The Time Dependence of Chemical Modification Reveals Slow Steps in the Folding of a Group I Ribozyme†

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ABSTRACT: L-21 *Sca*I ribozyme is a linear form of the self-splicing intron from the precursor of the *Tetrahymena thermophila* LSU intron. The time scales for tertiary folding of L-21 *Sca*I were investigated in two ways after bringing it from a partially denatured state at 60 °C and 1 mM Mg^{2+} to a renatured state at 15 °C and 10 mM Mg^{2+}. First, formation of a catalytically active structure was monitored by measuring the kinetics of the reaction: p*CUCUA_3 + G ⇌ p*CUCU + GA_3. This reaction mimics the first step of splicing. After 1 min of folding time, the catalytic rate is roughly 10% of the rate attained after 8 h of folding. This indicates that much of the structure refolds quickly. Also, at least two time scales of folding are observed, separated by a lag time of about 30 min. To define the regions folding on various time scales, all the guanosines of L-21 *Sca*I were probed with kethoxal at 15 °C while folding was in progress. Based on folding time scales, the guanosines can be placed into at least four classes. These are guanosines that (1) are already protected at 60 °C in 1 mM Mg^{2+} or which fold immediately, (2) fold during the lag time, (3) continue to fold after 1 h, and (4) never fold. These results give insight into the folding pathway of a group I ribozyme at nucleotide resolution. This provides useful information on the regions whose foldings are important for catalytic function of the ribozyme. The method may also provide a general way to suggest regions of an RNA that may interact with each other to form tertiary structure.

The most definitive way to determine a three-dimensional structure of a natural RNA molecule is to solve a crystal structure. For reasons yet unknown, however, only crystal structures of tRNAs have been solved to atomic resolution for natural RNAs (Kim et al., 1974; Robertus et al., 1974). Hence a combination of alternative experimental methods have been utilized to model the structures of RNA molecules (Michel & Westhof, 1990; Puglisi et al., 1992; Stern et al., 1989b; Brimacombe et al., 1988). One potentially useful approach is to elucidate the folding pathway of an RNA. In principle, such studies can suggest regions involved in tertiary structural interactions, since these are likely to fold more slowly than regions involved only in secondary structure (Crothers et al., 1974; Bevilacqua et al., 1992). The kinetics of folding induced by thermal or salt renaturation may further suggest which regions of an RNA might interact with each other to form tertiary structure, thus guiding site-directed mutagenesis studies.

Unfortunately, detailed investigations of folding pathways have been carried out only for tRNAs (Crothers et al., 1974; Riesner & Römer, 1973; Hilbers et al., 1976; Rhodes, 1977), viroids (Reisner, 1987; Steger et al., 1984), and 5S rRNA (Kjems et al., 1985). Various experimental methods that have been employed to investigate steps of folding include UV melting (Cole et al., 1972; Steger et al., 1984; Banerjee et al., 1993), NMR (Crothers et al., 1974), chemical probing (Celander & Cech, 1991; Banerjee et al., 1993; Laggerbauer et al., 1994), and kinetics (Crothers et al., 1974; Bevilacqua et al., 1992; Zarrinkar & Williamson, 1994).

In previous work, thermal denaturation of the L-21 *Sca*I ribozyme, a linear form of the self-splicing intron of *Tetrahymena thermophila* (Zaug et al., 1988; Inoue & Kay, 1987), was followed by UV absorption and chemical mapping in 50 mM Na^+ and 10 mM Mg^{2+} at pH 7.5 (Banerjee et al., 1993). UV melting experiments identified two major transitions with midpoints at 65 and 73 °C. Chemical mapping data indicated the 65 °C transition is primarily due to disruption of tertiary structure, whereas the 73 °C transition is due to disruption of secondary structure (Banerjee et al., 1993). Experiments on another group I intron are consistent with this hierarchy of folding (Jaeger et al., 1993).

In this paper, the time scales of the tertiary folding are investigated. This was done by first following the time dependence of thermal renaturation with an assay of enzyme activity. The time scales of folding for each guanosine were then probed by chemical modification to determine the regions important for enhancing catalytic activity. The method provides information similar to that provided by hydrogen exchange experiments for following protein folding (Roder et al., 1988; Udgaonkar & Baldwin, 1988).

MATERIALS AND METHODS

Ribozymes. L-21 *Sca*I ribozyme was prepared by T7 RNA polymerase transcription of pT7L-21 plasmid cut with *Sca*I endonuclease (Zaug et al., 1988). The ribozyme was purified by gel electrophoresis on a 4% polyacrylamide/7 M urea gel. The RNA was visualized by UV shadowing and eluted into 0.1% SDS, 0.1 mM Na_2EDTA, and 40 mM ammonium acetate at pH 8.0 by the crush and soak method (Barfod & Cech, 1988). After elution, the ribozyme was run through a G50 Sephadex column, precipitated in ethanol, and stored at −20 °C in sterile water.

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Oligonucleotides. Primers and oligoribonucleotide, CUCUA$_3$, were prepared by the phosphoramidite method and then purified by high-performance liquid chromatography and stored at −20 °C in sterile water (Beaucage & Caruthers, 1981; Matteuchi & Caruthers, 1981; Barone et al., 1984; Kierzek et al., 1986). Primers and CUCUA$_3$ were 5′-end labeled with [γ-^{32}P]ATP from New England Nuclear and T4 polynucleotide kinase from New England Biolabs. 5′-Guanosine monophosphate (5′-GMP) was from Sigma.

Reagents and Enzymes. Kethoxal (β-ethoxy-α-ketobutyraldehyde) was from United States Biochemicals. The buffer 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was from Aldrich. Avian myeloblastosis virus reverse transcriptase was from Life Sciences Incorporated. The deoxyribo-, dideoxyribo-, and ribonucleoside triphosphates were from Pharmacia. Gel solutions were prepared from ultrapure acrylamide, *N*,*N*′-methylenebisacrylamide, and boric acid from Bio-Rad.

Kinetics of Catalytic Activity. To investigate if the tertiary folding of the ribozyme is reflected in catalytic activity, a reaction mimicking the first step of splicing was monitored for different folding times of the ribozyme. The reaction

$$\text{p*CUCUA}_3 + \text{pG} \leftrightharpoons \text{p*CUCU} + \text{pGA}_3 \qquad (1)$$

was followed at 15 °C in 80 mM HEPES (made with 40 mM Na^+ salt and 40 mM free acid) at pH 7.5, 10.5 mM $MgCl_2$, 0.5 mM Na_2EDTA, and 10 mM NaCl (melting buffer). L-21 *Sca*I ribozyme was used to initiate the above reaction. L-21 *Sca*I at 7 μM was incubated for 5 min at 60 °C in 50 μL of melting buffer, except $[Mg^{2+}] = 1.5$ mM. UV melting experiments indicate that these conditions disrupt tertiary structure (Banerjee et al., 1993). The preincubation tube was immersed completely in a Lauda RC6 bath maintained at 60 °C. The volume containing ribozyme was then immediately transferred to 300 μL of melting buffer preincubated for at least 5 min at 15 °C, thus allowing tertiary folding to commence. The half-life for the temperature of the folding mixture to equilibrate from 60 °C back to 15 °C is about 22 s as measured by a thermistor in the solution. Reaction of 3.2 μL of ~1 nM p*CUCUA$_3$ and 1 mM pG at 15 °C was initiated by adding 32 μL melting buffer containing 1 μM of ribozyme after various folding times. While detailed measurements were not made, it is likely that the concentration of ribozyme is below K_d for p*CUCUA$_3$ binding (Bevilacqua et al., 1994) and the concentration of pG is also below its K_d for binding to the folded ribozyme (Moran et al., 1993; McConnell et al., 1993). Thus, the rate of reaction should be sensitive to folding of both binding sites as well as the catalytic site.

In order to obtain rates for reaction 1, aliquots of the reaction were taken at various times and quenched with an equal volume of 20 M formamide, 0.1% bromophenol blue, 0.1% xylene cyanol, 90 mM Tris-borate, and 2 mM EDTA (loading buffer) in a dry ice/ethanol bath. The reactant and product were then separated in 20% acrylamide/7 M urea gels and visualized by autoradiography. Bands were quantitated by counting in Ecosinct A scintillation fluid in a Packard Tri-Carb 4530 scintillation counter. No hydrolysis was observed when the above reaction was repeated in the absence of pG.

Fraction reaction is defined as $F = P/(P + R)$, where P and R are counts of product and reactant bands, respectively. Rates of reaction of p*CUCUA$_3$ and pG were calculated by fitting the data to

$$F_t = F_\infty (1 - e^{-kt}) \qquad (2)$$

Here F_t is the fraction reacted at time t, and F_∞ is the fraction reacted at equilibrium.

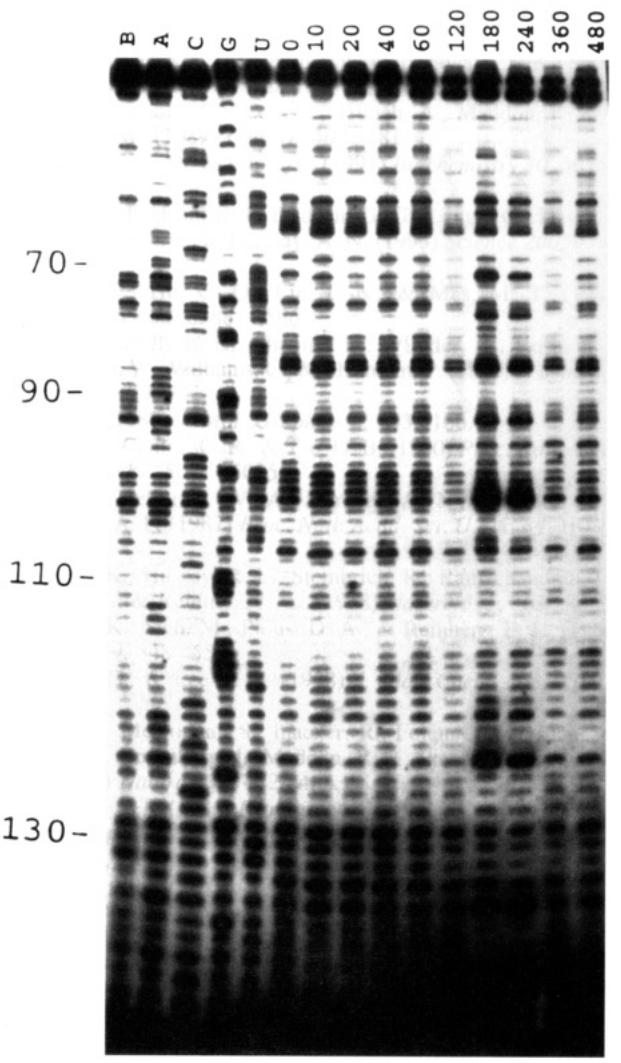

FIGURE 1: Autoradiogram of a sequencing gel of L-21 *Sca*I showing the modification of guanosines by kethoxal after allowing different folding periods (listed in minutes above appropriate lane) of the ribozyme at 15 °C in melting buffer. The $t = 0$ lane represents an aliquot taken within 5 s of kethoxal addition, and other times are relative to the time this first aliquot was taken. Region shown is near the 5′-end of ribozyme using primer 1P141.

Kinetics of Chemical Mapping. Ribozyme (100 pmol) was preincubated at 60 °C in 30 μL of melting buffer except that $[Mg^{2+}] = 1.5$ mM. After 5 min, the 30 μL was quickly transferred into the 250 μL of reaction mixture that had been preincubated for at least 5 min at 15 °C. The final volume of 280 μL contained melting buffer with 6 mM kethoxal reacting with 0.4 μM ribozyme.

Reaction of ribozyme with kethoxal was stopped by quenching 25-μL aliquots after 5 s, 10, 20, 40, 60, 120, 180, 240, 360, and 480 min as previously described (Stern et al., 1987; Banerjee et al., 1993). The concentration of kethoxal was chosen to give observable modification patterns while retaining significant full-length extension of reverse transcripts (Figure 1). Modification reactions were detected by primer extension and analyzed as described by Stern et al. (1987) and Banerjee et al. (1993). Individual bands were

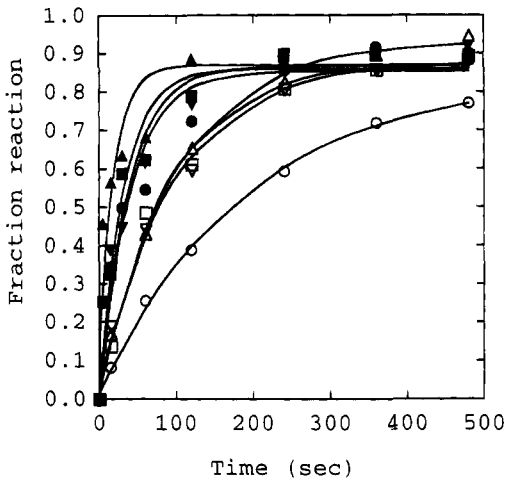

FIGURE 2: Typical plot of fraction product of reaction between ~1 nM p*CUCUA$_3$ and 0.09 mM pG in the presence of 1 μM L-21 *Sca*I ribozyme vs different folding periods of ribozyme at 15 °C in 80 mM HEPES (made with 40 mM Na^+ salt and 40 mM free acid) at pH 7.5, 10.5 mM $MgCl_2$, 0.5 mM Na_2EDTA, and 10 mM NaCl (melting buffer). Folding periods of 1, 10, 20, 30, 60, 120, 180, and 360 min are represented by ○, ▽, □, △, ●, ▼, ■, and ▲, respectively.

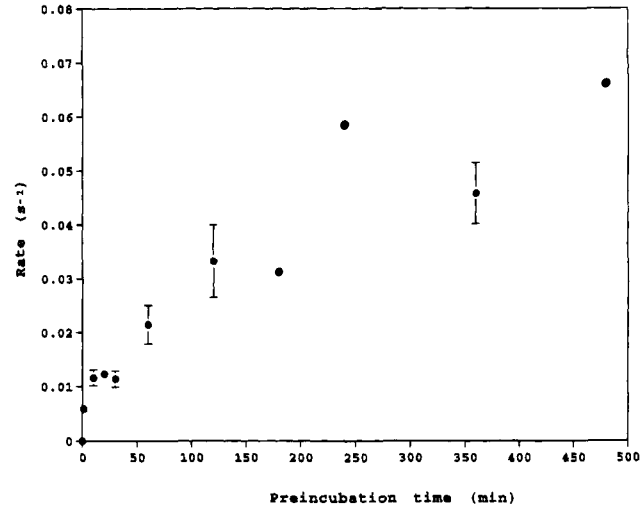

FIGURE 3: Rates of reaction from exponential fits of two different plots (one of which is shown in Figure 2) vs time periods allowed for L-21 *Sca*I ribozyme to fold at 15 °C. Points are averages of two experiments, except for 240 and 480 min. Error bars show range of measured rates. Error bars for preincubation times of 0, 1, 20, and 180 min are negligible. A plot of initial rates shows a similar pattern.

quantified with a Molecular Dynamics 425 PhosphorImager running ImageQuant software.

The fraction modified, f, for each guanosine band was calculated by dividing the counts of that particular band by the total number of counts in the lane. Assuming pseudo-first-order kinetics, the time dependence of this fraction formed is given by:

$$f_t = [k_R/(k_R + k_F)][1 - e^{-k_F t} + k_R t e^{-k_F t}] + f_0 \quad (3)$$

where f_t is the fraction reacted at time t and k_F is the first-order rate constant for folding of the guanosine in the ribozyme. The pseudo-first-order rate constant for reaction of guanosine with kethoxal is denoted by k_R, where $k_R t \ll 1$ since the reaction conditions chosen ensure less than one modification per molecule. The term f_0 is the fraction modified at time $t = 0$. In the ideal case, $f_0 = 0$ (see Appendix for derivation for this case). In a chemical mapping experiment, however, there is background due to spontaneous cleavage. Therefore, an aliquot was removed from the reaction mixture about 5 s after the addition of kethoxal. The fraction reacted in this sample was set equal to f_0. The time, t, for subsequent points is then the time after this first aliquot was taken.

For guanosines which do not become unreactive with time, since $k_F = 0$, eq 3 reduces to

$$f_t = k_R t + f_0 \quad (4)$$

For guanosines that become protected with time, the fraction modified is

$$f_t = [k_R/(k_R + k_F)][1 - e^{-k_F t}] + f_0 \quad (5)$$

since $k_R t \ll 1$ because the rate of guanosine modification by kethoxal is much slower than the rate of folding for guanosines that become protected.

RESULTS

Kinetics of CUCUA$_3$ and G. To determine the rates for catalytic activity of ribozyme after thermal renaturation, the time course of reaction of p*CUCUA$_3$ and pG in the presence of L-21 *Sca*I ribozyme was followed at 15 °C. Figure 2 shows typical plots of fraction product vs reaction time following different periods of ribozyme folding. Rates of reaction from exponential fits of such plots vs times allowed for ribozyme to fold at 15 °C are shown in Figure 3. The rate of catalysis increases as folding proceeds.

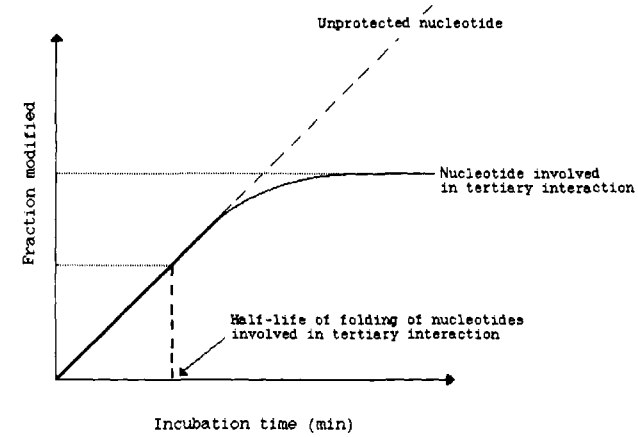

FIGURE 4: Comparison of expected plots of modification of two types of guanosines vs incubation time of L-21 *Sca*I ribozyme. Guanosines that always remain single stranded exhibit linear increase in modification with time, whereas those involved in a folding transition show exponential increase with time.

Time-Dependent Probing of Guanosines. To determine the folding time scales of various regions of the ribozyme, guanosines were probed during folding at 15 °C. Figure 4 shows a comparison of the expected plots of modification for two types of guanosine against incubation time of L-21 *Sca*I ribozyme. For a guanosine that remains single stranded, the fraction modified is expected to increase linearly with time (eq 4). For a guanosine involved in a folding transition with a single exponential rate, the fraction modified is expected to increase exponentially and then level off (eq 5). Plots of fraction of guanosines modified against preincubation times of L-21 *Sca*I are shown in Figures 5–7 and in

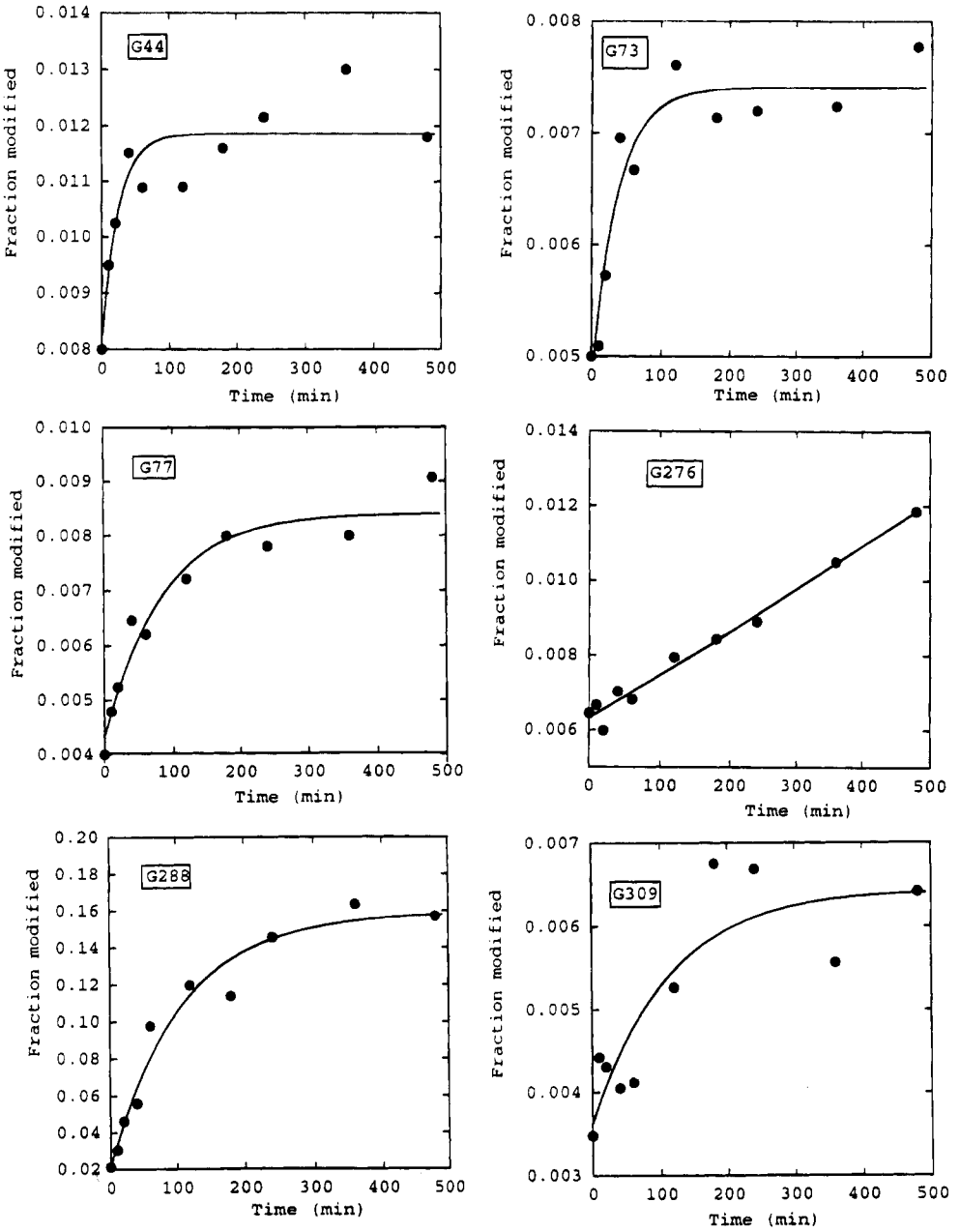

FIGURE 5: Plots of fraction of guanosines modified vs incubation times of L-21 *Sca*I ribozyme at 15 °C in melting buffer.

the supporting material (see paragraph at end of paper regarding supporting material). Both the expected and more complicated behavior are observed. For guanosines whose modification levels off with time, the plot of fraction modified with time was fit to a single exponential according to eq 5. Rates and half-lives for these guanosines are listed in Table 1. Figure 8 summarizes these results.

DISCUSSION

The catalytic activity of the ribozyme provides a reporter of renaturation upon changing conditions from 60 °C, $[Mg^{2+}]$ = 1 mM, to 15 °C, $[Mg^{2+}]$ = 10 mM. Previous UV melting experiments suggest this folding involves primarily tertiary interactions (Banerjee et al., 1993).

As shown in Figure 3, the rate of reaction for $CUCUA_3$ and pG at the shortest folding time of 1 min is about 10% of the rate that is attained after 8 h of folding. Evidently, most of the structure refolds within the first minute. Further folding that affects catalytic activity is observed after 1 min however. At least two time scales of folding are observed that are separated by a lag time of about 30 min. These folding events have half-lives of approximately 1 and >60

Table 1: Folding Rates of Guanosines Based on Kinetics of Reaction with 6 mM Kethoxal at 15 °C in Melting Buffer[a]

guano-sines	rate (min^{-1} × 100)	$t_{1/2}$ (min)	guano-sines	rate (min^{-1} × 100)	$t_{1/2}$ (min)
368	18	4	349	1.8	38
360	4.8	14	357	1.6	43
44	4.1	17	378	1.3	55
384	3.3	21	77	1.2	58
382	2.9	24	288	0.9	74
73	2.7	25	309	0.9	77
341	2.8	25	327	0.8	85
358	2.5	28	388	0.8	87

[a] Positions of guanosines that are folded at first time point or that never fold are shown in Figure 8.

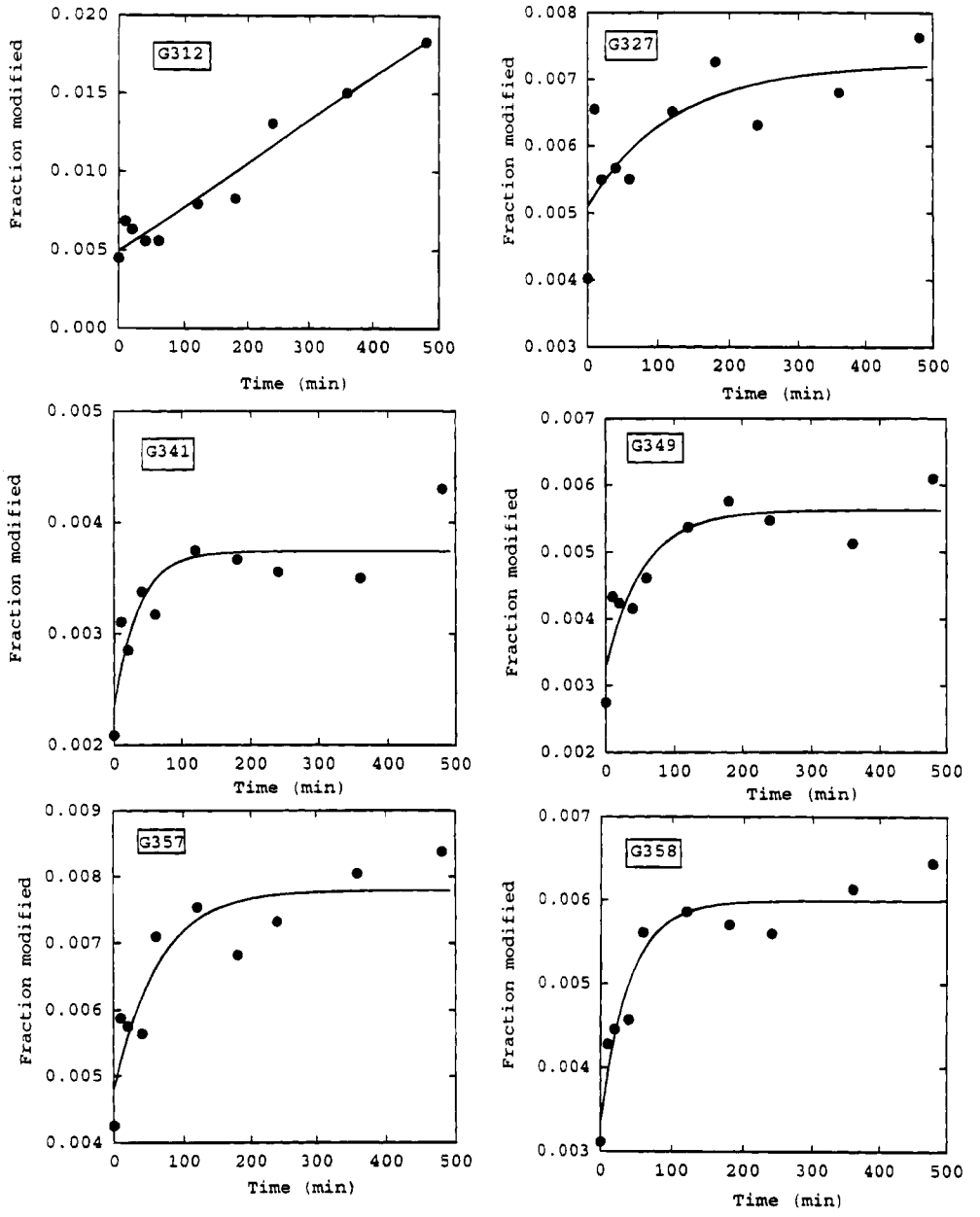

FIGURE 6: Plots of fraction of guanosines modified vs incubation times of L-21 *ScaI* ribozyme at 15 °C in melting buffer.

min. Some three-dimensional structural changes may be occurring during the lag time that allow the ribozyme to refold in some new conformation that exhibits more efficient catalysis.

To define the regions folding on various time scales, all the guanosines of the ribozyme were probed with 6 mM kethoxal at 15 °C during folding. Based on the folding time scales, the guanosines can be classified into at least four different categories. Guanosines already protected at 60 °C in 1 mM Mg^{2+} or which fold immediately show essentially no modification. This first class includes most of the guanosines (Figure 8).

The second class of guanosines fold with half-lives similar to the lag time of about 30 min observed in the activity assay. These guanosines are at positions 44, 73, 341, 349, 357, 358, 360, 382, and 384 (Figure 8). Some of them are probably involved in structural changes that occur during the lag time. The data for nucleotides 44 and 349, which are in complementary hairpins, could also be interpreted as having a time dependence for modification that is similar to the time dependence for enhancement of catalytic activity (compare to Figure 3). Unfortunately, the signal-to-noise ratio is not sufficient to make this distinction. The G's with apparent folding times of about 30 min are in regions L2, L2.1, L9.1, L9.1a, P9.2, and L9.2, suggesting that folding during the lag time may involve tertiary interactions between the 5′ and 3′ ends of the molecule. Loops L9.1a and L2.1 have 12 potential tertiary pairings, whereas four nucleotide complementarity exists between loops L9.1a and L2. Laggerbauer et al. (1994) have suggested that the 3′ end of the ribozyme helps stabilize part of the catalytic core. Interaction of the 5′ and 3′ ends could do this by essentially cyclizing the ribozyme.

The third class of guanosines fold with half-lives between 55 and 90 min. These guanosines are at positions 77, 288, 309, 327, 378, and 388. Interestingly, the time dependence of modification for G309 (Figure 5) also appears similar to the time dependence for enhancement of catalytic activity

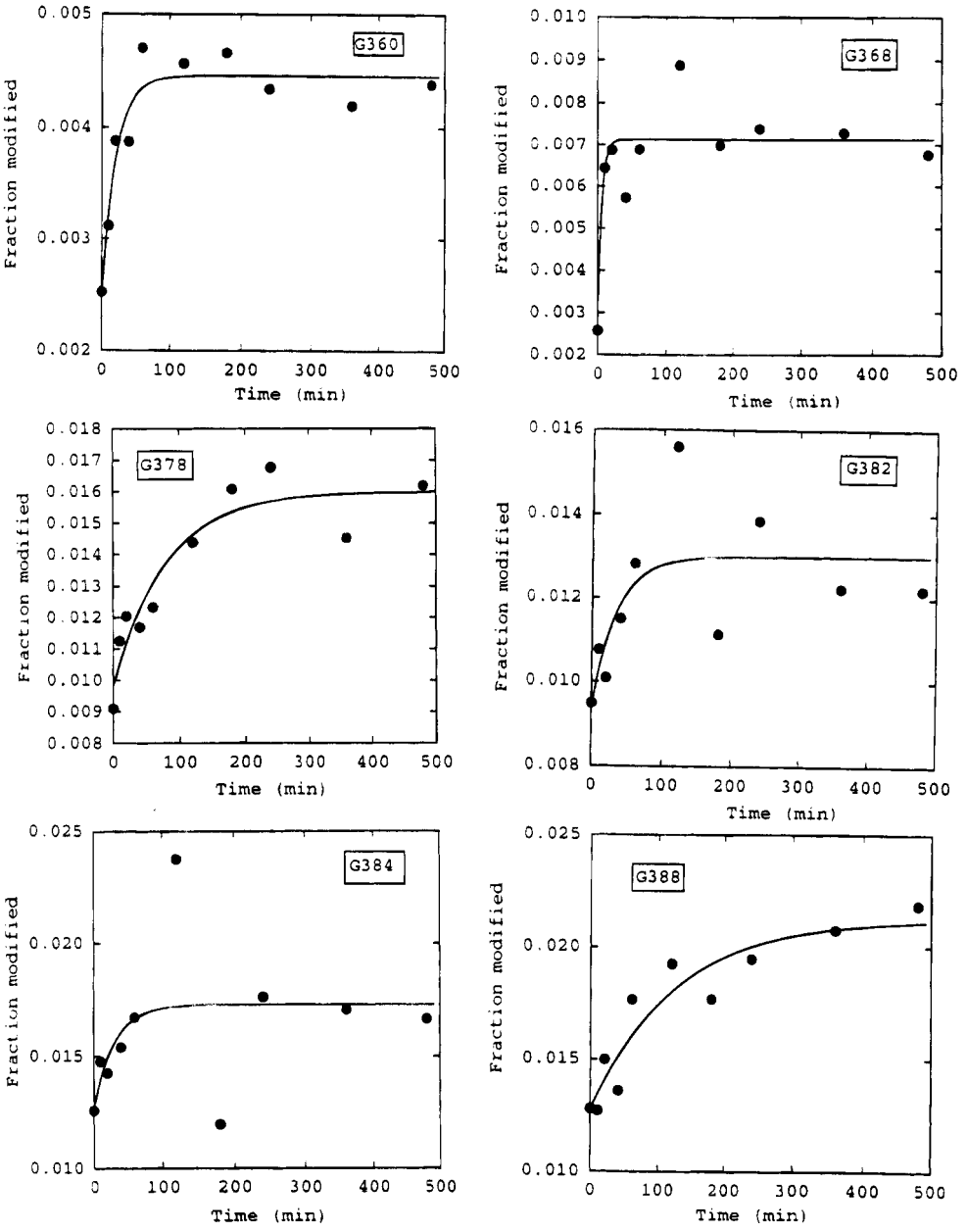

FIGURE 7: Plots of fraction of guanosines modified vs incubation times of L-21 *Scal* ribozyme at 15 °C in melting buffer.

(Figure 3). This suggests that P7 may close, reopen, and reclose during folding. Although G100 also showed a slow time-dependent increase in reaction with kethoxal, the change in the amount of modification is relatively small compared to other nucleotides with long half-lives. Hence, the folding time of this particular guanosine is not reported.

The fourth class of guanosines shows linear increases in modification with time, indicating that they remain single stranded. These are at positions 22−27 (which could not be quantitated individually), 220, 276, 279, 303, 312, and 313. All are either unpaired or at helix termini except for G276 in P3. This implies that in the absence of substrate, the pseudoknot involving P3 may never fold completely at 15 °C, under the conditions used. Interestingly, G220, G279, G312, and G313, which are near the pseudoknot in the three-dimensional model of Michel and Westhof (1990) also never become unreactive with time. Evidently, the formation of the P3/P7 pseudoknot is a difficult step in the folding pathway at 15 °C. A slow, but complete folding of P3 was observed by Zarrinkar and Williamson (1994) however when folding was initiated by the addition of Mg^{2+}. Similar complex steps in folding have been found during the binding of the pseudoknot of α-RNA from *Escherichia coli* to ribosomal protein, S4 (Tang & Draper, 1989; Spedding et al., 1993). Following the time dependence of folding may be a way to identify pseudoknots or other complex tertiary structure. This information could then be useful in modeling the RNA structure.

The inference that pseudoknot folding is difficult under the conditions reported here does not, however, imply it will be difficult *in vivo*. Presumably, folding *in vivo* occurs during transcription. Thus P3 may form before subsequent nucleotides are transcribed. Thus, for example, nucleotides 303−307 can pair with nucleotides 270−274 of P3 in the full-length transcript but not if P3 forms before 303−307 are transcribed. Interestingly, the rate for opening a pairing between nucleotides 303−307 and 270−274 is expected to be independent of Mg^{2+} concentration (Williams et al., 1989;

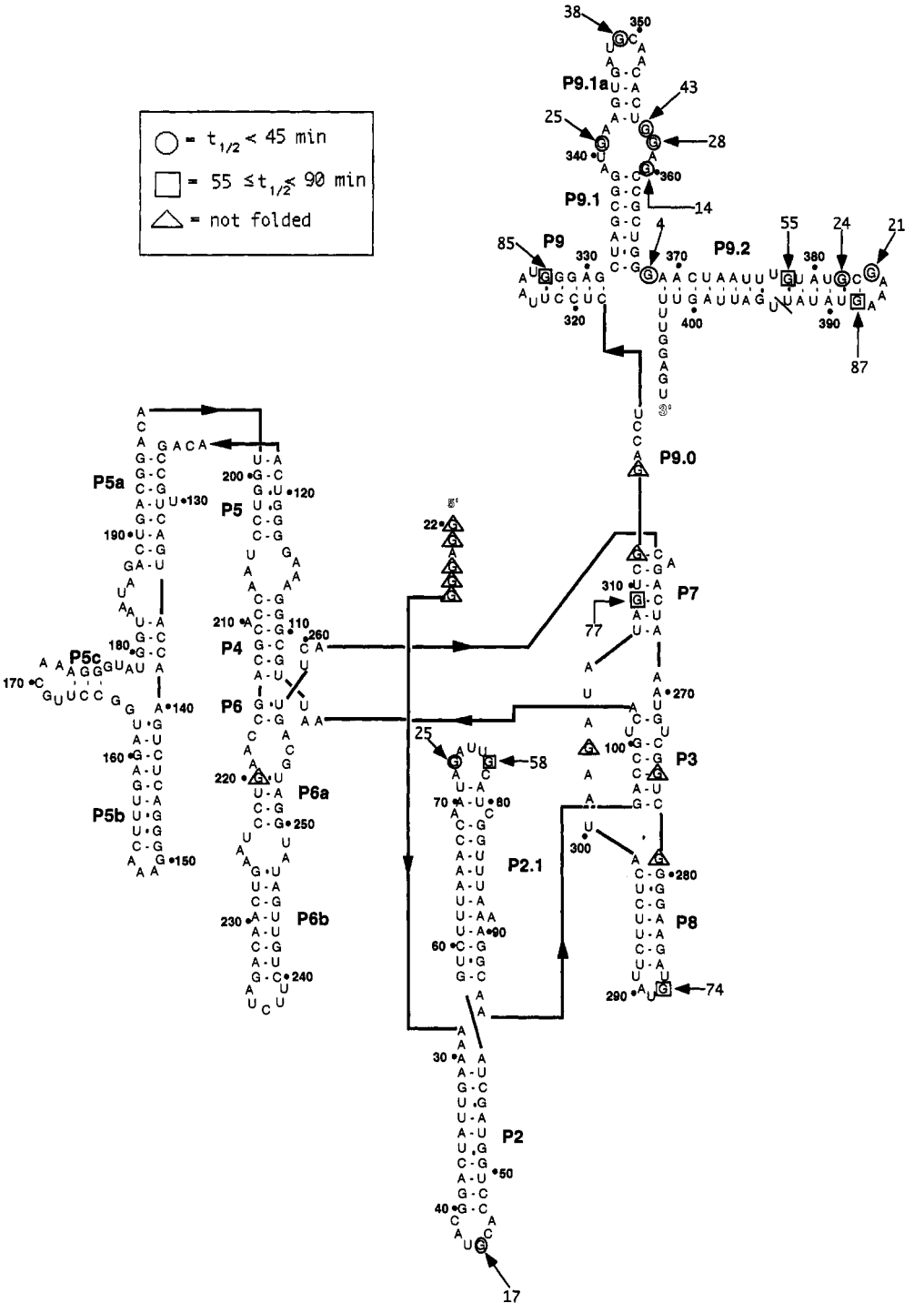

FIGURE 8: Half-lives of folding of guanosines at 15 °C in minutes. Unlabeled G's fold immediately (i.e., $t_{1/2}$ < 1 min) or were already folded at high temperature.

Pörschke et al., 1973). Thus, such opening could account for the Mg^{2+} independent step in folding observed by Zarrinkar and Williamson (1994). The only nucleotide that cannot be included in the above folding classes is G368, which folds with a half-life of about 4 min.

The time dependence of chemical modification during folding provides a way to follow folding pathways at nucleotide resolution. In this work, folding steps in the minute time range have been studied. Extension to faster processes should be straight forward with the use of higher concentrations of modfication reagent, or photogenerated modification reagents such as OH· (Macgregor, 1992) in conjunction with a rapid mixing apparatus. Such experiments may provide insight into regions that exhibit similar rates of folding and hence may interact.

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APPENDIX

Derivation of Rate Equations for Time Dependence of Modification. Consider the reaction scheme:

$$F \xleftarrow{k_F} U \xrightarrow{k_R} M \quad \text{(A1)}$$

where U is unfolded, unmodified guanosine; F is folded guanosine; M is modified guanosine; k_F is first-order rate constant for folding of a particular guanosine; and k_R is pseudo-first-order rate constant for reaction of a particular guanosine with kethoxal. The rate of loss of an unfolded, unmodified guanosine, dU/dt, is given by

$$dU/dt = -k_F U - k_R U = -U(k_F + k_R) \quad \text{(A2)}$$

Integrating both sides

$$\int_{U_0}^{U} dU/U = -\int_0^t (k_F + k_R)\, dt \quad \text{(A3)}$$

$$\Rightarrow \ln U \Big|_{U_0}^{U} = -(k_F + k_R)t\Big|_0^t = -(k_F + k_R)t \quad \text{(A4)}$$

$$\Rightarrow \ln U - \ln U_0 = \ln(U/U_0) = -(k_F + k_R)t \quad \text{(A5)}$$

$$\Rightarrow (U/U_0) = e^{-(k_F+k_R)t} \quad \text{(A6)}$$

$$\Rightarrow U = U_0(e^{-(k_F+k_R)t}) \quad \text{(A7)}$$

where U_0 is the initial concentration of the particular unfolded, unmodified guanosine.

Also, the rate of formation of the modified form of a given guanosine, dM/dt, is given by

$$(dM/dt) = k_R U \quad \text{(A8)}$$

$$= k_R U_0(e^{-(k_F+k_R)t}) \quad \text{(A9)}$$

Integrating both sides

$$\int_0^M dM = k_R U_0 \int_0^t (e^{-(k_F+k_R)t})\, dt \quad \text{(A10)}$$

$$\Rightarrow M = -[k_R/(k_F + k_R)]U_0 e^{-(k_F+k_R)t}\Big|_0^t \quad \text{(A11)}$$

$$\Rightarrow M = U_0[k_R/(k_F + k_R)][1 - e^{(k_F+k_R)t}] \quad \text{(A12)}$$

Similarly,

$$F = U_0[k_F/(k_F + k_R)][1 - e^{-(k_F+k_R)t}] \quad \text{(A13)}$$

Equation A12 can be rewritten as

$$f_t = (M/U_0) = [k_R/(k_F + k_R)][1 - (e^{-k_F t})(e^{-k_R t})] \quad \text{(A14)}$$

where f_t is the fraction of a given guanosine that has reacted at time t.

Since all mapping experiments were limited to less than one modification per molecule

$$k_R t \ll 1$$

Expanding $e^{-k_R t}$ by Taylor series and ignoring higher order terms, we get

$$\Rightarrow f_t = [k_R/(k_F + k_R)][1 - (e^{-k_F t})(1 - k_R t)] \quad \text{(A15)}$$

$$\Rightarrow f_t = [k_R/(k_F + k_R)][1 - e^{-k_F t} + k_R t e^{-k_F t}] \quad \text{(A16)}$$

Case I: For guanosines which do not become unreactive with time, i.e., $k_F = 0$, eq A16 becomes

$$f_t = k_R t \quad \text{(A17)}$$

Case II: For guanosines that become protected with time under the condition that $k_R t \ll 1$ (This condition can be met because the rate of guanosine modification by kethoxal is much slower than the rate of folding), eq A16 becomes

$$f_t = [k_R/(k_R + k_F)][1 - e^{-k_F t}] \quad \text{(A18)}$$

SUPPLEMENTARY MATERIAL AVAILABLE

Plots of fraction reacted vs time for G22−27, G220, G279, G303, and G313 (1 page). All plots are linear. Ordering information is given on any current masthead page.

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