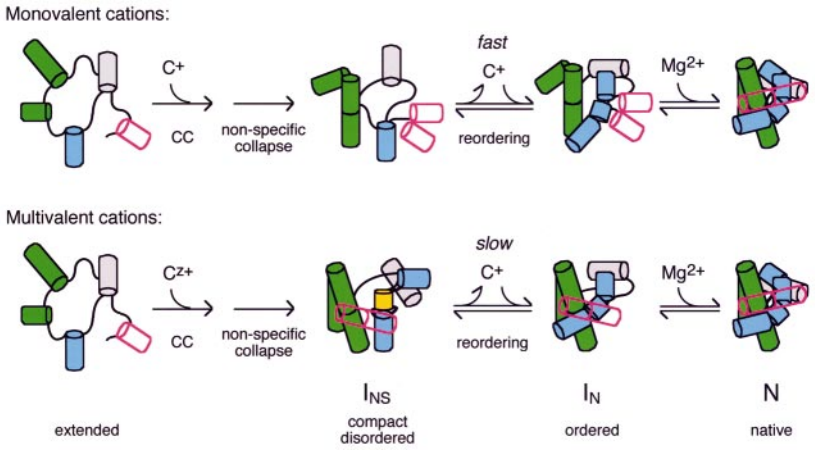

Figure 7. Model for counterion-induced RNA folding. Counterion condensation (CC) leads to compact RNA conformations. The initial state is structurally disordered (I_{NS}). It slowly rearranges to an ordered intermediate (I_N) that readily forms the native structure (N) in the presence of $MgCl_2$. Exchange between I_{NS} and I_N involves partial unfolding of the RNA and coupled dissociation and rebinding of counterions, and may occur in several steps. Monovalent salts and polyamines are proposed to produce more extended intermediates (top) than multivalent metal ions (bottom). As a result, the transition from I_{NS} to I_N is slower in the presence of multivalent metal cations.

terion radius.[41] For the counterions considered here, τ_{CC} ranges from 0.2 to 5 μs at the transition midpoints (Table 1). After counterion condensation, non-specific collapse is predicted to occur on a timescale of 100-400 μs.[11] This is only slightly longer than the relaxation times of small hairpins,[5,42] but is less than the shortest time constants (20-100 ms) for forming stable tertiary structure.[18,19,43,44]

As native interactions compete with non-native contacts, few of the initial structures resemble the native state. Under the conditions of our experiments, collapse is non-specific and produces a disordered state containing many conformations (I_{NS}) (Figure 7). As a result, many of the molecules become trapped in metastable intermediates that require long times (one second to 100 minutes) to be converted to the native structure. The idea that large RNAs may be compact yet conformationally disordered was suggested by native gel electrophoresis and gel filtration chromatography experiments on the yeast bI5 group I ribozyme.[45] These experiments showed that the Stokes radius of the RNA approaches that of the native structure under conditions in which little coherent tertiary structure is detectable.

Kinetic folding mechanism

The dependence of the folding kinetics on the cation concentration is consistent with the interpretation that reorganization of the intermediates involves partial unfolding of the RNA and the coupled release of some bound counterions. This is further supported by the fact that urea increases the rate of folding in saturating Mg^{2+} [15,25,36] and in $[Co(NH_3)_6]^{3+}$ (Figure 5). The observed rate constants shown in Figure 4 can be accounted for by a mechanism involving the exchange between two states, I_{NS} and I_N (see Materials and Methods). Both states are assumed to be stabilized by counterions, but some bound counterions are released during the transition from I_{NS} to I_N. Because increased counterion concentration drives the folding equilibrium toward I_N, a greater number of bound ions are linked to I_N than I_{NS}. Although we cannot exclude more complex models, the data are not explained by mechanisms in which the transition from I_{NS} to I_N does not involve the transient release and rebinding of counterions.

Because the electrostatic interactions between the counterions and the RNA are not described well by mass-action equilibria, the parameters of the fits are only semi-empirical measures of the change in k_{obs} value with counterion concentration. Moreover, some features of the data, such as the small increase and decline in k_{obs} values around 1 M NaCl, are not reproduced very well (Figure 4(d)). As I_{NS} encompasses many structures, the system is more complex than indicated in Figure 7. The transition from I_{NS} to I_N may occur in several steps, each involving the dissociation and reassociation of some of the bound counterions. If several ions must dissociate for the ribozyme to unfold significantly, the RNA will remain effectively trapped in metastable structures, even if individual ions are highly mobile.[46] Despite these caveats, this simple model qualitatively fits the counterion dependence of the folding kinetics.

The effect of urea on transitions induced by other counterions can be anticipated, depending on whether the observed folding rate increases or decreases with higher counterion concentration. For example, the folding rate decreases with increasing Ba^{2+} concentration over the range of concentrations shown in Figure 4(c). We expect folding in Ba^{2+} to be accelerated by the addition of urea, because the overall rate is determined by the I_{NS} to I_N transition. The precise extent of enhancement or reduction in folding rates will depend on the concentration and the nature of the counterions. It will be interesting to explore RNA folding kinetics as a function of both counterion and urea concentration in the future.

Counterion charge

As the transition from I_{NS} to I_N or N requires partial unfolding of the RNA, the rate of folding depends on the relative free energy of the intermediates, as well as the free energy of the transition states linking I_{NS} and I_N. We find that folding transitions occur much more rapidly in monovalent ions and a polyamine than in multivalent metal ions. These results can be explained in two ways: (1) multivalent ions stabilize I_{NS} more than monovalent ions; or (2) the free energy of the transition state in monovalent ions is lower than that in the presence of multivalent counterions.

The fact that folded RNA conformations are stabilized by divalent or trivalent ions is supported by a large body of experimental work.[2] Multivalent ions interact more strongly than monovalent ions with the dense electronegative field created by folded structures. As a result, folding of the RNA is coupled to the uptake of multivalent counterions and the displacement of monovalent salts.[2] Poisson-Boltzmann simulations suggest that the favorable entropy gained by the release of monovalent ions contributes significantly to the stabilization of tertiary structure by Mg^{2+}.[47]

An alternative view of the stabilizing effect of multivalent counterions is that they form Coulombic interactions with more than one phosphate group at a time.[28] These favorable electrostatic interactions, which operate over relatively long distances, tend to compact the RNA. Compact structures are likely to contain a greater number of energetically favorable interactions. Hence, the average free energy of the folding intermediates (I_{NS}) is expected to be lower in divalent and trivalent salts than in monovalent salts. Since our data suggest that the RNA must unfold somewhat during the transition from I_{NS} to I_N, the relative stability (and compactness) of the folding intermediates can account for the inverse correlation

between the folding kinetics and the valence of the counterions.

Counterion release and activation entropy

The second possibility, namely that the free energy of the transition state is lower in monovalent ions than in multivalent ions, can be rationalized if one considers that the transition from I_{NS} to I_N involves the release and rebinding of counterions. If most of the counterions are released before the transition state, the activation free energy will be reduced by a favorable entropy term. This term can be estimated from $\Delta S_{cation} = rR\ln(C_{condensed}/C_{bulk})$, where r is the number of ions displaced from I_{NS} and C is the concentration of ions in the condensed phase and bulk solution, respectively.[31] A larger number of monovalent than multivalent ions must be released per charge equivalent, resulting in a lower activation free energy for transitions in monovalent salts. However, very high concentrations of monovalent ions (0.5 M-1 M) are needed to drive folding transitions in the ribozyme. In practice, little entropy may be gained by the transfer of counterions from the condensed phase to bulk solution ($C_{condensed} \approx 2$ M for a monovalent salt interacting with *A*-form RNA[12]). Information about the temperature dependence of the folding rates will be needed to assess if arguments that rely on entropic barriers are adequate to explain the trends in the data.

Counterion shape

The effect of counterion size and shape on the rate of folding is vividly demonstrated by comparing spermidine and cobalt hexammine. Although both are trivalent cations, the folding rate is tenfold higher in spermidine (Table 1), and the equilibrium midpoint is fourfold higher. This suggests that spermidine is less effective than $[Co(NH_3)_6]^{3+}$ in stabilizing folded structures. If the compactness of the RNA correlates with the fractional charge remaining on the RNA, as we have suggested,[28] spermidine should produce more extended folding intermediates than $[Co(NH_3)_6]^{3+}$. Indeed, this expectation is consistent with experiments on DNA condensation by metal ions and polyamines.[48,49]

The differences between spermidine and $[Co(NH_3)_6]^{3+}$ can be rationalized in terms of the density at which counterions coat the RNA. Because of excluded volume interactions, two condensed counterions must be separated by a distance (2σ) that reflects the spatial correlation between the ions. The value of σ is determined by a combination of factors, including the size of the counterion, interactions between the counterion and RNA, and the distance dependence of interactions between counterions. Close packing of the counterions along the backbone becomes more difficult as σ increases. If σ is large, then a less negative charge will be neutralized, favoring more extended structures. Thus, the distance between counterions helps determine the extent of counterion-induced collapse, and hence the stability of the resulting structures.

We expect σ to be larger for spermidine^{3+} (~13 Å long) than cobalt (III) hexammine (diameter <9.4 Å).[50] This suggests that the latter should be a more efficient condensing agent, in accord with our observations. These observations are consistent with experimental and theoretical work on condensation of DNA by multivalent metal ions and polyamines.[49]

Transition state for counterion-induced folding

Information about the native character of the transition state can be gleaned from relative effect of counterions on the activation free energy and the overall free energy of the reaction. This is analogous to linear free energy relationships in physical organic chemistry, which have been applied to protein folding.[51] The free energy of the ribozyme folding transitions is linearly dependent on counterion concentration, over a range surrounding the midpoint. As a result, the free energy can be expressed as $\Delta G = \Delta G_{max} + m_C(C_{sat} - C)$, where C is the concentration of counterion, C_{sat} is the counterion concentration required to saturate the folding transition, and m_C reflects the change in the free energy with counterion.[34] In the transition from I_{NS} to I_N (or I to N), the activation free energy may be written as $\Delta G^{\ddagger} = \Delta G^{\ddagger}_0 - m^{\ddagger}_C C$. Notice that by this definition, $m^{\ddagger}_C < 0$ under most conditions, so that the energy barrier reaches a maximum at high counterion concentration. The ratio $\beta_C = m^{\ddagger}_C/m_C$ reflects the degree to which the transition state varies with counterion. For a simple two-state transition, the transition state is taken to resemble the native structure when $\beta \approx 1$, and the unfolded state when $\beta \approx 0$.

Application of this analysis to counterion-induced transitions of the ribozyme revealed that β_C values are small (Table 2). Hence, the gap between the free energies of the transition state and I_{NS} changes very little with counterion concentration, compared with the overall stabilization of I_N. This suggests that counterion-induced changes in the RNA conformation primarily occur in the early stages of folding, before the rate-determining step (which in this case involves reorganization of non-native intermediates). In Ba^{2+} or Na^+, β_C is less than zero, because the folding rate decreases with counterion concentration, even as the stability of I_N increases. The increase in the height of the free energy barrier can be explained either by stabilization of I_{NS} by the counterions, or by the fact that less entropy is gained from release of counterions in the transition state as the bulk ion concentration rises. The decrease in β_C with the valence of the counterion suggests a more compact transition state in counterions with greater

Table 2. Estimated free energy parameters for counterion-dependence of folding

Cation	m_C[a] kcal mol^{-1} mM^{-1}	$m_C^{\ddagger}$[b] kcal mol^{-1} mM^{-1}	β_C[c]
$[Co(NH_3)_6]^{3+}$, low[d]	116	28	+0.2
$[Co(NH_3)_6]^{3+}$, high[d]	(116)	−1.7	−0.02
Spermidine^{3+}	23	−0.4	−0.02
Ba^{2+}	3.0	−1.3	−0.4
Na^{+}	0.0071	−0.003	−0.4

[a] The change in the folding equilibrium with respect to cation concentration was determined from the best linear fit to ln$K$ *versus* C, where $K = f_N/f_I$, C is cation concentration, and the slope of the line is $-m_C/RT$. The temperature was 303.15 K. Data were taken from Heilman-Miller *et al.*[28]

[b] Determined from the best linear fit to ln(k_{obs}) *versus* C, where the slope $= -m_C^{\ddagger}/RT$.[34] This value was computed for limited regions of the curves in Figure 4 for which the folding rate varies exponentially with ion concentration.

[c] $\beta_C = m_C^{\ddagger}/m_C$.

[d] Low concentration range is from 12-100 μM; high range is from 100-500 μM.

charge density, although additional experiments are required to establish this.

Specificity of ion binding

Since all the counterions tested can induce I_N, it seems unlikely that this transition involves highly specific coordination of individual ions.[28] Nonetheless, a small subset of ions may occupy discrete binding sites in the native RNA, leading to specific stabilization of native interactions. Thus, both delocalized and site-specific interactions with counterions ultimately contribute to the stability of the native state and the folding kinetics.

High affinity sites are usually more selective for particular ions than delocalized electrostatic interactions, and may involve direct coordination of the metal ion by the RNA.[2,3] For example, as many as five divalent metal ions are coordinated by the P5abc subdomain of the ribozyme, while other sites are occupied by cobalt hexammine.[52,53] As a result, tertiary folding of P5abc is very sensitive to Mg^{2+} concentration. Rapid formation of tertiary structure in P5abc decreases the overall folding rate of the riboyzme by stabilizing intermediates in which the P3-P7 domain is misfolded.[16,22] Thus, binding of Mg^{2+} to specific sites in P5abc and elsewhere in the ribozyme may explain why the folding kinetics is much slower in Mg^{2+} than in Ba^{2+} or in Ca^{2+}. This is consistent with the general notion that destabilizing the interactions that lead to rapid formation of the P4-P6 domain should enhance overall folding rates.[54]

It is of interest to note that monovalent salts and even spermidine accelerate folding of the ribozyme, even though they do not strongly stabilize the tertiary structure of the RNA. In retrospect, it should not be surprising that *in vitro* refolding of RNA is most efficient in mixtures of counterions (K^+, Mg^{2+} and polyamines) that best approximate physiological conditions.[12] Finally, the fact that the RNA folds more rapidly in spermidine than expected for a trivalent cation raises the question of whether some proteins could facilitate the folding of RNAs merely by presenting a surface of regularly spaced groups with +1 charge.

Materials and Methods

Preparation of RNA

L-21 Sca ribozyme RNA[55] and stock reagents were prepared as described.[28]

Kinetics of ion-dependent folding transition

Non-renatured ^{32}P-labeled ribozyme RNA was incubated at 30 °C in HE buffer (50 mM Na-Hepes, 1 mM EDTA (pH 7.5)) plus other salts and 10 % (v/v) glycerol, 0.1 % (w/v) xylene cyanol. Where stated, the reaction mixture also contained 0.8 M urea. Reactions were begun by adding RNA to premixed buffer. Aliquots were removed at various times up to 240 minutes and loaded directly onto a native gel as described above. Gels were run continuously during the experiment. If necessary, power was temporarily increased while loading samples to ensure that the RNA entered the gel matrix within 15-30 seconds. Plots of f_N *versus* time were adequately fit by a single exponential equation $f_N(t) = f_N(0) + A_0(1 - e^{-kt})$, where A_0 is the amplitude of the folding transition, k is the observed rate constant of folding, and $f_N(0)$ is the fraction of native RNA at time zero.

The observed rate of folding as a function of cation concentration was fit to a semi-empirical equation derived from the two-state model below. For simplicity, we assume that the conversion from I_{NS} to I_N occurs in one step, and that all the RNA in the I_N state is converted to N when Mg^{+2} is added to the system, so that $f_{IN} = f_N$ in the native gel. The transition from I_{NS} to I_N involves the release of r counterions (C) and reassociation of s counterions:

$$I_{NS} \cdot C_r^{z+} \underset{k_u}{\overset{k_f}{\rightleftharpoons}} I_N \cdot C_s^{z+}$$

$$k_{obs} = \frac{k_f C^{s-r}}{K_I[1 + (C/C_{m,I})^r]} + k_u \qquad (1)$$

The data were fit to equation (1), where k_f is the rate of folding, k_u is the unfolding rate, C is the counterion concentration, $C_{m,I}$ is the dissociation constant for binding of

counterion to I_{NS}, s and r are the number of ions linked to forming I_N and I_{NS}, respectively, and K_I expresses the equilibrium between I_{NS} and I_N. Although the convergence of the fits was good, the uncertainty in individual parameters was very high in some cases. As a result, the curves should be only interpreted as a qualitative guide. The data are also described by a three-state model (U, I_{NS}, I_N), but are insufficient to handle additional parameters.

Acknowledgments

We thank Doug Turner and David Draper for helpful discussion, Ross Shiman for comments on the manuscript, and Yong Cao for the gift of T7 RNA polymerase. This work was supported by grants from the NSF (S.W. and D.T.) and the NIH (GM46686 to S.W.).

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Edited by J. Doudna

(Received 18 January 2001; received in revised form 28 March 2001; accepted 28 March 2001)