A new method to monitor the rate of conformational transitions in RNA

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ABSTRACT

Many RNAs need Mg2+ to produce stable tertiary structures. Here we describe a simple method to measure the rate and activation parameters of tertiary structure unfolding that exploits this Mg2+ dependence. Our approach is based on mixing an RNA solution with excess EDTA in a stopped-flow instrument equipped with an absorbance detector, under conditions of temperature and ionic strength where, after chelation of Mg2+, tertiary structure unfolds. We have demonstrated the utility of this method by studying phenylalanine-specific transfer RNA from yeast (tRNA\textsubscript{Phe}) because the unfolding rates and the corresponding activation parameters have been determined previously and provide a benchmark for our technique. We find that within error, our stopped-flow method reproduces both the rate and activation enthalpy for tertiary unfolding of yeast tRNA\textsubscript{Phe} measured previously by temperature-jump relaxation kinetics. Since many different RNAs require divalent magnesium for tertiary structure stabilization, this technique should be applicable to study the folding of other RNAs.

INTRODUCTION

While significant advances have been made in studying native RNA structure (1–7), relatively little is known about the rates of tertiary folding and unfolding, particularly on timescales faster than seconds. Such data are essential for developing mechanistic models of RNA folding and are particularly important in the context of present efforts to design RNA molecules that adopt stable structures possessing specific binding and catalytic properties (8). Recent approaches to investigate tertiary folding have used the intrinsic properties of native structure to report on folding transitions, such as self-splicing for ribozymes (9–11), as well as indirect techniques like oligonucleotide hybridization (12,13), UV cross-linking (14) and chemical modification (10,15,16). With the exception of recent stopped-flow fluorescence experiments that monitor conformational changes that can occur upon substrate binding to ribozymes (17,18), these methods cannot observe transitions that occur faster than about several events per minute.

At present, temperature-jump (T-jump) relaxation is the only general method capable of directly measuring folding transitions in RNA that occur faster than seconds. Indeed, much of our understanding of RNA folding comes from the elegant work of Crothers (19–22), Maass (23–27) and Biltonen (28) who used T-jump to study the folding and unfolding kinetics of transfer RNAs (tRNA). These studies established that denaturation of tRNA tertiary structure to a ‘cloverleaf’ is the first step in the unfolding pathway of tRNAs, and that this conformational change happens on millisecond timescales. It was also found that under the appropriate conditions, the various elements comprising a cloverleaf melt in a sequence-dependent fashion after tertiary structure has been disrupted (19–28). While T-jump is an extremely powerful technique, it does have some limitations. For example, T-jump equipment is expensive and not widely available, current commercial instrumentation uses relatively large sample volumes, and photobleaching can lead to sample degradation. These latter points are particularly important when using RNAs that are only available in small quantities such as synthetic constructs possessing site-specific modifications (29).

Here, we describe a simple non-invasive method to determine the rate and activation parameters associated with unfolding of native RNA tertiary structure. This method exploits the fact that formation of native RNA structure often requires Mg2+ or other divalent metals (30–40), and it entails mixing an RNA solution containing Mg2+ with excess EDTA in a stopped-flow instrument under conditions of temperature and ionic strength where tertiary structure unfolds after chelation of Mg2+. Low millisecond resolution can be achieved, and the unfolding transition can be observed by standard absorbance measurements. We chose to develop this method using phenylalanine specific transfer RNA from yeast (tRNA\textsubscript{Phe}) as a model system because the unfolding rates and the corresponding activation parameters for this molecule are already known and provide a standard for comparison (23–25,41). Our results show that the stopped-flow technique is robust and versatile, which in principle can be used to examine a range of systems.

MATERIALS AND METHODS

Sample preparation

Yeast tRNA\textsubscript{Phe} was obtained from Sigma and was purified by denaturing polyacrylamide gel electrophoresis (8% polyacrylamide,
29:1 acrylamide/bis-acrylamide, 8 M urea, 31.0 cm × 38.5 cm × 0.8 mm). Product bands were excised from the gels and soaked in 4× TAE (450 µl, 160 mM Tris, 80 mM acetic acid, 8 mM EDTA, pH 7.5) for 12 h. The tRNA samples were ethanol precipitated and stored at −20°C. Prior to use, samples were dialyzed against buffer 1 (600 ml, 2 h, 5 mM Na2HPO4, 22 mM NaCl, 3 mM Na2EDTA, pH 6.8) to remove any residual Mg2+ and then dialyzed against buffer 2 (600 ml, 2 h, 5 mM Na2HPO4, 22 mM NaCl, pH 6.8) to remove the EDTA. Lastly, the tRNA was dialyzed into the folding buffer (1.6 l, 6.5 h, 5 mM Na2HPO4, 22 mM NaCl, 1 mM MgCl2, pH 6.8). The dialyzed tRNA was diluted with folding buffer to a final concentration of 0.5 µM. No more than 6 h prior to use, aliquots of this solution (1 ml) were heated to 72°C for 5 min and then cooled to room temperature over 30 min to properly fold the tRNA. Mg2+-free yeast tRNA Phe was prepared following the method of Levy et al. (28). Briefly, samples were dialyzed against buffer 1 (600 ml, 2 h) and buffer 2 (1 l, 4 h) and contained <0.5 molecules of Mg2+ per tRNA as determined by atomic absorption analysis with ICP detection.

**Kinetic measurements**

Kinetic experiments were performed using an Applied Photophysics Ltd model SX18MV stopped-flow spectrophotometer. In a typical experiment, a solution of folded tRNA (0.5 µM) and an EDTA solution (ranging from 0.5 to 9 mM) in the same folding buffer were each equilibrated in the stopped-flow instrument at the desired temperature for 15 min prior to mixing. Mixing was commenced (deadtime of 1.4 ms) using equal volumes (50 µl) of the two solutions and the unfolding transition was monitored at 268 nm (rather than 260 nm to reduce the background absorbance of EDTA). A minimum of seven kinetic traces were obtained at each temperature and each concentration of EDTA. All kinetic data obtained under a given set of conditions were averaged, and the average trace was analyzed using the Kinetic Spectrometer Workstation Software provided with the instrument. Errors to these fits were <6% of the rate. Standard deviations for the rates were calculated from exponential fits to each of the traces that were included in the average rate. To determine activation parameters, Arrhenius plots were constructed for each concentration of EDTA as described by Cole et al. (21).

**UV thermal denaturation experiments**

UV spectra were measured on a Cary 3 spectrophotometer equipped with a Varian Peltier. tRNA samples were prepared by dialysis against the requisite buffer. Aliquots of dialyzed tRNA were diluted with the same degassed buffer to a final volume of 1 ml in 10 mm long self-masking cuvettes and a final tRNA concentration of 0.5 µM. Samples were heated at 85°C for 1 min and then cooled to 5°C at a rate of 3°C/min. The tRNA samples were equilibrated at 5°C for 30 min and then heated at a rate of 0.5°C/min while monitoring the absorbance.

**RESULTS AND DISCUSSION**

**Experimental design and selection of buffer conditions**

This stopped-flow unfolding experiment was first proposed nearly 30 years ago (28), yet the instrumentation of that era had relatively long dead times (e.g., 10 ms) and low sensitivity which made the approach impractical. However, hardware advances have circumvented these limitations and the basic outline of our experiment is shown in Figure 1. While it should be possible to study folding by rapidly adding Mg2+ to an RNA solution void of divalent metals, we have concentrated on measuring the rates and energetics of unfolding because unfolding measurements start from the most defined state (a folded structure) and alternate/unproductive folding pathways due to misfolded intermediates are eliminated. Indeed, the advantages of using unfolding kinetics to examine folding pathways and transition-state structure have been addressed by Fersht and others in studies of protein folding (42,43).

Three requirements exist for use of our stopped-flow technique. First, tertiary unfolding must be accompanied by a spectral change so that the conformational transition can be monitored. While the fluorescence spectrum of yeast tRNA Phe does not change upon tertiary unfolding (24), there is an increase in hyperchromicity upon conversion of the native structure to a cloverleaf. For example, in low [Na+] buffers, the hyperchromicity increases by ~6% when the native tertiary structure unfolds to the cloverleaf (26,44). Therefore, we elected to use UV absorption to follow the unfolding transition. The second condition for use of our method is that dissociation of Mg2+ from the RNA must not be rate limiting. At 37°C, EDTA binds Mg2+ with an association constant (Ka) of 106 M−1 (45) while the Ka values for Mg2+ binding to tRNA Phe, corrected for the polyelectrolyte effect, are ~102 M−1 (44,46–48). The corresponding on-rates for complexation of Mg2+ by EDTA and tRNA Phe are 1.2 × 106 and 5.5 × 105 s−1 M−1, respectively (45,49). The off-rate of Mg2+ (and Mn2+) from yeast tRNA Phe is in the microsecond range (47,49), while the off-rate for Mg2+ from EDTA is 0.55 s−1 (45). Thus, at sufficiently high [EDTA], k1 should be negligible and k−2 will not be significant within the time course of our experiment (<0.5 s). The rate of tertiary unfolding for yeast tRNA Phe determined by T-jump measurements conducted at
37°C is 220 s⁻¹ which suggests that unfolding will be the slow step (k₃) in our experiment (23). The deadtime of our stopped-flow spectrophotometer is 1.4 ms so that the processes associated with k₁, k₋₁ and k₂ cannot be observed.

The last condition for use of our method is that in the presence of Mg²⁺ the native conformation must be stable at temperatures where unfolding would occur in the absence of Mg²⁺. Furthermore, to study just tertiary unfolding, all measurements must be conducted under conditions (e.g., temperature, ionic strength and pH) where unfolding of the native structure is isolated from denaturation of the secondary structure elements that comprise the cloverleaf.

With respect to solution conditions, Urbanke et al. report that in pH 6.0, 20 mM phosphate buffer containing either 0.1–0.3 M CsCl or 0.1 M KCl, native yeast tRNA⁰° unfold to the cloverleaf between 15 and 40°C (23). Addition of Mg²⁺ (1 mM) to a solution of yeast tRNA⁰° in these buffers increases the melting temperature (Tₘ) of the unfolding transition by 30°C (data not shown). Normalized UV melting spectra measured in these CsCl and KCl buffers both in the presence and absence of Mg²⁺ reveal that between 15 and 40°C, the relative absorbance differences between the native structure and the cloverleaf are very small (<1% over most of this temperature range). Although these small absorbance differences can be detected in our stopped-flow experiments using relatively concentrated tRNA solutions (e.g. 2.5 µM), concentrated tRNA solutions also give rise to a large background absorbance which leads to considerable uncertainty in the rate measurements. In these buffers unfolding rates can only be measured accurately at ~40°C, which is the point where the absorbance difference between the native folded structure and the cloverleaf is at a maximum and the rate data show acceptable variance from run-to-run. Specifically, we find that at 39.2°C, the rate of tertiary unfolding measured by our stopped-flow method is 294 (± 10) s⁻¹, and this value is in close agreement with a rate of 285 s⁻¹ measured at 39.5°C by T-jump (23).

In other studies, it has been shown that in pH 6.8 buffer with a total [Na⁺] of 32 mM, thermal denaturation of yeast tRNA⁰° is characterized by five conformational transitions (24,41,50,51). The five transitions occur in a sequential order (25,41,50), the first transition spans 15–35°C and corresponds to conversion of the native folded tertiary structure to the cloverleaf (24–26,41), and the subsequent transitions that occur at higher temperature represent denaturation of the various secondary structure units that comprise the cloverleaf (24–26,41). That tertiary unfolding is isolated from denaturation of the cloverleaf in this buffer is based on several observations. For example, denaturation of the anticodon and acceptor branches can be monitored by observing changes in the fluorescence spectrum of both the Y-base which is in the anticodon stem, and of a formycin derivatized analog of yeast tRNA⁰° where the label is on the 3' terminus (24). Over the temperature range of the tertiary unfolding transition, no resolved relaxation is observed in the fluorescence intensity of either the Y-base or of the formycin label which indicates that denaturation of the anticodon and acceptor stems does not occur with tertiary unfolding (24). The anticodon and acceptor stems of yeast tRNA⁰° melt simultaneously after the tertiary structure has denatured, beginning at ~36°C (24).

Upon addition of Mg²⁺ (1 mM) to a solution of yeast tRNA⁰° in pH 6.8, 32 mM Na⁺ buffer, thermal denaturation of the secondary and tertiary structure is coupled resulting in a single cooperative melting transition with a Tₘ ~70°C (25,26). Thus, in the presence of Mg²⁺ the tertiary structure is stable at temperatures where in its absence, unfolding would occur. Normalized UV thermal denaturation spectra obtained in this Na⁺ buffer, both in the presence and absence of Mg²⁺, show a 3% difference in hyperchromicity between the native structure and the cloverleaf at 28°C, and a 5% difference at 35°C (just below 28°C the relative absorbance differences between the native structure and the cloverleaf decrease to <1%; data not shown). For a 0.5 µM tRNA solution, the relative hyperchromicity differences measured at 28 and 35°C correspond to absolute absorbance increases of 4 and 15 mOD, respectively, and such changes can be measured without interference from the background. Therefore, we selected this 32 mM Na⁺ buffer and a [tRNA] of 0.5 µM for our rate measurements. As described below, working between 28 and 35°C provides a large enough window to accurately measure the rates and activation parameters of tertiary unfolding for comparison with the values obtained previously by T-jump.

**Kinetics of yeast tRNA⁰° unfolding**

In preliminary stopped-flow experiments, we observed an increase in hyperchromicity when folded yeast tRNA⁰° is mixed with a large excess of EDTA (e.g., 6-fold). The rates obtained from these measurements range from 51 (± 8) s⁻¹ at 28.5°C to 127 (± 8) s⁻¹ at 34.8°C (Fig. 2). The relative absorbance changes observed in the stopped-flow experiments match the UV melting data described above to within 5%. In addition, the maximum hyperchromicity increase observed in the stopped-flow experiments is ~6% and this determination agrees with the value measured previously by Leroy et al. under similar solution conditions (26,44).

We performed several control experiments to verify that the hyperchromic shift is due to tertiary unfolding rather than non-specific effects. First, when EDTA is replaced by NaOAc no hyperchromic change is observed (Fig. 2). Second, in buffer containing 150 mM Na⁺, the tertiary structure of yeast tRNA⁰° is stable without Mg²⁺ (up to ~40°C; 41,50,52), and under these
conditions we do not observe a hyperchromic change upon chelation of Mg\(^{2+}\). Third, mixing either an RNA tetraloop hairpin that does not require Mg\(^{2+}\) for folding (53) or Mg\(^{2+}\)-free tRNA (28) with excess EDTA, does not produce a hyperchromic shift. Fourth, a hyperchromic change is not observed when [EDTA] < [Mg\(^{2+}\)]. Lastly, previous work has shown that Mn\(^{2+}\) and Ca\(^{2+}\) can substitute for Mg\(^{2+}\) in stabilizing the folded structure of yeast ΔtRNA\(_{\text{Phe}}\) (54), and chelation of these metals from low salt buffer affords the same rates and activation enthalpy (Δ\(H^\circ\)) of unfolding as measured with buffers containing Mg\(^{2+}\) (vide infra).

Next, we studied tertiary unfolding as a function of [EDTA] and temperature. Arrhenius plots for each [EDTA] were constructed using rate data derived from fits to the average of at least seven experiments. In the absence of EDTA, double exponential kinetics are observed between 28 and 35°C, and the activation enthalpy measured in these conditions is 5.4 kcal/mol (Fig. 3).

Based on T-jump experiments, it was reported that the kinetics of the native-cloverleaf interconversion is a single exponential process with a Δ\(H^\circ\) of 30 (± 5) kcal/mol, and our determinations at the higher [EDTA] are within error of this value (23). At the higher [EDTA], we find that varying the [Mg\(^{2+}\)] 2-fold, or increasing the [tRNA] 3-fold, does not affect changes in rates of unfolding or values of Δ\(H^\circ\). Collectively, these data indicate that when the [EDTA] is ~3.5-fold the [Mg\(^{2+}\)] unfolding of tertiary structure is cleanly being monitored and that tertiary unfolding, not dissociation of Mg\(^{2+}\) ions from the tRNA, is rate limiting.

In summary, we have described a simple approach to measure the rates and activation parameters associated with the unfolding of RNA tertiary structure. With present stopped-flow equipment low millisecond resolution is easily achieved and, because the unfolding transition is monitored directly by UV absorption, possible alterations in the unfolding pathway that can occur in more invasive methods like chemical modification (10, 15, 16) and oligonucleotide hybridization (12, 13) are eliminated. Our method is not limited to studies of tertiary unfolding since chelating Mg\(^{2+}\) at higher temperature in principle could allow other conformational transitions to be examined. Stopped-flow instrumentation is relatively inexpensive and widely accessible; if appropriate, conformational transitions can be observed by other methods like fluorescence or circular dichroism; very little material is needed for these experiments (0.6 ng for a single rate measurement); and the method is non-destructive. Based on these considerations and the properties of other RNAs (16, 30–40, 55), it appears that this technique should be useful to study a range of other RNAs.

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