A Thermodynamic Framework and Cooperativity in the Tertiary Folding of a Mg^{2+}-Dependent Ribozyme†

Xingwang Fang, Tao Pan,* and Tobin R. Sosnick*

Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

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ABSTRACT: The folding thermodynamics of the catalytic domain from the *Bacillus subtilis* RNase P RNA is analyzed using circular dichroism and fluorescence spectroscopies, hydroxyl radical protection, and catalytic activity. Folding of this 255-nucleotide ribozyme can be described with three populated species: unfolded (U), intermediate (I), and native (N) states. The U-to-I transition primarily involves secondary structure formation, whereas the I-to-N transition is dominated by tertiary structure formation. The I-to-N transition is highly cooperative as indicated by the coincidence of the four probes applied here. Two isothermal methods are used to determine the stability of the N state relative to the I state at 10 and 37 °C. The first method measures the extent of Mg^{2+}-induced folding without urea or at constant urea concentrations. The second method measures the extent of urea-induced unfolding at constant Mg^{2+} concentrations. Via application of a cooperative binding analysis, the Mg^{2+} transition midpoint (K_{Mg}), the Hill constant (n), and the urea-dependent surface burial parameter (m value) determined by both methods are identical, indicating that they report the same, reversible folding event. Three conclusions can be drawn from these results. (i) The folding free energy of a Mg^{2+}-dependent tertiary RNA structure can be described by the K_{Mg} and n parameters according to a cooperative Mg^{2+} binding model. (ii) The Hill constant for this tertiary RNA structure probably represents the differential number of Mg^{2+} ions bound in the I-to-N transition. (iii) Under physiological conditions, the stability of this large ribozyme is similar to that of small globular proteins.

The elucidation of the thermodynamic properties of a biopolymer is one of the central aspects in understanding its folding behavior. A number of thermodynamic studies on tertiary RNA structures have been conducted (*1*−*4*). Two significant factors must be considered in the Mg^{2+}-dependent folding of tertiary RNAs.

First, a thermodynamic analysis requires the identification of multiple thermodynamic states because the folding free energy is defined as the logarithm of the ratio of two populations. For example, the stability of the RNA secondary structure can be described as the comparison between unpaired, unstacked RNA and paired, stacked RNA in a duplex (*1*). In our previous study on the ribozyme from the *Bacillus subtilis* ribonuclease P (denoted P RNA),[1] we identified two less structured states, the unfolded state and an intermediate state, in addition to the native state (*2*). Hence, the thermodynamics of P RNA folding involves the relative stability among at least three populated states.

The second consideration is the Mg^{2+} requirement for tertiary RNA folding. Due to the differential interaction of Mg^{2+} among the different structural states, the stability of the native state is explicitly dependent on the Mg^{2+} concentration. A Mg^{2+}-dependent folding transition may be described by a binding model in which the number of cations that bind cooperatively is correlated with an empirical parameter, the Hill constant n (*3*, *4*). The Hill constant is often interpreted as the minimal number of Mg^{2+} ions bound in the folding transition from a less structured state to the native state. Whether this Hill-type, cooperative binding model describes the underlying mechanism of Mg^{2+} stabilization, and hence can be used to define the free energy of tertiary folding, is unclear. To properly interpret the Hill constant and the validity of a Mg^{2+} binding model, a method independent of the assumptions in this model is needed to measure the folding free energy at constant Mg^{2+} concentrations.

To investigate the free energy and its relationship to the Hill constant in tertiary RNA folding, we have measured the folding free energy of the catalytic or C-domain of P RNA using two separate methods, Mg^{2+} and urea titrations. This independently stable, 255-nucleotide domain contains the entire active site and is fully active in the catalytic cleavage of an in vitro-selected substrate (*5*, *6*). Unlike the full-length P RNA, the C-domain contains a single tertiary structure and its kinetic folding pathway is free of traps (*7*), making this ribozyme a good model system for investigating the thermodynamics of tertiary RNA folding. The Mg^{2+}- and

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* Corresponding authors. Phone: (773) 702-4179. Fax: (773) 702-0439. E-mail: taopan@midway.uchicago.edu and trsosnic@midway.uchicago.edu.

[1] Abbreviations: CD, circular dichroism; C-domain, catalytic domain of the *B. subtilis* RNase P RNA containing nucleotides 240−409 and 1−85; ΔG, free energy of the I-to-N transition in the absence of urea; K_{Mg}, Mg^{2+} concentration at the midpoint of an RNA folding transition; m, free energy dependence of the I-to-N transition on urea concentration; n, Hill constant; P RNA, ribozyme component from *B. subtilis* RNase P; S-domain, specificity domain of the *B. subtilis* RNase P RNA containing nucleotides 86−239.

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urea-dependent folding equilibrium of the C-domain is examined by hydroxyl radical footprinting, catalytic activity, circular dichroism (CD), and fluorescence spectroscopies at constant temperatures. We apply a framework to quantitate the stability of tertiary RNA structures based upon the Mg^{2+} and urea dependence of the folding transition. This work, together with the accompanying paper (*29*), represents a systematic thermodynamic study of RNA folding using techniques and concepts commonly applied in protein folding studies.

MATERIALS AND METHODS

Preparation of the C-Domain. The C-domain of the *B. subtilis* P RNA was derived from a circularly permuted P RNA with the 5′-end at nucleotide 240 (*8*). The C-domain construct contains the T7 RNA polymerase promoter, nucleotides 240−409 and 1−85 of the P RNA, and 14 3′-nucleotides which include a *Fok*I restriction site. Transcription was carried out using the standard in vitro transcription procedure with the T7 RNA polymerase (*9*) using *Fok*I-cut plasmid DNA as the template. The transcription mixture contained 50 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 1 mM spermidine, 50 μg/mL bovine serum albumin, 2 mM DTT, each nucleoside triphosphate at 4 mM, and 40 μg/mL T7 RNA polymerase. The C-domain containing a fluorescein at the 5′-end was also prepared by in vitro transcription in the same buffer with 14 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.5 mM GTP, and 0.75 mM 5′-fluorescein-G. The 5′-fluorescein-G compound was synthesized by Dharmacon Research (Boulder, CO) using fluorescein phosphoramidite purchased from Glen Research (Sterling, VA). Unreacted fluorescein-G was removed by performing two phenol/chloroform extractions on the completed transcription mixture. The C-domain transcript was precipitated with ethanol, redissolved in 7 M urea and 100 mM EDTA loading buffer, purified on a polyacrylamide gel containing 7 M urea and 2 mM EDTA (to chelate residual cations), and stored in water at −20 °C.

In all experiments, the purified C-domain transcript was first heated in 20 mM Tris-HCl (pH 8.1) at 85−90 °C for 2 min followed by incubation at ambient temperature for 3 min. The C-domain at this stage was designated as the U state.

Mg^{2+} Titration Monitored by Hydroxyl Radical Protection. The fraction of the RNA protected against hydroxyl radical attack was determined by the Fe(II)−EDTA footprinting method (*10*). The procedure at 37 °C was the same as described for the full-length P RNA with 1 mM $Fe(NH_4)_2(SO_4)_2$ and 1.2 mM EDTA (*11*). For measurements at 10 °C, varying concentrations of Mg^{2+} were added to the U state and the mixture was incubated at 10 °C for 15 min. Ascorbic acid and DTT were then added to final concentrations of 1 and 5 mM, respectively, and the reaction was initiated by the addition of a 10× mixture of 0.5 mM $Fe(NH_4)_2(SO_4)_2$ and 0.6 mM EDTA (pH 8.0). The reaction proceeded at 10 °C for 14 h and was quenched upon addition of 10 mM thiourea. The reaction mixture was separated on a denaturing polyacrylamide gel containing 7 M urea and the result quantitated with a Molecular Dynamics phosphorimager.

Mg^{2+} Titration Monitored by Catalytic Activity. The fraction of the catalytically competent C-domain as a function of Mg^{2+} concentration at 10 °C was determined by the amount of cleavage of an in vitro-selected substrate (*6*). Varying concentrations of Mg^{2+} were added to the U state, and the mixture was incubated at 10 °C for 10 min. The folding half-life at this temperature was 25−60 s, depending on the Mg^{2+} concentration (*7*). The ribozyme was then mixed with an equal volume of renatured substrate in 50 mM Tris-HCl, 200 mM $MgCl_2$, and 2 mM spermine. The cleavage reaction proceeded for 7−15 s, and the reaction was stopped upon adding twice the volume of 9 M urea and 90 mM EDTA. The reaction product was separated from the unreacted substrate on 15% denaturing polyacrylamide gels and the fraction of the product quantitated by phosphorimaging. Compared to a control reaction mixture with the C-domain preincubated at 37 °C, >70% of the RNA incubated at 10 °C was catalytically active.

Mg^{2+} and Urea Titration Monitored by Spectroscopy. The U state was obtained as described above. When needed, Mg^{2+} was added to appropriate concentrations and the RNA incubated at 25 °C for 5 min to obtain the I state and the N state. The absorbance, CD, and fluorescence ($\lambda_{ex} = 493 \pm 5$ nm and $\lambda_{em} > 500$ nm) measurements were conducted with the Jasco J715 spectropolarimeter interfaced with a Hamilton electronic titrator.

Data Analysis. The two Mg^{2+}-dependent transitions were described according to a semiemperical cooperative binding model

$$U \xleftrightarrow{K_{Mg}^{UI}, n_1} I \xleftrightarrow{K_{Mg}^{IN}, n_2} N$$

where n and K_{Mg} are the Hill constant and Mg^{2+} midpoint of each transition, respectively (*12*). Because the two transitions were well separated in this system, each transition was fit independently according to

$$\frac{[I]}{[U]+[I]} = \frac{[Mg^{2+}]^{n_1}}{[Mg^{2+}]^{n_1} + (K_{Mg}^{UI})^{n_1}} \quad (1)$$

$$\frac{[N]}{[I]+[N]} = \frac{[Mg^{2+}]^{n_2}}{[Mg^{2+}]^{n_2} + (K_{Mg}^{IN})^{n_2}} \quad (2)$$

The K_{Mg} and n parameters were used to define the Mg^{2+}-dependent stability of the C-domain. For the transition between the I and N states where n Mg^{2+} cations were bound cooperatively, the (Mg^{2+}-dependent) free energy of the N state relative to the I state was written as

$$\Delta G_{IN}([Mg^{2+}]) = -RT \ln([N]/[I]) = -nRT \ln([Mg^{2+}]/K_{Mg}) \quad (3)$$

where R is the gas constant and T is the absolute temperature.

The K_{Mg} values of both transitions increase significantly in the presence of urea, but the Hill constants remain unchanged (ref *2* and this work). The shift in the midpoint in the presence of urea was used to calculate the change in stability according to

$$\Delta\Delta G = -nRT \ln[K_{Mg}(\text{urea})/K_{Mg}] \quad (4)$$

As is frequently done in protein folding studies (*13*, *14*), the folding free energy was approximated with a linear

dependence on denaturant concentration:

$$\Delta G(\text{urea}) = \Delta G + m[\text{urea}] \quad (5)$$

The K_{Mg}, and hence ΔG, values obtained from the Mg^{2+} titrations at two urea concentrations were used to calculate the denaturant response parameter, the *m* value, according to

$$m = -nRT \ln(K_{Mg2}/K_{Mg1})/([\text{urea}]_2 - [\text{urea}]_1) \quad (6)$$

obtained by combining eqs 4 and 5. In the urea unfolding measurement, ΔG and *m* values at a fixed Mg^{2+} concentration were obtained by fitting the observed signal, *S*, as the sum of the signal from the I (fraction f_I, signal S_I) and N (fraction f_N, signal S_N) states according to

$$S(\text{urea}) = f_I S_I + f_N S_N = \frac{S_I + S_N e^{-(\Delta G + m[\text{urea}])/RT}}{1 + e^{-(\Delta G + m[\text{urea}])/RT}} \quad (7)$$

Extraction of the free energy from urea titrations does not require knowledge of the Hill constant. However, the Mg^{2+}-dependent change in stability obtained from urea titrations can be used to calculate the Hill constant upon a rearrangement of eq 3:

$$n = (\Delta G_2 - \Delta G_1)/RT \ln([Mg^{2+}]_1/[Mg^{2+}]_2) \quad (8)$$

where ΔG_1 and ΔG_2 are the stabilities at Mg^{2+} concentrations $[Mg^{2+}]_1$ and $[Mg^{2+}]_2$, respectively.

Data analysis was performed using the Microcal Origin version 5.0 nonlinear fitting routine. Unless otherwise noted, errors listed are the standard deviation calculated by the fitting algorithm and reflect the statistical uncertainty of the fitted parameters.

RESULTS

Choice of the Tertiary RNA. The C-domain used in this study originates from a circularly permuted version of the P RNA having the 5′- and 3′-ends at nucleotides 240 and 85, respectively (Figure 1A; *8*). This domain folds independently as determined by the hydroxyl radical protection method (Figure 1B). In this method, nucleotides in RNA secondary structures are readily cleaved, and only those in regions with a reduced level of solvent exposure due to tertiary folding are protected (*3*, *10*). The protected regions in the C-domain are essentially identical to their counterparts in the full-length P RNA (*11*). For example, the J18/2, J18/4, J3/4, and P4 regions that form the active site (*15*, *16*) are well protected.

We chose to carry out a thermodynamic analysis of this RNA based on three observations. (i) This ribozyme folds without kinetic traps from 2 to 37 °C (*7*). Therefore, the observed intermediate state is likely to be on-pathway and productive, thus avoiding ambiguities in the interpretation related to misfolded structures. (ii) This ribozyme is fully active in the cleavage of an in vitro-selected substrate. Therefore, the native state can be unambiguously assigned and correlated to the folding transitions observed by spectroscopy and hydroxyl radical protection. (iii) This ribozyme has two well-separated structural transitions in the thermodynamic folding pathway, and the folding and unfolding can be studied over a wide range of Mg^{2+} and urea concentrations. To fully elucidate the applicability of our thermodynamic analysis, folding of this ribozyme is examined at 10 and 37 °C.

Mg^{2+}-Dependent Folding Monitored by Four Probes. Four structural and spectroscopic probes are applied here in examining the Mg^{2+} and urea dependence of the equilibrium folding transitions. In protein folding studies, a cooperative, two-state folding process without significantly populated intermediates implies that all probes report the same transition. We apply multiple probes in investigating the cooperativity of the folding of the C-domain.

Hydroxyl radical protection provides site-specific information about the folding of individual regions of the RNA. The relative level of protection as a function of Mg^{2+} concentration is the same for different regions of the C-domain (Figure 2A,B). This behavior indicates that these regions fold cooperatively in a single structural transition. This transition can be fit with a cooperative binding, Hill-type model (eq 2) (*12*), yielding two parameters, K_{Mg} (the Mg^{2+} concentration required to fold 50% of this RNA) and *n* (differential ion binding term or Hill constant). The K_{Mg} is 0.4 mM at 10 °C and increases to 1.2 mM at 37 °C. The Hill constant of about 3, however, remains constant at these temperatures (Table 1).

Catalytic cleavage of an in vitro-selected substrate provides a second probe for the N state. To assess the catalytically active fraction, the C-domain is first incubated with varying concentrations of Mg^{2+}. Then prefolded substrate is added at a high Mg^{2+} concentration, and the cleavage reaction is carried out for 7−15 s. Because the folding half-life of the U-to-N transition at 10 °C is ∼30 s, catalytic activity is primarily due to prefolded molecules. At 37 °C, however, folding occurs in <1 s (*7*) and additional molecules fold during the activity assay. Hence, the amount of molecules folded prior to the assay cannot be determined accurately at 37 °C. The folding transition monitored by catalytic activity is in excellent agreement with that monitored by hydroxyl radical protection (Figure 2C and Table 1).

CD provides an effective method for monitoring additional folding transitions that occur at lower Mg^{2+} concentrations (*2*). In the Mg^{2+} titration of the C-domain, pronounced changes occur in the near-UV CD spectrum at 260 and 287 nm (Figure 3). These signal changes can be explained with a U-to-I and an I-to-N transition, just as observed for the full-length P RNA. The absorbance signals at 260 and 287 nm mirror the U-to-I transition identified by CD. The U-to-I transition occurs below 100 μM Mg^{2+} at 10 and 37 °C and accounts for most of the A_{260} (hypochromicity due to base stacking) and $\Delta\epsilon_{260}$ (helix formation) change. These spectroscopic probes indicate that the I state has a near-native amount of secondary structure (Figure 3). Because both transitions are well separated, each can be fit independently with a Hill-type equation (eq 2). The focus of the study presented here, the I-to-N transition, can be readily observed with $\Delta\epsilon_{287}$ and is coincident with the I-to-N transition observed by the hydroxyl radical cleavage and catalytic activity (Table 1).

To enhance our ability to monitor folding with spectroscopic techniques, a fluorescein was covalently linked to the 5′-end of the C-domain. This fourth probe is sensitive to the two transitions observed by CD (Figure 4). The fluorescence signal decreases for the U-to-I transition, but increases for

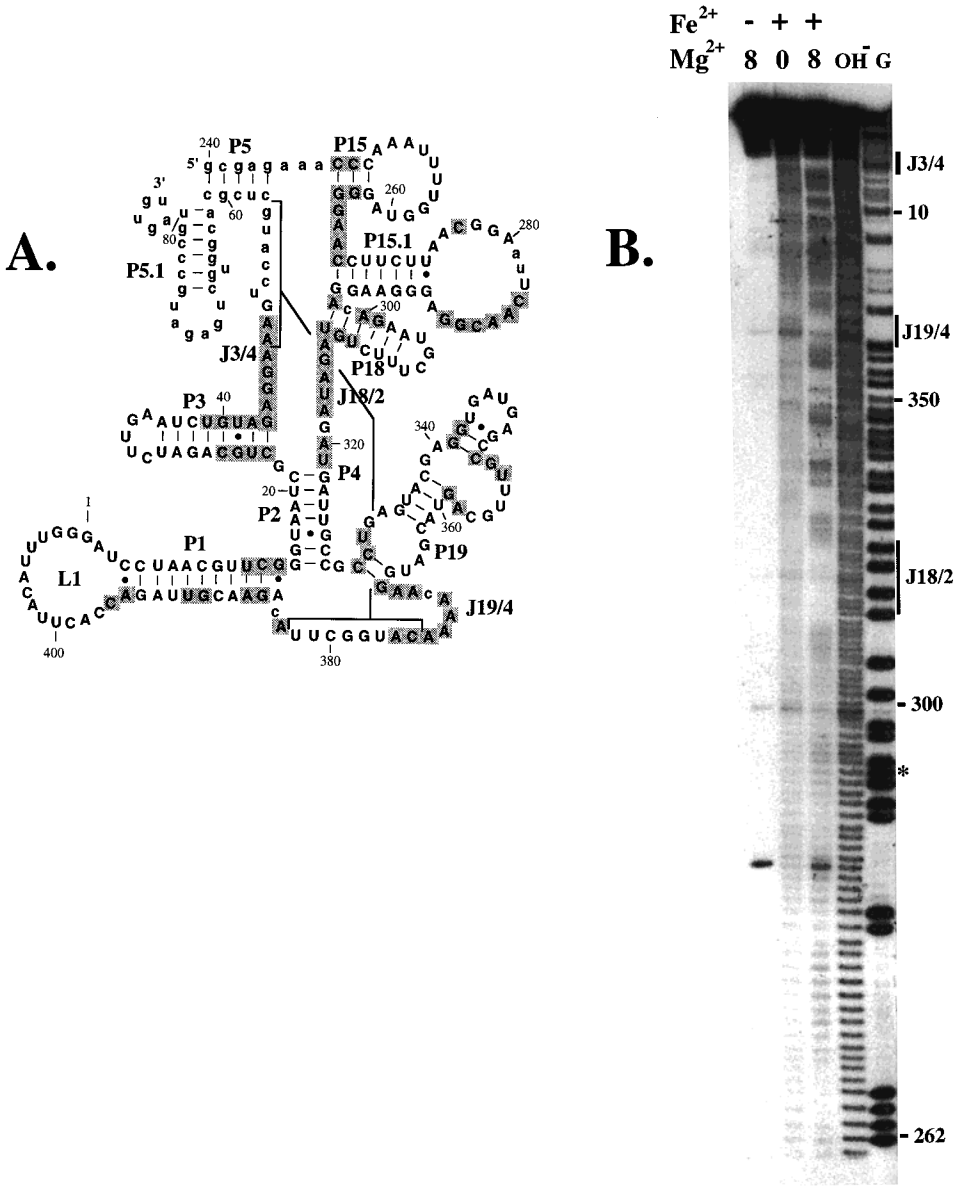

FIGURE 1: (A) Sequence of the C-domain construct and summary of the hydroxyl radical protection data at 37 °C. The nucleotide numbering is according to the phylogenetically derived secondary structure of the *B. subtilis* P RNA (*28*). Nucleotide residues with a protection factor of >1.5 are shaded. Residues that cannot be analyzed due to gel resolution are shown in lowercase letters. The proposed active site of P RNA is composed of residues in the J18/2, J19/4, J3/4, and P4 regions (*15*, *16*). (B) Hydroxyl radical protection of the C-domain at 37 °C in the absence or presence of 8 mM $MgCl_2$. The asterisk indicates an A292 → G mutation in the construct used in this work. This mutation changes an A-U base pair to a G-U pair and has no effect on the catalytic activity of the C-domain (data not shown). OH^- and G lanes represent partial alkaline hydrolysis and nuclease T1 digestion of the same RNA.

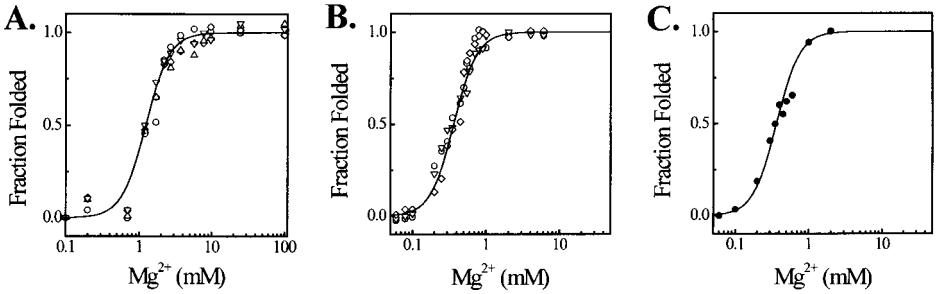

FIGURE 2: Mg^{2+} dependence of C-domain folding monitored by hydroxyl radical protection and catalytic activity. (A) Hydroxyl radical protection at 37 °C for residues 286−288 (○), 314−316 (◇), 373−375 (△), and 43−46 (▽). (B) Hydroxyl radical protection at 10 °C for residues 286−288 (○), 373−375 (▽), and 263−268 (◇). (C) Catalytic activity at 10 °C. All data are fit to the Hill equation (eq 2).

the I-to-N transition. This change in sign clearly distinguishes the two folding transitions, particularly the U-to-I transition identified by A_{260} and $\Delta\epsilon_{260}$. Folding monitored by fluorescence yields K_{Mg} and n values for the I-to-N transition identical to those determined by the three other methods (Table 1).

Table 1: Thermodynamic Parameters of the I-to-N Transition Obtained from Mg^{2+} Titrations

	K_{Mg} (mM)		n	
probe	37 °C	10 °C	37 °C	10 °C
hydroxyl radical	1.2 ± 0.2	0.42 ± 0.06	2.8 ± 0.3	2.7 ± 0.6
catalytic activity	ND[a]	0.40 ± 0.01	ND[a]	2.8 ± 0.1
$\Delta\epsilon_{287}$	1.1 ± 0.2	0.43 ± 0.04	3.5 ± 0.4	2.8 ± 0.5
Flr_{493}	1.1 ± 0.1	0.43 ± 0.02	3.6 ± 0.8	2.4 ± 0.7

[a] Not determined due to significant folding during assay.

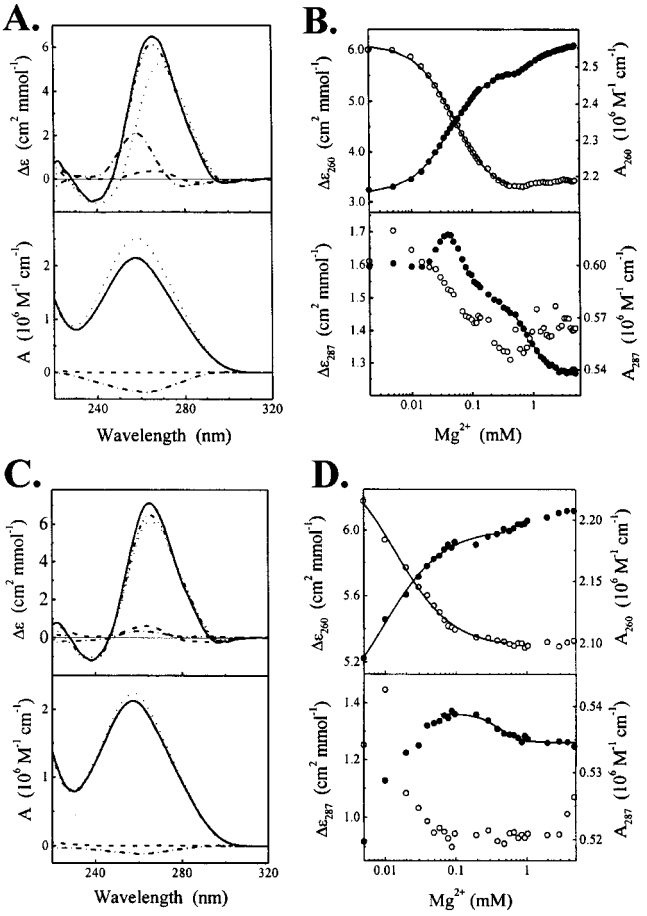

FIGURE 3: (A) CD (top) and absorbance (bottom) spectra of the C-domain at 37 °C. Mg^{2+} concentrations are 0, 0.4, and 5 mM, levels necessary to form the U (···), I (-···-), and N (—) states, respectively. The difference spectra between the U and I states (-··-) exhibit significant CD and absorbance changes at 260 nm, whereas the difference spectra between the I and N states (- - -) exhibit a significant change in CD only at 287 nm. (B) Mg^{2+} titration of the C-domain at 37 °C monitored by CD (●) and absorbance (○) at 260 (top) and 287 nm (bottom). The U-to-I transition is monitored at 260 nm by CD and absorbance, and the data are fit to eq 1. The I-to-N transition is monitored at 287 nm by CD, and the data are fit to eq 2. (C) CD and absorbance spectra of the C-domain at 10 °C. The Mg^{2+} concentration is 0, 0.15, and 5 mM for the U (···), I (-···-··), and N (—) states, respectively. Compared to that at 37 °C, the magnitude of signal change between U and I states is much smaller at 10 °C, suggesting that the U state has more secondary structure at the lower temperature. (D) Mg^{2+} titration of the C-domain at 10 °C. The data are treated in a manner identical to that of the data depicted in panel B.

In summary, the K_{Mg} and n values for the I-to-N transition are essentially identical as measured by all four probes. This overlapping behavior for different probes demonstrates that no other species are significantly populated and the folding transition is highly cooperative. These observations are consistent with our kinetic data (*7*).

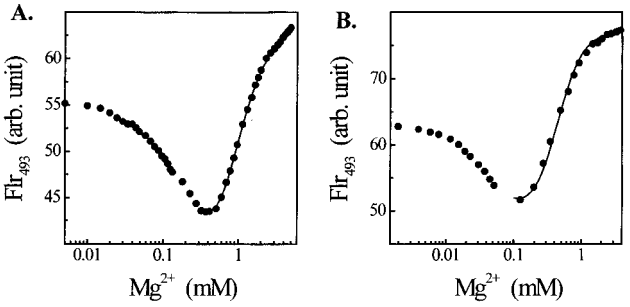

FIGURE 4: Fluorescence-monitored Mg^{2+} titration of the 5′-fluorescein-labeled C-domain at (A) 37 and (B) 10 °C. The U-to-I and I-to-N transitions are well separated as indicated by the change of sign.

Table 2: Thermodynamic Parameters of the I-to-N Transition Obtained from Mg^{2+} Titrations Carried Out in the Presence of 3 M Urea[a]

temp (°C)	K_{Mg} (mM)	n	m (kcal mol^{-1} M^{-1})[b]
37	6.5 ± 1.2	2.7 ± 0.4	1.1 ± 0.1
10	2.9 ± 0.4	3.1 ± 0.4	1.0 ± 0.1

[a] Monitored by CD at 287 nm. [b] Calculated according to eq 6.

Folding and Unfolding in the Presence of Urea. Urea denaturation has been used extensively in protein folding where the free energy is often linearly related to the urea concentration (eq 5). This same linear relationship also applies to RNA folding (*29*). The dependence of free energy on urea concentration is described by the m value which correlates to the amount of surface area buried in the folding transition of protein (*17*) and RNA (*29*). In this work and the accompanying paper, we show that the Mg^{2+}-dependent tertiary folding transition, I-to-N, can be characterized by three parameters: K_{Mg}, n, and m. All three parameters can be obtained from Mg^{2+} titrations at constant urea concentrations as well as from urea titrations at constant Mg^{2+} concentrations.

Mg^{2+}-Dependent Folding in the Presence of Urea. The Mg^{2+} midpoints for the U-to-I transition (not shown) and the I-to-N transition (Table 2) increase in the presence of urea, but the Hill constants remain unchanged, similar to those for the folding of the full-length P RNA (*2*) and tRNA (*29*). The invariance of the Hill constant for the I-to-N transition suggests that the structures of the I and N states are largely unchanged in the presence of urea with respect to Mg^{2+} binding. We calculated the m value for the I-to-N transition from the decrease in the free energy (obtained from the Mg^{2+} titrations) upon addition of urea according to eq 6. The m value for this transition is $\sim$1.0 kcal mol^{-1} M^{-1}, equivalent to the surface buried in the formation of $\sim$12 base pairs in an RNA helix (see Figure 6 in ref *29*). This relatively small m value indicates that the I state already has a significant amount of surface burial, consistent with this state having a near-native amount of secondary structure as indicated by UV absorbance.

Urea-Dependent Unfolding at Constant Mg^{2+} Concentrations. An alternative method for measuring the folding free energy is urea titration at fixed Mg^{2+} concentrations. This method does not require knowledge of the Hill constant in determining the folding free energy. Two parameters can be obtained from a single urea titration, the free energy at 0 M

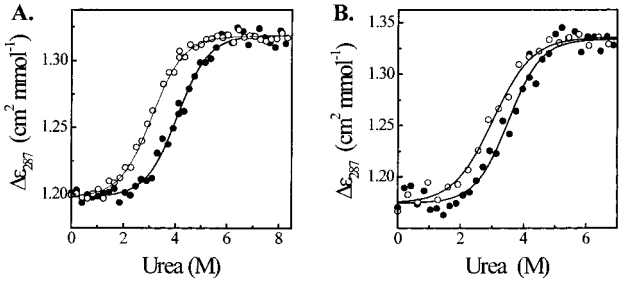

FIGURE 5: Urea titration of the C-domain (A) with 6 (○) and 12 mM $MgCl_2$ (●) at 37 °C and (B) with 2 (○) and 4 mM $MgCl_2$ (●) at 10 °C. Folding is monitored by CD at 287 nm.

Table 3: Thermodynamic Parameters of the I-to-N Transition Obtained from Urea Denaturation at Constant Mg^{2+} Concentrations[a]

temp (°C)	$[Mg^{2+}]$ (mM)[b]	ΔG (kcal/mol)[c]	m (kcal mol^{-1} M^{-1})	n[d]
37	6.0	3.0 ± 0.2 (3.0 ± 0.1)	1.0 ± 0.1	2.8 ± 0.4
	12.0	4.2 ± 0.3 (4.3 ± 0.2)	1.0 ± 0.1	
10	2.0	2.7 ± 0.3 (2.7 ± 0.2)	0.9 ± 0.1	2.8 ± 0.5
	4.0	3.8 ± 0.4 (3.9 ± 0.3)	1.1 ± 0.1	

[a] Monitored by CD at 287 nm. [b] Mg^{2+} concentrations equivalent to $\sim 5K_{Mg}$ and $\sim 10K_{Mg}$ of the I-to-N transition. [c] In parentheses are listed ΔG values calculated according to eq 3 with data from Mg^{2+} titrations (Table 1). [d] Calculated according to eq 8.

urea, ΔG, and the m value (Figure 5). As expected for Mg^{2+}-dependent folding, the stability increases with increasing Mg^{2+} concentrations (Table 3). The m value, however, is independent of the Mg^{2+} concentration and depends only on the level of surface burial between the I and the N states (Table 3).

Several lines of evidence indicate that the urea titration and the Mg^{2+} titration monitor the same I-to-N transition. (i) The ΔG values are identical (Table 3). (ii) The m values are identical (Tables 2 and 3). (iii) The ΔG values obtained from the urea titrations at different Mg^{2+} concentrations can be used to calculate the Hill constant according to eq 8. The Hill constant so obtained is in excellent agreement with that obtained directly from the Mg^{2+} titrations (Tables 1 and 3).

In summary, by performing Mg^{2+} or urea titrations at different concentrations of the complementary variable, one can obtain the three thermodynamic parameters, K_{Mg}, n, and m. These parameters can be used to calculate the stability at any given Mg^{2+} or urea concentration. By performing both a urea unfolding titration and a Mg^{2+} folding titration, one can also confirm the reversibility of the folding transition. Because the determination of the free energy from urea titration does not rely on the same assumptions as those used in Mg^{2+} titration, the agreement of ΔG, n, and m obtained from these two methods provides strong support for the binding model formalism applied here.

DISCUSSION

We have applied chemical, enzymatic, and spectroscopic probes in characterizing thermodynamic states and examining the cooperativity of tertiary folding of a large RNA under isothermal conditions. Using Mg^{2+} and urea titrations, we have implemented a cooperative Mg^{2+} binding model to quantify the folding free energy. Our results consolidate some previous conclusions (*18*, *19*) and provide some new insights regarding the stability of tertiary RNA structures.

A meaningful discussion of the folding free energy must include the identification of a thermodynamic reference state. For small globular proteins, the reference state is typically the unfolded or denatured state. For the C-domain of P RNA, however, the energy level nearest to the native form is the I state. The I state has a near-native amount of secondary structure as indicated by the absorbance at 260 nm. Just like the spectrum of the N state, the spectrum of the I state is relatively insensitive to temperature and urea, indicating that the I state is a well-defined thermodynamic entity. The U state is less well-defined and is sensitive to temperature and urea, presumably due to the presence of residual secondary structures.

Tertiary RNA Folding Described by a Mg^{2+} Binding Model. Two types of Mg^{2+} ions are often considered in RNA folding studies, specifically bound and delocalized (*19*, *20*). According to polyelectrolyte theory (*21*), delocalized cations preferentially associate with folded structures having increased charge density. Both the I and N states probably have comparable charge density because both have comparable amounts of secondary structure (A_{260}) and surface burial (small m value for the transition; see below). Hence, delocalized cations presumably interact similarly well with both the I and N states. Therefore, by using the I state as the reference state, the effect of the delocalized Mg^{2+} ions on tertiary RNA stability is likely to be minimal and is not considered further.

In the cooperative binding model applied in this study, a Mg^{2+}-dependent RNA folding transition is characterized by two parameters: the Mg^{2+} concentration at the midpoint of transition, K_{Mg}, and the Hill constant, n. At a given Mg^{2+} concentration, the stability can be calculated according to $\Delta G([Mg^{2+}]) = -RT \ln([N]/[I]) = -nRT \ln([Mg^{2+}]/K_{Mg})$. The fraction of N increases and the fraction of I decreases with increasing Mg^{2+} concentrations as described by a Hill equation (eq 2) so that the folding free energy becomes increasingly negative (i.e., more stable). At the transition midpoint (i.e., $[Mg^{2+}] = K_{Mg}$), the populations of N and I are equal and ΔG is zero. Under physiological conditions (37 °C with 5−10 mM Mg^{2+}), the stability of this tertiary RNA is on the order of 2−4 kcal/mol, comparable to the stability of many small globular proteins.

The Hill constant, or the differential ion binding term, of a folding transition has been called the minimum number of Mg^{2+} ions bound in the transition (*3*, *22*). The experimentally determined Hill constant in several cases can be lower than the actual number of binding sites. For example, a Hill constant of unity observed for the U-to-I transition probably represents multiple but independent binding events. Alternatively, a transition may not occur in an all-or-none fashion. A notable example for the latter case is the oxygen binding to hemoglobin where the maximum slope in the nonlinear, sigmoidal Hill plot corresponds to a Hill constant of 2.8, even though four oxygen molecules are bound (*12*, *23*, *24*). Close inspection of the binding behavior shows that the binding of the first and last oxygen can be separated, each with a Hill constant of unity at extremely low and high oxygen concentrations, respectively. This incompletely cooperative, stepwise binding results in a maximum Hill constant that is less than the total number of oxygen molecules bound.

A method for resolving the ambiguity over the interpretation of the Hill constant and for justifying the applicability

of a cooperative binding model is to measure the stability with a method that does not require knowledge of the Hill constant. The urea titration provides this option because the free energy is obtained from the data fitting using eq 7. The change in stability determined from urea titrations conducted at different Mg^{2+} concentrations can be used to calculate the Hill constant. The resulting Hill constant is identical to that derived from the Mg^{2+} titrations. These results argue that the Hill constant in this system probably represents the number of Mg^{2+} cations bound cooperatively in the I-to-N transition.

The present thermodynamic framework for the stability of tertiary RNA structures is similar to the analysis used in a recent study of group I intron mutants (*25*). Another recent study on the group I intron precursor, however, proposed that the folding free energy increases linearly with Mg^{2+} concentration (*26*) as opposed to a logarithmic dependence in the Hill-type analysis described here. A linear relationship between ΔG and Mg^{2+} concentration implies that the folding of a ribozyme would not require Mg^{2+}, which is not the case for the C-domain.

The *m* value, obtained either from urea titration at a fixed Mg^{2+} concentration or from Mg^{2+} titration at a fixed urea concentration, provides a measure of the amount of RNA structure formed in a folding transition (*29*). For globular proteins where the thermodynamic reference state is the completely unfolded form, the *m* value directly correlates to the size of the protein (*17*). For a tertiary folding transition of RNA, however, folding can be referenced to the I state which is partially structured. In the two cases where the *m* values for the I-to-N transitions have been determined, the value for the 255-nucleotide C-domain [$\sim$1.0 kcal mol^{-1} M^{-1} (this work)] is significantly smaller than the *m* value for the 76-nucleotide $tRNA^{Phe}$ [$\sim$1.7 kcal mol^{-1} M^{-1} (*29*)]. For the C-domain and $tRNA^{Phe}$, these *m* values equate to the amount of surface buried upon the formation of 12 and 22 base pairs, respectively. These results indicate that the I state of the C-domain is more structured than the I state of $tRNA^{Phe}$, and the *m* value for tertiary RNA folding transitions cannot be predicted on the basis of the size of the RNA alone.

Tertiary RNA Folding and Cooperativity. For the folding of the C-domain, the I-to-N transition is a cooperative process because all probes give identical K_{Mg} values and Hill constants. This high degree of cooperativity parallels that observed for the U-to-N transition in protein folding. For proteins, cooperativity is partially due to the relative instability of isolated secondary structures. In contrast, most RNA helices are independently stable. Hence, the cooperativity seen for the C-domain is striking.

Cooperativity is frequently found in biological systems stabilized by weak, noncovalent interactions. Cooperativity occurs when each contact makes subsequent contacts easier to form through the increase in the local concentration of interacting groups (*27*). For proteins, the cooperative effect is most dramatic in cases where regions that are distant in sequence are brought together. The cooperativity in tertiary RNA folding, however, is observed for the binding of *n* Mg^{2+} ions. RNA cooperativity may therefore reflect the feature that initial binding events bring distant regions together to form subsequent Mg^{2+} binding sites (*20*).

Although cooperativity in thermodynamics reflects an organizational process where parts are assembled in the context of other parts, the assembly need not occur in a single kinetic step. In fact, our kinetic data for the I-to-N transition of the C-domain indicate that folding does occur in multiple, sequential Mg^{2+} binding steps (*7*). However, the multistep Mg^{2+} binding must still obey the thermodynamic rule in which the partially formed species are not stable in equilibrium relative to the beginning and ending states, although kinetic intermediates may accumulate transiently. Hence, cooperativity in RNA folding is a statement about the relative stability of RNA structures, not the temporal process of structural transitions.

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