Time-Resolved, Single-Molecule, Correlated Chemical Probing of RNA

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ABSTRACT: Capturing the folding dynamics of large, functionally important RNAs has relied primarily on global measurements of structure or on per-nucleotide chemical probing. These approaches infer, but do not directly measure, through-space structural interactions. Here we introduce trimethyloxonium (TMO) as a chemical probe for RNA. TMO alkylates RNA at high levels in seconds, and thereby enables time-resolved, single-molecule, through-space probing of RNA folding using the RING-MaP correlated chemical probing framework. Time-resolved correlations in the RNase P RNA—a functional RNA with a complex structure stabilized by multiple noncanonical interactions—revealed that a long-range tertiary interaction guides native RNA folding for both secondary and tertiary structure. This unprecedented nonhierarchical folding mechanism was directly validated by examining the consequences of concise disruption of the through-space interaction. Single-molecule, time-resolved RNA structure probing with TMO is poised to reveal a wide range of dynamic RNA folding processes and principles of RNA folding.

Chemical probing is widely used to monitor RNA folding reactions. Broadly, chemical probing involves reacting an RNA with a small molecule that is sensitive to the underlying nucleic acid structure.1−4 Chemical probing has revealed numerous features of RNA biology and allows complex RNA structures to be modeled with good to outstanding accuracy.4−7 However, most chemical probing strategies merely infer RNA structure from observed reactivity, but do not measure RNA structure directly.

RNA interaction groups analyzed by mutational profiling (RING-MaP) technologies directly detect through-space structural communication in RNA. In RING-MaP, RNAs are modified at high per-nucleotide rates such that multiple chemical adducts occur in the same RNA strand and are comprehensively detected by a processive relaxed fidelity reverse transcription (MaP) reaction.8−10 RING-MaP measures reagent-induced sequential unfolding of RNA molecules; however, every molecule is perturbed in a unique manner and perturbations average out over a population of molecules.5,10 Modification of one nucleotide in a dynamically exposed base pair or tertiary interaction can block local refolding, increasing the probability of subsequent modification of the pairing partner (Figure 1A). Through-space structural communication is detected as the subset of interdependent or correlated modification events in the same molecular strand of RNA (Figure 1A). RING-MaP chemical probing can be used to measure multiple features of RNA structure, depending on how correlation data are interpreted. RING-MaP has been used to directly detect RNA duplexes (termed PAIR-MaP),5,10 to define multiple conformational states in an RNA ensemble,5,9 and to reveal tertiary interactions and through-space structural communication5,11,12 (Figure 1B). In this study, we focus on the latter, examining through-space RNIGs, in a time-resolved way. To date, RING-MaP correlated chemical probing has been carried out using dimethyl sulfate (DMS).

DMS is widely used to examine interrelationships between RNA structure and cellular function, including in cells,1,13 DMS has high reactivity with unpaired adenosine and cytidine nucleotides, and our laboratory has developed reaction conditions that now enable DMS to modify uridine and guanosine at structurally informative levels (Supporting Methods and ref 10). DMS reacts slowly with RNA, with a reaction half-life of ~12 min (Figure 1C). We sought to identify a chemical probe with the advantages of DMS but much faster reactivity. Trimethyloxonium tetrafluoroborate (TMO)14 is highly soluble, reacts through RNA alkylation and self-quenching hydrolysis, and has a half-life in buffered solution of 7.5 s (Figure 1C, Supporting Figure 1). TMO thus reacts 90 times more rapidly than DMS, does not require an explicit quenching step, and probes RNA folding on the seconds time scale.

We compared TMO and DMS reactivities by probing the structure of the B. steaothermophilus ribonuclease P (RNase P) catalytic domain, an RNA with a complex secondary and tertiary structure.15,16 TMO and DMS both achieve the high level of reactivity required for correlated chemical probing (Figure 2A). TMO, like DMS, measures RNA structure in accurate and useful ways, as shown by multiple experiments.

First, superimposition of TMO and DMS reactivities on the secondary structure of RNase P (Figure 2B) and a second
model RNA (Supporting Figure 2) reveals that both reagents react with all four RNA nucleotides and preferentially with unpaired RNA regions. Second, TMO reactivities correlate strongly with those for DMS (Figure 2C) and, third, discriminate strongly and similarly between paired and unreactive per-nucleotide reactivity data suggested that P2, P5, the catalytic core, and maintenance of the overall RNA structure.4,10 When reactivities are converted to pseudo-free energy change restraints, DMS reactivity-informed modeling of both RNase P and other model RNA structures recovered both long- and short-range effects that fewer pairwise modiﬁcations are observed as nucleotides in these stable noncanonical interactions become unreactive. In sum, visualization of through-space folding networks (Figure 1B). Extensive changes in through-space structural communication occur as the RNase P RNA folds (Figure 3C, Supporting Figure 5). Time-resolved TMO probing revealed that nucleotides in the P2, P5, and P15 helices refolded relatively slowly upon Mg2+ addition, with nucleotides in the catalytic core showing even slower reactivity changes (Figure 3B). Conversely and intriguingly, loops L5.1 and L15.1 were relatively rapidly protected from TMO reaction, suggestive of a critical role for this element in the folding landscape. Global structural transitions of the RNase P catalytic domain have been monitored previously by their circular dichroism proﬁle. These studies revealed that the RNA folds through a, currently uncharacterized, metastable intermediate to form the equilibrium structure.17 Time-resolved TMO reactivity data suggested that P2, P5, the catalytic core, and the L5.1−L15.1 interaction might all contribute to formation of intermediates in the RNase P RNA folding pathway.

We next analyzed these same TMO data based on pairwise RING correlations (Figure 1A,B). Extensive changes in through-space structural communication occur as the RNase P RNA folds (Figure 3C, green lines and heatmap intensities). Interactions are relatively sparse in the absence of Mg2+, consistent with the role of Mg2+ in stabilizing tertiary RNA structures. Correlations linking P4 and P7, and P2 and P15.2 occurred in the absence of Mg2+ but disappeared in the ﬁnal structure, indicative of misfolding in the Mg2+-free structure. Correlations between the L5.1 and L15.1 loops appeared rapidly upon addition of Mg2+ and then decreased gradually as the RNA folded (Figure 3C, Supporting Figure 6). The decrease in correlation density in L5.1−L15.1 after 60 s likely reﬂects that fewer pairwise modiﬁcations are observed as nucleotides in these stable noncanonical interactions become unreactive. In sum, visualization of through-space folding (Figure 3C,D, Supporting Figure 6) reveals rapid formation of the tertiary L5.1−L15.1 loop−loop interaction followed by slower folding of the P2 pseudoknot and catalytic core.

Our study thus far suggested that the L5.1−L15.1 tertiary interaction guides native RNase P RNA folding. We made a
A concise two-nucleotide mutation that extends the P15.1 helix and disrupts noncanonical tertiary base pairing between L5.1 and L15.1 (Figure 4A). TMO probing showed that the native sequence and L15.1 mutant RNAs formed the same misfolded starting structure in the absence of Mg²⁺. Upon Mg²⁺ addition, TMO reactivities suggested that neither the L5.1–L15.1 tertiary interaction nor the P2–P4 pseudoknot formed fully in the mutant, as these nucleotides were more reactive than for the native sequence RNA (Figure 4B). RING correlations then directly showed that neither the L5.1–L15.1 interaction nor the P2 pseudoknot formed in the mutant (Figure 4C, Supporting Figure 7). Indeed, the final mutant structure resembles that observed for early folding stages of the native RNA. The L5.1–L15.1 loop–loop tertiary

Figure 2. Comparison of TMO and DMS reactivities. (A) Raw reactivity rates (top) for the RNase P catalytic domain RNA. RNA adducts were identified using MaP;8,10 median modifications per strand were 5–8. Loop (L) and junction (J) landmarks are emphasized; off-scale rates are labeled. Differential reactivity (bottom) occurs preferentially at sites of noncanonical base pairing (labeled nucleotides). (B) Reactivities superimposed on the RNase P secondary structure. Red, yellow, and black nucleotides indicate high, medium, and low reactivities, respectively. Dashes and circles connecting nucleotides show Watson–Crick and noncanonical base pairing, respectively. (C) Correlation between TMO and DMS reactivity. Points indicate individual nucleotides. Positions with preferential reactivity toward DMS (red) correspond to Hoogsteen-paired adenosine nucleotides and were excluded from R² calculation. (D) Chemical probing-directed secondary structure models for the RNase P catalytic domain based on TMO and DMS reactivities, modeled using the PAIR-MaP framework.10 Blue and green arcs indicate correctly predicted base pairs; gray arcs indicate missed base pairs relative to the accepted structure.
interaction thus coordinates RNA folding at both base-pairing and tertiary-structure levels.

RNA folding is broadly modeled as hierarchical, meaning that base-paired secondary structures form independently of and prior to higher-order tertiary structures. Within this framework, structural coupling is also observed such that disruption of a tertiary structure can destabilize secondary structure.

Figure 3. Time-resolved folding of the RNase P RNA. (A) Secondary structure. Regions undergoing folding transitions upon Mg\textsuperscript{2+} addition are emphasized in color (in all panels). (B) Time-dependent reactivity profiles. Individual per-nucleotide reactivities are shown as points. Red and black lines show best fits to averaged reactivities for nucleotides that form the L5.1−L15.1 interaction and for all other folding motifs. Rate constants are 0.030 and 0.011 s\textsuperscript{-1}, respectively. Representative unchanging region is shown with \( \times \) and gray line. (C) Pairwise through-space RING-MaP correlations as a function of folding time. Internucleotide correlations are shown with green lines superimposed on secondary structure; heatmaps of the same data are shown below each panel, with key regions boxed and labeled. (D) Three-dimensional structure of RNase P RNA,\textsuperscript{15} with superimposed RING correlations.
Single-molecule correlated probing directly visualizes time evolution of dozens of through-space interactions (Figures 3, 4; Supporting Figures 6, 7). Unanticipatedly, our study shows that RNase P folding is clearly not hierarchical. First, non-native interactions occur at early folding time points but then disappear, consistent with reorganization of both secondary and tertiary structures. Second, early formation of the long-range L5.1–L15.1 tertiary interaction drives native folding of both secondary and tertiary structures.

Time-resolved, correlated chemical probing experiments provide a powerful approach to characterize RNA folding landscapes. TMO has rapid, readily controlled, alkylation reaction kinetics and high reactivity rates, allowing for layering of single-molecule pairwise correlation data on per-nucleotide reactivities to reveal RNA folding intermediates and pathways directly. This study moves chemical probing beyond merely inferring dynamics of RNA structure formation, to enable simultaneous and direct measurement of multiple complex folding features for functionally important RNAs.

**REFERENCES**


