Altering the Intermediate in the Equilibrium Folding of Unmodified Yeast tRNA^{Phe} with Monovalent and Divalent Cations†

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ABSTRACT: The isothermal equilibrium folding of the unmodified yeast tRNA^{Phe} is studied as a function of Na^+, Mg^{2+}, and urea concentration with hydroxyl radical protection, circular dichroism, and diethyl pyrocarbonate (DEPC) modification. These assays indicate that this tRNA folds in Na^+ alone. Similar to folding in Mg^{2+}, folding in Na^+ can be described by two transitions, unfolded-to-intermediate-to-native. The I-to-N transition has a Na^+ midpoint of ∼0.5 M and a Hill constant of ∼4. Unexpectedly, the urea *m*-value, the dependence of free energy on urea concentration, for the I-to-N transition is significantly smaller in Na^+ than in Mg^{2+}, 0.4 versus 1.7 kcal mol^{-1} M^{-1}, indicating that more structure is formed in the Mg^{2+}-induced transition. DEPC modification indicates that the I state in Na^+-induced folding contains all four helices of tRNA and the I-to-N transition primarily corresponds to the formation of the tertiary structure. In contrast, the intermediate in Mg^{2+}-induced folding contains only three helices, and the I-to-N transition corresponds to the formation of the acceptor stem plus tertiary structure. The cation dependence of the intermediates arises from the differences in the stability of the acceptor stem and the tertiary structure. The acceptor stem is stable at a lower Na^+ concentration than required for the tertiary structure formation. The relative stability is reversed in Mg^{2+} so that the acceptor stem and the tertiary structure form simultaneously in the I-to-N transition. These results demonstrate that formation of the RNA secondary structure can be independent or coupled to the formation of the tertiary structure depending on their relative stability in monovalent and divalent ions.

The stability of a tertiary RNA is very sensitive to metal ions due to their interactions with the negatively charged phosphodiester backbone. Metal ions can stabilize an RNA structure through nonspecific interactions as well as specific binding to ligands in phosphate-lined pockets formed by the tertiary fold of the RNA (*1*, *2*). Although both Na^+ and Mg^{2+} ions can interact nonspecifically with RNA, the divalent cations are generally more effective in specific binding.

Transfer RNAs have been used as model systems in RNA folding for more than 3 decades. As measured by either absorbance or fluorescence spectroscopy, tRNAs with posttranscriptional modifications can fold into their native structure in the presence of Na^+ or Mg^{2+}, although much higher concentrations of Na^+ are needed for tertiary folding [reviewed in (*3*)]. These early results suggest that Na^+, like Mg^{2+}, can bind to the highly negatively charged pockets in the native tRNA structure. Most of these early studies focused on the analysis of the native structure, and specific information on the thermodynamic intermediates in either Na^+ or Mg^{2+} was largely not investigated. It was generally assumed that the thermodynamic intermediate in tRNA folding is the same in either Na^+ or Mg^{2+} and this intermediate resembles the cloverleaf secondary structure of a tRNA.

Our previous study on the unmodified yeast tRNA^{Phe} suggests that its penultimately populated intermediate in the Mg^{2+}-dependent equilibrium folding has a noncloverleaf structure (*4*). Applying the urea-dependent parameter, the *m*-value,[1] as a quantitative measure of structure formation, the intermediate in Mg^{2+}-induced folding contains less structure than the sum of the four helices in the secondary structure of a tRNA. In this previous study, the folding of tRNA^{Phe} was monitored solely as a function of Mg^{2+} concentration at a constant, low Na^+ concentration (∼10 mM). The nature of the Na^+-induced folding transitions was not investigated. It was unclear whether Na^+ alone would be sufficient to fold this unmodified tRNA, and whether the thermodynamic intermediate in tRNA^{Phe} folding would be the same in mono- and divalent ions.

The present work demonstrates that Na^+ alone can fold unmodified tRNA^{Phe} to its native structure. Surprisingly, the folding in Na^+ and in Mg^{2+} results in different thermodynamic intermediates, as monitored by site-specific chemical modification and circular dichroism (CD). The Na^+-induced intermediate contains all four helices, but at most three helices are present in the Mg^{2+}-induced intermediate. These

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[1] Abbreviations: CD, circular dichroism; DEPC, diethyl pyrocarbonate; *m*, dependence of free energy on urea concentration; *n*, Hill coefficient; PF, protection factor; $U_{4M\ urea}$, unfolded state in 4 M urea in 10 mM Na^+ and no Mg^{2+}.

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results demonstrate that the type of metal ion is an important factor in determining the structure of thermodynamic intermediates in RNA folding, and is likely due to the differential effects of Na^+ and Mg^{2+} on the relative stability of RNA secondary and tertiary structures.

MATERIALS AND METHODS

RNA Synthesis. Unmodified yeast $tRNA^{Phe}$ was synthesized using T7 RNA polymerase by standard in vitro transcription from a *Bst*NI cut plasmid DNA template (*5*). The transcript was precipitated with ethanol, redissolved in 9 M urea and 100 mM EDTA loading buffer, and purified on a polyacrylamide gel containing 7 M urea and 2 mM EDTA. The RNA was eluted from the gel by the crush and soak method in 50 mM potassium acetate and 200 mM KCl, pH 7, precipitated with ethanol, and stored in water at −20 °C.

Folding Monitored by Hydroxyl Radical Protection. The fraction of tRNA protected from hydroxyl radical cleavage was determined by the standard Fe(II)-EDTA footprinting method with 50 μM Fe(II) and 60 μM EDTA at 0.5 μM tRNA (*6*). The tRNA was heated in 20 mM sodium cacodylate (pH 6.6) at 90 °C for 2 min followed by incubation at 20−25 °C for 3 min. Varying concentrations of NaCl were added, and the solutions were incubated at ambient temperature for an additional 5 min. Ascorbic acid and dithiothreitol were added to 1 mM and 5 mM, respectively. The hydroxyl radical generation and cleavage reaction were initiated by the addition of a 10× solution of $Fe(NH_4)_2(SO_4)_2$ and EDTA (pH 8.0). The cleavage reaction was allowed to proceed at 37 °C for 2 h and was quenched by the addition of thiourea to a final concentration of 10 mM. The reaction mixture was then separated using denaturing polyacrylamide gels containing 7 M urea, and the amount of products was quantitated using a Molecular Dynamics phosphorimager and ImageQuant Software.

Folding Monitored by Circular Dichroism. Circular dichroism was measured using a Jasco J715 spectropolarimeter interfaced with a Hamilton titrator. The sample cuvette holder was outfitted with a magnetic stirrer and circulating water jacket for proper mixing and temperature control. CD data were collected at 260 nm with 2 nm bandwidth and an acquisition time of 15 s in order to minimize UV cross-linking observed for this molecule (*7*).

The $tRNA^{Phe}$ was refolded by heating in buffered solution (20 mM sodium cacodylate, pH 6.6, and varying concentrations of urea when appropriate) at 90 °C for 2 min followed by incubation at 20−25 °C for 3 min. The tRNA at this stage was designated as the U state.

Folding Monitored by DEPC Modification. The fraction of tRNA protected from modification by DEPC was determined by the published procedure with minor modifications (*8*). The tRNA was heated in 20 mM sodium cacodylate (pH 6.6) in varying concentrations of urea at 90 °C for 2 min followed by incubation at 20−25 °C for 3 min. Varying concentrations of NaCl were added, and the solutions were incubated at ambient temperature for an additional 5 min. DEPC was added to 0.34 M to a final volume of 10 μL, and the modification was allowed to proceed at 37 °C for 30 min and was quenched by the addition of 4 μg of *E. coli* tRNAs in 100 μL of cold potassium acetate/KCl (pH 7.0) buffer. The mixture was precipitated twice with ethanol and dried. To each dried reaction was added 10 μL of 1 M aniline (pH 4.5 in sodium acetate buffer) followed by incubation at 60 °C for 20 min. The RNA was precipitated with ethanol, briefly dried, and redissolved in 9 M urea and 100 mM EDTA loading buffer. The reaction mixture was then separated using denaturing polyacrylamide gels containing 7 M urea, and the amount of products was quantitated using a Fuji Film phosphorimager and Image Gauge software.

Data Analysis. The yeast $tRNA^{Phe}$ undergoes two Na^+-dependent structural transitions. As in the case of Mg^{2+} folding of this tRNA (*4*), we describe these transitions with a cooperative Na^+ binding model.

$$\text{Fraction folded} = \frac{[\text{I}]}{[\text{U}]+[\text{I}]} = \frac{[\text{Na}^+]^{n_1}}{[\text{Na}^+]^{n_1} + (K_{\text{Na1}})^{n_1}} \quad (1a)$$

$$\text{Fraction folded} = \frac{[\text{N}]}{[\text{I}]+[\text{N}]} = \frac{[\text{Na}^+]^{n_2}}{[\text{Na}^+]^{n_2} + (K_{\text{Na2}})^{n_2}} \quad (1b)$$

The CD data were fit as a linked equilibrium between these two transitions according to

$$\Delta\epsilon_{\text{obs}} = f_{\text{U}}\Delta\epsilon_{\text{U}} + f_{\text{I}}\Delta\epsilon_{\text{I}} + f_{\text{N}}\Delta\epsilon_{\text{N}} = \frac{\Delta\epsilon_{\text{U}} + \Delta\epsilon_{\text{I}}\left(\frac{[\text{Na}^+]}{K_{\text{Na1}}}\right)^{n_1} + \Delta\epsilon_{\text{N}}\left(\frac{[\text{Na}^+]}{K_{\text{Na1}}}\right)^{n_1}\left(\frac{[\text{Na}^+]}{K_{\text{Na2}}}\right)^{n_2}}{1 + \left(\frac{[\text{Na}^+]}{K_{\text{Na1}}}\right)^{n_1} + \left(\frac{[\text{Na}^+]}{K_{\text{Na1}}}\right)^{n_1}\left(\frac{[\text{Na}^+]}{K_{\text{Na2}}}\right)^{n_2}} \quad (2a)$$

$$f_{\text{U}} + f_{\text{I}} + f_{\text{N}} = 1 \quad (2b)$$

where $\Delta\epsilon_{\text{obs}}$ is the observed signal intensity, $\Delta\epsilon_{\text{U}}$, $\Delta\epsilon_{\text{I}}$, and $\Delta\epsilon_{\text{N}}$ are the spectroscopic signals for the U, I, and N states, respectively, K_{Na1} and K_{Na2} are the Na^+ midpoints, and n_1 and n_2 are the Hill coefficients of the U-to-I and I-to-N transitions, respectively. DEPC modification revealed that the U-to-I transition probably corresponded to several overlapping transitions. Therefore, only the thermodynamic parameters for the second transition were interpreted for the CD data. The hydroxyl radical protection data were fit only to the I-to-N transition using eq 1b, since the protection did not change for the U-to-I transition for this tertiary RNA.

The quantitative extent of DEPC modification of RNA relative to the modification in the presence of 8 M urea and 10 mM Na^+ is presented as a protection factor (PF). The parameter 1/PF is the fraction of RNAs modified by DEPC and is directly related to the fraction folded.

Data analysis was performed using the Microcal Origin version 5.0 nonlinear fitting. Errors listed are the standard deviation calculated by the fitting algorithm and reflect the statistical uncertainty of the fitted parameters.

RESULTS

Na^+-Induced Tertiary Folding of Unmodified Yeast $tRNA^{Phe}$. Hydroxyl radical protection is a commonly used probe to identify the ribose moieties that are buried in the tertiary RNA structure (*9*). In Mg^{2+}-induced folding, significant protection was observed in two regions of $tRNA^{Phe}$ consistent with the crystal structure. We observe the same pattern and extent of protection for unmodified $tRNA^{Phe}$ at saturating Na^+ (>1 M) as at saturating Mg^{2+} (>0.05 mM)

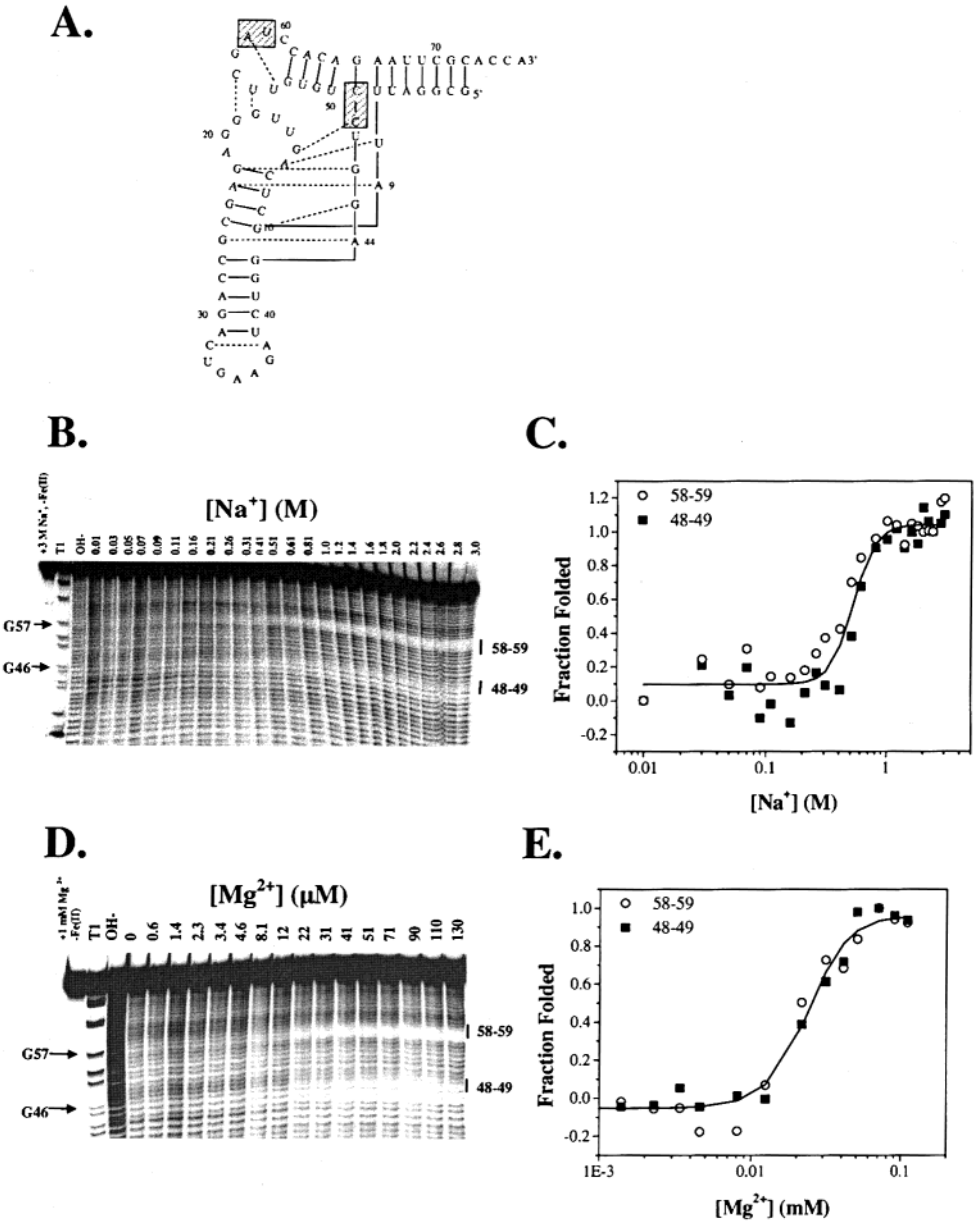

FIGURE 1: Folding of the tertiary structure of the unmodified yeast tRNA^{Phe} in 20 mM sodium cacodylate, pH 6.6 and 37 °C, monitored by hydroxyl radical protection. (A) The L-presentation of the yeast tRNA^{Phe}. The protected residues are shaded and include nucleotides 58, 59, 48, and 49. (B) Protection as a function of Na^+ concentration. Lane T1: partial T1 ribonuclease digestion under denaturing conditions. Lane OH-: partial alkaline hydrolysis of the same RNA. (C) Fraction folded versus [Na^+]. The plot of the fraction folded versus Na^+ concentration is fit with eq 1b to obtain K_{Na2} and n_2. (D) Protection as a function of Mg^{2+} concentration. (E) Fraction folded versus [Mg^{2+}].

(Figure 1). No additional protection upon the addition of 10 mM Mg^{2+} to 1 M Na^+ sample was observed (data not shown). We conclude that unmodified tRNA^{Phe} folds to the native structure in Na^+ alone.

The folding transition of tRNA^{Phe} with either metal ion can be fit with a Hill-type equation (eq 1b). The midpoint of the transition in Na^+ and Mg^{2+} is 520 ± 30 mM (K_{Na}), and 0.022 ± 0.001 mM (K_{Mg}), respectively. The Hill constant, however, is very similar for folding in Na^+ (4.6 ± 1.0) as it is in Mg^{2+} (4.1 ± 0.6).

A second probe, circular dichroism (CD), is applied to monitor Na^+-dependent folding in the presence of urea. As with Mg^{2+}-induced folding, Na^+-induced folding has two folding transitions as monitored by CD at 260 nm ($\Delta\epsilon_{260}$) (Figure 2). These two transitions are clearly separated only in the presence of urea. In the absence of urea, residual structure in the denatured state obscures the first transition that has already begun in the initial conditions (~10 mM Na^+). The value of $\Delta\epsilon_{260}$ for the native state at saturating Na^+ concentrations is very similar at all urea concentrations studied, just as for folding in Mg^{2+}. Likewise, the magnitude of the CD change of the I-to-N transition is relatively insensitive to urea concentration.

The two folding transitions monitored by CD can be fit with a linked equilibrium formula (eq 2) to obtain K_{Na} and n for each transition (Table 1). Because DEPC modification indicates that the U-to-I transition probably corresponds to several overlapping transitions (see below), only the thermodynamic parameters for the second, I-to-N transition are interpreted for the CD data. Under all urea conditions, the Hill constant for the I-to-N transition is essentially the same as that obtained for tRNA^{Phe} folding in the absence of urea

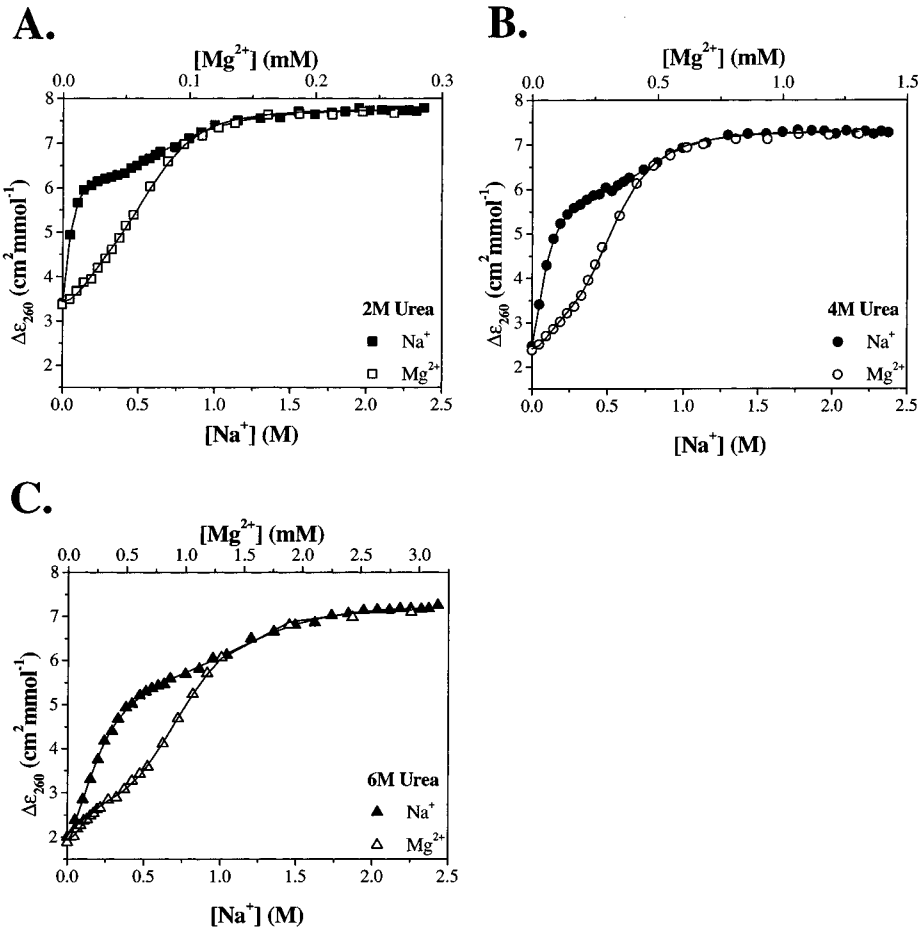

FIGURE 2: Folding of 0.5 μM unmodified yeast tRNA^{Phe} as a function of Na^+ or Mg^{2+} concentration in 20 mM sodium cacodylate, pH 6.6 and 37 °C, monitored by circular dichroism at 260 nm. (A) 2 M urea. (B) 4 M urea. (C) 6 M urea. The data are fit with eq 2 as a linked equilibria. The Mg^{2+} titration data are from (*4*).

Table 1: Na^+ Titration of the Unmodified Yeast tRNA^{Phe} at Constant Urea Concentrations[a]

[urea] (M)	K_{Na1} (M)	n_1	K_{Na2} (M)	n_2
0			0.52 ± 0.03[b]	4.6 ± 1.0
			0.46 ± 0.02[c]	4.9 ± 1.0
2			0.76 ± 0.02[d]	3.4 ± 0.2
4			0.85 ± 0.02[d]	5.1 ± 0.4
	0.21 ± 0.02[c,e]	1.5 ± 0.1	0.73 ± 0.03[c]	3.6 ± 0.5
6			1.3 ± 0.03[d]	5.0 ± 0.4
	0.28 ± 0.01[c,e]	1.9 ± 0.2	0.82 ± 0.03[c]	4.6 ± 0.8
8	0.53 ± 0.09[c,e]	2.3 ± 0.6		

[a] Conditions: 20 mM sodium cacodylate, pH 6.6, 0.5 μM tRNA, 37 °C. [b] Hydroxyl radical protection. [c] DEPC modification. [d] Circular dichroism. [e] Acceptor stem folding.

determined by hydroxyl radical protection. The uniform change in both the CD signal and Hill constant for the I-to-N transition indicates that a well-defined intermediate exists in Na^+, as well as in Mg^{2+}, although the two species, I_{Na} and I_{Mg}, could be structurally distinct.

To determine when individual helices and tertiary structures form, DEPC modification is used to analyze the folding in Na^+ and Mg^{2+} (Figures 3 and 4). DEPC carbethoxylates the N^7 position of adenosines. Adenosines that are stacked, as in a base-paired region, or have the N^7 involved in tertiary interactions, are protected from modification by DEPC. In the case of tRNA^{Phe}, modification of the exposed adenosines in the anticodon loop, A35/A36/A38, is not sensitive to the presence of Na^+ or Mg^{2+}, and these residues serve as excellent controls for the modification reaction.

All 14 adenosines monitored (from a total of 18 in tRNA^{Phe}) are fully modified in the presence of urea at concentrations of 4 M or higher. Eleven are protected in the native structure, but not the three exposed adenosines in the anticodon loop (Figure 3). At least one adenosine in each of the four helices is sensitive to DEPC modification: A23 in the D stem, A29/A31 in the anticodon stem, A62/A64 in the T stem and A66/A67 in the acceptor stem. Therefore, the cation-dependent formation of every helix in this tRNA can be monitored by DEPC modification of these seven adenosines. The formation of the tertiary structure can be monitored by modification of four adenosines, A14/A21 in the D loop, A58 in the T loop, and A44 in the variable loop. DEPC modification data for all adenosines within a particular stem or loop were fit separately. Averaged data and their fits are shown in Figures 3 and 4 for each stem and loop region.

Similar to the hydroxyl radical protection and CD measurements, protection from DEPC modification depends on the Na^+ and Mg^{2+} concentration (Figures 3 and 4). The extent of protection of the residues involved in tertiary structure can be fit with the same Hill-type equation (eq 1b) to obtain the cation midpoints and Hill constants (Figure 3). As expected, the K_{Mg} is significantly lower than the K_{Na}, but the Hill constants are the same for both cations and are

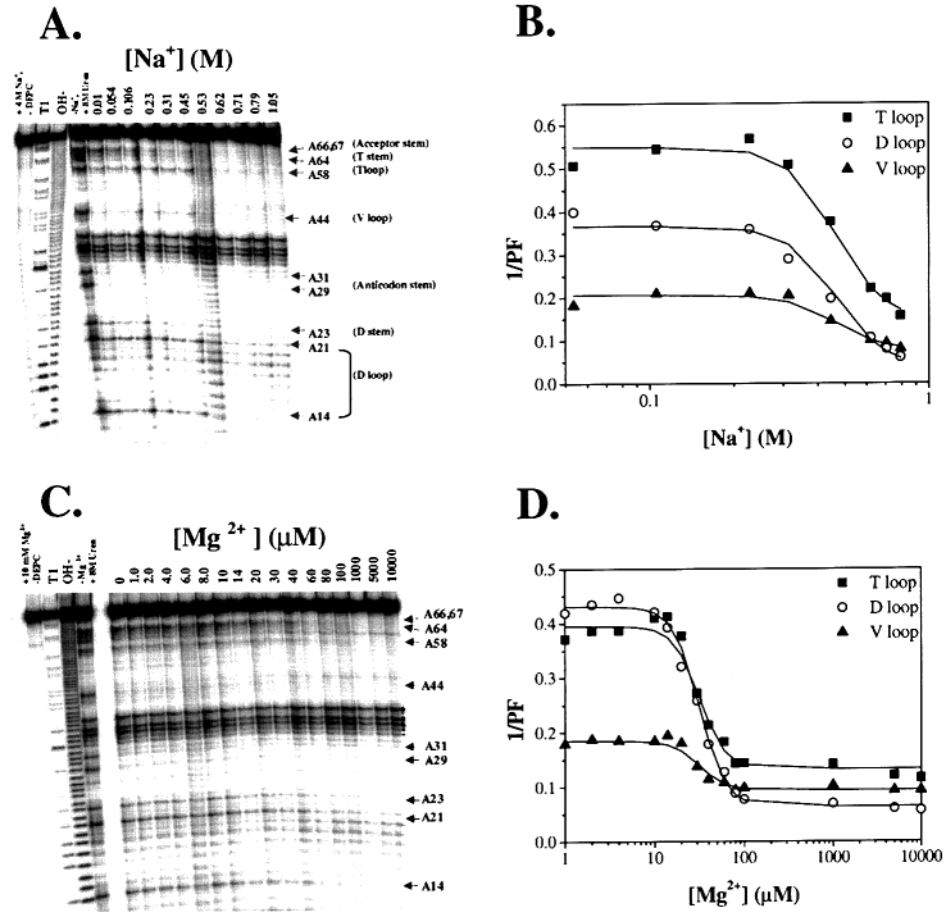

FIGURE 3: Tertiary structure formation as monitored by DEPC modification in 20 mM sodium cacodylate, pH 6.6 and 37 °C. (A) Modification as a function of Na^+ concentration. The modification of 14 adenosine residues is determined. Lane T1: partial T1 ribonuclease digestion under denaturing conditions; lane OH-: partial alkaline hydrolysis of the same RNA. (B) 1/PF versus [Na^+] for loop residues fit to a single I-to-N transition (eq 1b) to obtain K_{Na2} and n_2. (C) Modification as a function of Mg^{2+} concentration. (D) 1/PF versus [Mg^{2+}] for the loop residues.

independent of urea concentration (Tables 1 and 2). The DEPC modification data provide further support that unmodified tRNA^{Phe} can fold to the native state in both Na^+ and Mg^{2+}.

Residual Structure in 10 mM Na$^+$. In the absence of urea, DEPC modification of the helical residues in Mg^{2+}-induced folding does not change until the I_{Mg}-to-N transition, suggesting that some of the helices either are not present or are already formed in the starting condition which contains ~10 mM Na^+ (Figure 4B). In the starting condition, the anticodon, D, T, and acceptor stems have protection factors of about 5, 2.5, 2, and 1.3, respectively. Complete DEPC modification (PF = 1.0) occurs in 4 M urea where the residual structure is disrupted, consistent with our previous studies (*4*, *10*). The high PF value for the anticodon stem indicates that it is formed while the intermediate values for the D and T stems suggest that they are formed as well.

To accurately identify the helical complement of the starting condition, in particular the status of the T and acceptor stems, DEPC modification is carried out with a bimolecular construct that contains just these two regions (nucleotides 1−9 and 49−73 of tRNA^{Phe}). In this construct, the "T" stem region is part of a single, contiguous strand whereas the "acceptor" stem requires binding of the two pieces (Figure 4C). As with the full-length tRNA^{Phe}, the protection factors for A62/A64 in the "T" stem and A66/A67 in the "acceptor" stem are measured. Importantly, the initial and final (high Mg^{2+}) levels of protection for the "T" and "acceptor" stems in the bimolecular construct are very similar to the corresponding values measured in the full-length tRNA^{Phe}. This identity of PFs indicates that this construct is a faithful analogue of the T and acceptor stems in the context of the full-length tRNA^{Phe}.

From a comparison of the Mg^{2+} dependence of PF of this bimolecular construct [yF(1−9) and yF(49−73)] to that of the "T stem" containing piece [yF(49−73)], we conclude that for tRNA^{Phe} the acceptor stem is not formed, but the T stem is formed in the starting condition, based upon the following results. In the absence of the complementary strand, the PF is ~1.2 for A66/A67 in the "acceptor" portion, indicating that this level of protection represents that of an unstructured region. Upon addition of about 10 mM Mg^{2+} in the presence of the complementary strand, the PF sharply increases from ~1.2 to 5 for the "acceptor" stem, indicating that a high PF is the signature of the formation of the "acceptor" stem. This same change is observed for the acceptor stem in the full-length tRNA^{Phe} in the I-to-N transition. The "T" stem, however, only experiences a mild increase in PF from 2.5 to 3 at 10 mM Mg^{2+}. We interpret this mild increase to result from base stacking of the newly formed "acceptor" stem on top of the "T" stem. In summary, these data in conjunction with those for the full-length

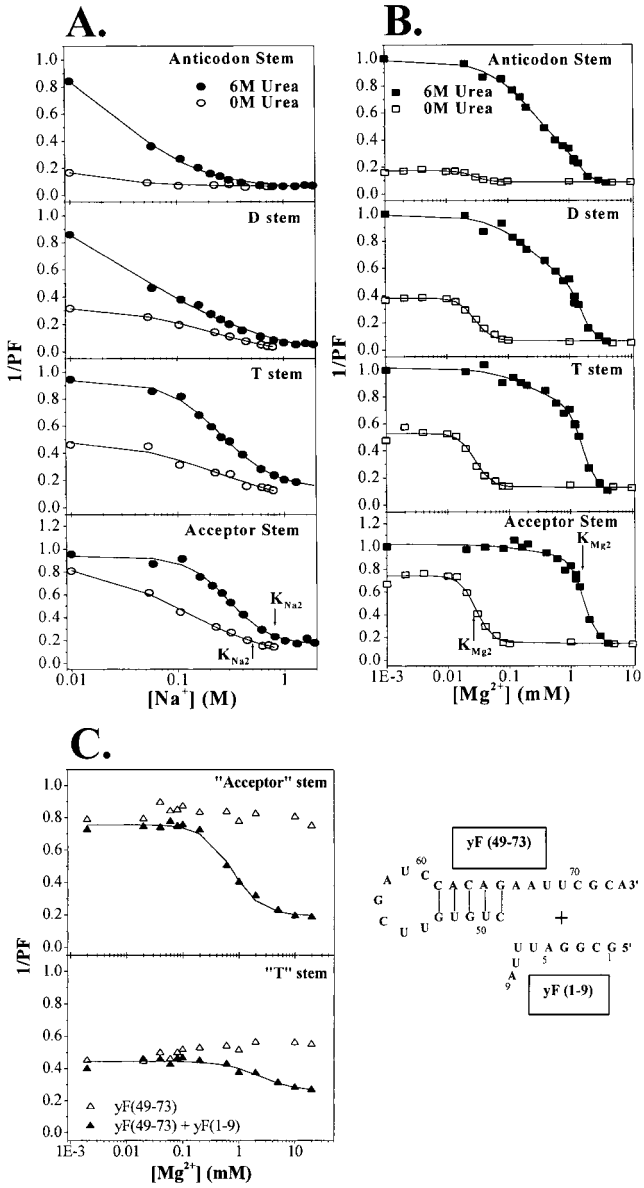

FIGURE 4: Secondary structure formation as monitored by DEPC modification. (A) Modification as a function of Na^+ concentration. The plot of 1/PF versus Na^+ concentration in 6 M urea is fit to a single U-to-I transition (eq 1a) to obtain K_{Na1} and n_1. (B) Modification as a function of Mg^{2+} concentration. The plot of 1/PF versus Mg^{2+} concentration in 6 M urea is fit to a linked equilibria (eq 2) to obtain K_{Mg1}, K_{Mg2}, n_1, and n_2. (C) Modification of a model construct containing nucleotides 49−73 and 1−9 of the yeast $tRNA^{Phe}$ at 20 mM sodium cacodylate, pH 6.6, 1 M urea, and 23 °C.

$tRNA^{Phe}$ indicate that the acceptor stem is not formed but the other three stems are present in the starting, 10 mM Na^+, condition.

Structural Differences between I_{Na} and I_{Mg}. The above results demonstrate that the equilibrium folding pathway in the presence of either cation contains a thermodynamic intermediate. Do the intermediates in the different cations have the same or distinct structures? This question is addressed in two ways. The secondary structural differences are visualized with DEPC modification. The quantitative extent of the magnitude of the structural change in the I-to-N transition is characterized by the urea *m*-value. The *m*-value corresponds to the change in free energy, ΔG_{I-to-N}, upon the addition of a molar of denaturant (units of kcal mol^{-1} M^{-1}). This quantity correlates with the differential accessible surface area burial accompanying a folding transition for both RNAs and proteins (*4*, *11*).

Table 2: Mg^{2+} Titration of the Unmodified Yeast $tRNA^{Phe}$ at Constant Urea Concentrations[a]

[urea] (M)	K_{Mg1} (mM)	n_1	K_{Mg2} (mM)	n_2
0			0.022 ± 0.001[b]	4.1 ± 0.6
			0.028 ± 0.001[c]	3.3 ± 0.2
2			0.065 ± 0.008[d]	3.1 ± 0.5
4	0.061 ± 0.036[d]	1.9 ± 1.1	0.32 ± 0.005[d]	3.4 ± 0.1
6	0.23 ± 0.09[d]	1.4 ± 0.3	1.0 ± 0.01[d]	3.8 ± 0.1
	0.28 ± 0.06[c]	1.1 ± 0.2	1.6 ± 0.05[c]	3.8 ± 0.4

[a] Conditions: 20 mM sodium cacodylate, pH 6.6, 0.5 μM tRNA, 37 °C. [b] Hydroxyl radical protection. [c] DEPC modification. [d] Circular dichroism data from (*4*).

DEPC modification indicates that the secondary structure content of I_{Na} is greater than that of I_{Mg} (Figure 4). In the absence of urea for Na^+-induced folding, protection of all helices continually increases until $[Na^+] \sim 0.1-0.2$ M, the point where I_{Na} is the major species. This conclusion is based on a similar PF value of the clearly identifiable I_{Na} species in the presence of 6 M urea (Figure 4). The level of protection, however, for each helix is different. Importantly, the protection factor of the acceptor stem, the only helix not formed at the starting condition, increases from ~1.2 to about 3, indicating that it also is present in I_{Na}.

Different behavior is observed for Na^+-induced folding at 6 M urea because the starting condition lacks all four helices. In 4 and 6 M urea, the two folding transitions monitored by DEPC modification of residues involved in tertiary structures can be fit with eq 2 to obtain K_{Na} and n for each transition. K_{Na2} and n_2 values determined from DEPC data (Table 1) are average values for all tertiary residues. In 6 M urea, the approximate Na^+ level for the formation of all four helices is about 0.4 M, well below the midpoint of the I-to-N transition which is close to 1 M. The DEPC protection factors for anticodon and D stems continuously increase whereas the PFs for the T and acceptor stems have a sharper transition near 0.3 M NaCl. These data indicate that the entire cloverleaf secondary structure is present in I_{Na} even in the presence of urea.

In the absence of urea, the level of modification of the helices in the I_{Mg} state is the same as in the starting conditions; i.e., the anticodon and D and T stems are present, but the acceptor is not. The two transitions, U-to-I_{Mg}-to-N, still occur in the presence of 4−6 M urea although at elevated Mg^{2+} levels. Our previous study using small-angle X-ray scattering (*12*) indicates that I_{Mg} has the same dimension in the absence and presence of urea. DEPC modification now indicates that the I_{Mg} in the presence and absence of urea contains the same helical structure (Figure 4B). Hence, the I_{Mg} is structurally very similar in the absence and presence of urea. I_{Mg} differs from I_{Na} in at least one aspect, the absence of the acceptor stem.

The change in the $\Delta\epsilon_{260}$ signal for I_{Na}-to-N transition is significantly smaller compared to that for the I_{Mg}-to-N transition (Figure 2). In Na^+, the $\Delta\epsilon_{260}$ signal change is ~2 cm^2 $mmol^{-1}$, compared to ~4 cm^2 $mmol^{-1}$ for its folding in Mg^{2+}. This result suggests that more structure forms in the I_{Mg}-to-N transition, consistent with the DEPC data that I_{Mg}

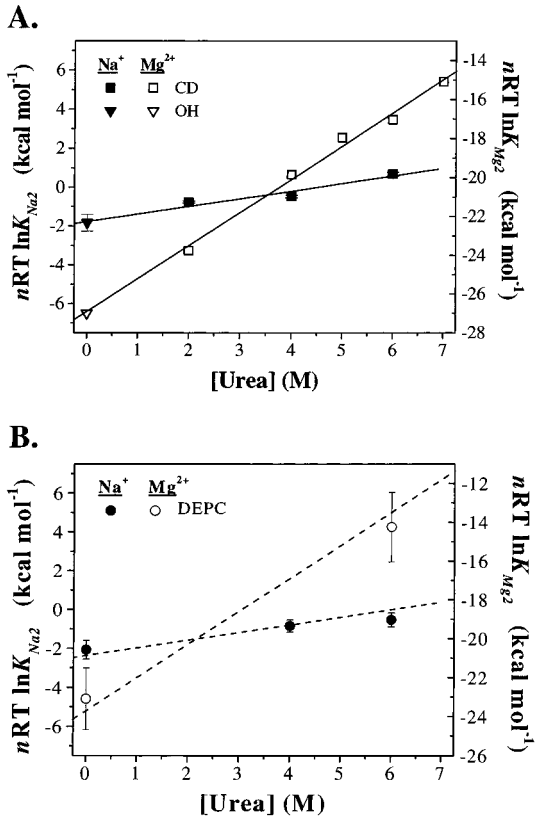

FIGURE 5: Stability as a function of urea concentration. The *m*-value of the I-to-N transition is obtained from the slope of the plot of *nR*T ln *K* versus [urea], normalized to a standard cation concentration of 1 M. (A) The values of *K* are obtained from CD and hydroxyl radical protection data [Mg^{2+}-dependent CD data from Shelton et al. (*4*)]. The resulting *m*-values for Na^+ and Mg^{2+} are 0.39 ± 0.06 kcal mol^{-1} M^{-1} ($r^2 = 0.959$) and 1.70 ± 0.10 kcal mol^{-1} M^{-1} ($r^2 = 0.989$), respectively. (B) The values of *K* are obtained from DEPC modification. The slightly lower K_{Na} or slightly higher K_{Mg} probably reflects the presence of 0.34 M DEPC. Nevertheless, similar *m*-values can be obtained from the DEPC data compared to CD and hydroxyl radical protection data (dashed line).

contains one less helix than I_{Na}, and an extra helix forms in the I_{Mg}-to-N transition.

The urea *m*-value for the I-to-N transition indicates that I_{Na} is much more structured than I_{Mg}. The *m*-value is determined from the slope of the trace of $-nRT \ln(K_{Na})$ or $-nRT \ln(K_{Mg})$ versus [urea], normalized to a standard state concentration of 1 M (Figure 5), where K_{Na} (K_{Mg}) and *n* are determined by CD, hydroxyl radical protection, or DEPC modification. The *m*-value for the Na^+-induced I-to-N transition is only 0.39 ± 0.06 kcal mol^{-1} M^{-1}, whereas it is 1.7 ± 0.1 kcal mol^{-1} M^{-1} for the Mg^{2+}-induced transition. The significantly smaller *m*-value for Na^+-induced folding indicates that less structural change accompanies the I-to-N transition in Na^+ compared to that in Mg^{2+}.

Because the native structure of tRNA^{Phe} is the same in Na^+ and in Mg^{2+}, the differential *m*-value must reflect the structural differences of the equilibrium intermediates. The m_{Na}-value is consistent with the calculated surface area burial that includes only the formation of the tertiary structure between D, T, and variable loops. This estimate suggests that I_{Na} contains all secondary structures plus the structures within the anticodon, D, and T loops, but it has no loop−loop interactions. The larger m_{Mg}-value is consistent with the surface area burial accompanying the formation of the tertiary structure plus the acceptor stem and the structures within anticodon, D, and T loops. Hence, I_{Mg} probably contains only the anticodon, D, and T stems.

DISCUSSION

Folding of Tertiary RNAs with Monovalent Ions. We have shown that unmodified yeast tRNA^{Phe} can fold in the presence of Na^+ alone, albeit at concentrations greater than 0.5 M. As with folding in Mg^{2+}, folding in Na^+ can be described by two transitions, U-to-I-to-N, and a well-defined thermodynamic intermediate exists. This intermediate in Na^+-induced folding requires ~0.1 M Na^+, and it contains all four helices of the tRNA^{Phe}. The Na^+ requirement and the cloverleaf secondary structure are similar to those observed in the folding of the modified yeast tRNA^{Phe} in the presence of monovalent ions alone (*13*, *14*), except that higher Na^+ concentration is needed for the I-to-N transition for the unmodified tRNA^{Phe}. This result provides additional evidence that modified tRNAs are generally more stable than the corresponding unmodified version.

Several other tertiary RNAs have been shown to fold with monovalent ions alone. The L11 binding site of rRNA folds with NH_4^+ (*15*, *16*). The hammerhead, hairpin, and VS ribozymes are functional at high concentrations of monovalent ions alone (*17*). The presence of K^+, Na^+, or NH_4^+ is sufficient to fold the pseudoknot structure involved in translational frameshifting (*18*, *19*). Clearly, monovalent ions alone are sufficient to fold the tertiary structure of some small RNAs, albeit at much higher ion concentrations compared to folding with divalent ions.

It is unclear whether the monovalent ions bind to the same sites as the divalent ions in tertiary RNA structures. The crystal structures of the tRNA^{Phe} with different divalent ions (*20*−*22*) show that other divalent ions can occupy the same sites as Mg^{2+}, as well as new sites. Even trivalent ions such as Sm^{3+} can occupy some of the same sites as Mg^{2+}, suggesting that it is possible to substitute a specifically bound Mg^{2+} with an ion of a different valence.

Previous analysis of the Mg^{2+}-induced folding of the unmodified tRNA^{Phe} suggests that the observed Hill constant of 4 represents the number of Mg^{2+} ions that bind in the I-to-N transition (*4*). The change in stability determined from urea titrations conducted at different Mg^{2+} concentrations was the same as that determined from Mg^{2+} titrations. Furthermore, the Hill constant was the same determined from the urea and Mg^{2+} titrations. The most parsimonious conclusion is that the Hill constant in this system represents the number of Mg^{2+} cations bound cooperatively in the I-to-N transition.

The Hill constant in the Na^+-induced folding is identical to that observed in the Mg^{2+}-induced folding. Unfortunately, the low *m*-value of the I-to-N transition in Na^+-induced folding prevents accurate determination of the free energy of the native structure in Na^+ by urea titration (unpublished results). Hence, we cannot compare the stability based upon urea titration to that based upon Na^+ titration for this RNA. Therefore, it is unclear how many and where Na^+ ions bind in the I-to-N transition.

Parallel versus Sequential Pathways. The different thermodynamic intermediates observed in Na^+- and Mg^{2+}-induced folding suggest two possible scenarios for the

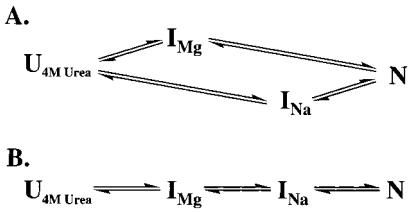

FIGURE 6: Equilibrium folding models of the unmodified yeast $tRNA^{Phe}$. (A) Parallel pathway. (B) Sequential pathway. All residual structures are disrupted in 20 mM sodium cacodylate, pH 6.6, 4 M urea, 37 °C, represented as $U_{4M\ Urea}$ (*4*, *12*).

equilibrium folding pathway of this tRNA. In the first scenario, these intermediates fold directly to the native state via two structurally distinct, parallel pathways (Figure 6A). In this case, the thermodynamic pathway is different in monovalent and divalent metal ions. In the second scenario, these intermediates fold sequentially to the native state via the same pathway (Figure 6B). In this case, I_{Mg} and I_{Na} are two intermediates that differentially populate depending on the solution condition. In Mg^{2+}-induced folding, I_{Na} is less stable than both I_{Mg} and N at all Mg^{2+} concentrations, so that I_{Na} does not populate. In Na^+-induced folding, I_{Na} is more stable than I_{Mg} at intermediate Na^+ concentrations, so that I_{Na} does populate.

Our results favor the sequential pathway model for $tRNA^{Phe}$ folding in Na^+. The structure of I_{Mg} is largely the same as the starting condition of the Na^+-dependent pathway. The I_{Mg} exhibits no increase in DEPC protection nor a change in the radius of gyration upon the addition of Mg^{2+} (*12*). In essence, the entire structural component of I_{Mg} is already in place prior to the addition of the divalent cation. Even in the presence of urea where Mg^{2+} is required for the formation of I_{Mg}, the I_{Mg} structure is the same as the structure in the absence of urea in the starting condition. Hence, the Na^+-dependent pathway essentially begins from a species structurally equivalent to I_{Mg}, providing evidence for a sequential pathway.

Equilibrium Folding of the Unmodified Yeast $tRNA^{Phe}$. The main structural difference between the two intermediates is the presence of the acceptor stem in I_{Na}. This difference is likely to be attributed to how Na^+ and Mg^{2+} stabilize the acceptor stem, a secondary structure, relative to the tertiary structure of tRNA. When the acceptor stem is more stable than the tertiary structure, as is the case for Na^+-induced folding, this helix forms at lower Na^+ concentration, and a distinct thermodynamic intermediate, I_{Na}, populates containing this helix. In Mg^{2+}-induced folding, the acceptor stem is unstable without the tertiary structure, so that this helix forms only in conjunction with the tertiary structure and the comparable intermediate to I_{Na} does not populate.

The differential stability of a secondary structure and the tertiary structure as a function of monovalent and divalent metal ions suggests that the thermodynamic pathway of RNA folding can be an intricate interplay between the formation of the secondary and tertiary structures. The population of thermodynamic intermediates depends on the nucleotide sequence of the secondary structure as well as the ionic conditions, even though the native structure can stay the same. This interplay has implications for kinetic studies where pathways are dependent upon the starting ionic conditions (*23*−*26*).

Implications of Switching the Intermediate on Stability. The free energy of a tertiary RNA, as defined by ΔG_{I-to-N}, is proportional to the logarithm of the ratio of the native population to the intermediate population. The structure and energetics of the intermediate must be considered in any prediction of the stability of the native state. Thus, the differential cation binding to the I and N states is the relevant quantity, not just the binding to the N state. The present study also emphasizes that the structure of the intermediate can vary, and predictions of stability in different cations need to account for this structural change.

The cation dependence of the intermediate has implications for mutational studies. Even though the native structure is the same for $tRNA^{Phe}$ folding in Na^+ and Mg^{2+}, the intermediate is different, and mutations may have different effects on the stability depending on the cation used. If a mutation destabilizes a particular region which is formed in both I_{Na} and N states to the same degree, the free energies of both states are shifted by the same amount, and the difference, ΔG_{I-to-N}, will not change. However, if this region is not present in I_{Mg}, only the stability of the N state may be altered, and ΔG_{I-to-N} can change in Mg^{2+}. Hence, the thermodynamic effects of mutations can depend on the metal ion used to fold the RNA.

An additional issue highlighted by the present study is the possibility of the reference state shifting upon mutation. For example, if a mutation sufficiently stabilizes the acceptor stem so that it now forms in I_{Mg}, the reference state is altered and comparing the Mg^{2+}-dependent free energy of the mutant to the wild-type tRNA would not be meaningful. On the other hand, because the acceptor stem already forms in the reference state when folding in Na^+, the reference state for the native structure remains the same, so that comparing the Na^+-dependent free energy of the mutant to the wild-type tRNA now would be meaningful. Conversely, if a mutation selectively destabilizes the acceptor stem when folding in Na^+, a different intermediate without the acceptor stem may become the reference state, again changing the interpretation of the stability of such RNA mutants. In these cases, one should ensure that the equilibrium folding of the mutant and the wild-type tRNA follows the same pathway, for example, as indicated by the same urea *m*-value for the I-to-N transition.

Comparisons to Thermal Unfolding of tRNAs. The cation-induced thermodynamic folding of the yeast $tRNA^{Phe}$ has similarities but some striking differences to the thermal unfolding sequence of several tRNAs. These differences largely can be explained by the altered stability of the helices due to their differential GC content. For the *E. coli* $tRNA^{fmet}$ in 0.17 M Na^+, the first structures to unfold at increasing temperature are the tertiary structure plus the D stem (*27*). The T stem and the anticodon stem unfold next, with the acceptor stem unfolding at the highest temperature. For the yeast $tRNA^{Asp}$ in 0.5 M Na^+, the first structures to unfold at increasing temperature are the tertiary structure plus the D and acceptor stems (*28*). The anticodon stem unfolds next, with the T stem unfolding at the highest temperature. For the yeast $tRNA^{Gly}$ in 0.15 M Na^+, the first structures to unfold at increasing temperature are the tertiary structure plus the D and anticodon stems (*29*). The acceptor stem unfolds next, with the T stem unfolding at the highest temperature. For the helical stems of these tRNAs, the order of thermal

unfolding has a good correlation with the GC content of the particular helices. The ultimate helix to unfold contains 100% GC, and the penultimate helix to unfold contains 70−80% GC, whereas the helix that unfolds with the tertiary structure contains less than 60% GC.

For the modified yeast tRNA^{Phe} in 0.03 M Na^+, the first structure to unfold at increasing temperature is the tertiary structure, followed by the acceptor and the anticodon stems (*30*). The T stem unfolds next, with the D stem unfolding at the highest temperature. This order of thermal unfolding also follows the GC content of this tRNA: 75% for the D stem, 60% for the T stem, 60% for the anticodon stem, and 43% for the acceptor stem.

These thermal unfolding studies indicate that the nucleotide sequence of the helical stem is a significant factor in the equilibrium folding pathway of a tRNA. The acceptor stem of the yeast tRNA^{Phe}, composed of three GC, one GU, and three AU base pairs, has the lowest GC content among all four helical stems in this tRNA. This low GC content of the acceptor stem may in part explain the observed equilibrium folding of this tRNA.

The fact that any helix can fold first implies there is no single equilibrium folding pathway for the generic tRNA structure, although there may be a dominant pathway for a given sequence. The multiplicity of pathways may occur only up to the formation of the highly structured I_{Na} species where all four helices are present. Additional folding, however, may become so conformationally restricted that, effectively, there may be only a single pathway from the I_{Na} state to the N state.

Comparisons to Ion-Induced Folding of Other tRNAs. The equilibrium folding of the unmodified yeast tRNA^{Phe} in Na^+ is reminiscent of the phase diagram for *E. coli* tRNA^{fmet}, tRNA^{Tyr}, tRNA^{Phe}, and tRNA^{Val} at moderate temperatures (*31*). At constant temperatures, these tRNAs fold through two transitions: "extended forms"-to-"cloverleaf or close variants" and "cloverleaf or close variants"-to-"native". These transitions may be analogous to the I_{Mg}-to-I_{Na} and the I_{Na}-to-N transitions in the ion-induced folding of tRNA^{Phe} (Figure 6B). An NMR study of the unmodified *E. coli* tRNA^{Val} revealed that tertiary and D stem interactions were disrupted, but the other stems were intact under low Na^+ and low Mg^{2+} conditions (*32*). This result provides additional evidence for a noncloverleaf I_{Mg} in another tRNA. An NMR study of the unmodified yeast tRNA^{Phe} showed the presence of an intact acceptor stem at 0.1 M NaCl and low Mg^{2+}, consistent with the present result (*33*).

Although many modified tRNAs can fold in Na^+ alone, the addition of Mg^{2+} at moderate Na^+ concentrations increases the stability of the tRNA. For example, at 0.17 M Na^+, the melting temperature for *E. coli* tRNA^{Tyr} is increased from ∼27 °C in the absence of Mg^{2+} to ∼37, ∼55, and ∼70 °C in the presence of 0.1, 1, and 10 mM Mg^{2+}, respectively (*31*). Folding of the modified yeast tRNA^{Phe} requires ∼0.2 mM Mg^{2+} at a Na^+ concentration (∼0.03 M) too low to fold this tRNA (*34*).

The modified Barley tRNA^{Phe} appears to be unable to fold into the native structure in the absence of Mg^{2+} at 21 °C, even with Na^+ concentration as high as 2 M (*35*). The Mg^{2+} concentration required to fold this tRNA increases from ∼0.007 mM in the absence of Na^+ to ∼0.01, ∼0.05, and ∼4 mM in the presence of 0.01, 0.1, and 2 M Na^+, respectively. Such an increase in the Mg^{2+} requirement is similar to the folding of the unmodified yeast tRNA^{Phe} where the Mg^{2+} requirement increases from 0.02 to 0.3 mM upon increasing the Na^+ concentration from 0.01 to 0.15 M (unpublished results).

CONCLUSIONS

The unmodified yeast tRNA^{Phe} folds to the native tertiary structure both in Na^+ and in Mg^{2+} alone. The thermodynamic intermediate in Mg^{2+}-induced folding, which contains the anticodon, D, and T stems, but not the acceptor stem, is less structured than the Na^+-induced species, which contains all four helices as well as structures within the anticodon, D, and T loops. This result can be explained by a differential stability of a secondary structure, the acceptor stem, and the tertiary structure as a function of monovalent and divalent metal ions. Hence, the equilibrium folding of tertiary RNAs can be an intricate interplay between the formation of the secondary and tertiary structures, and the pathway can have different intermediates, depending on the ionic conditions.

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