Probing the Structure of an RNA Tertiary Unfolding Transition State

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The functional properties of RNA are often linked to specific tertiary structures and conformational changes that they undergo.[1] Fully understanding RNA function therefore requires defining the factors governing RNA folding and stability. A complete description of folding requires knowing the structure and energetics of each conformational state for a given RNA, from the unfolded state to the native functional form. Although aspects of folding have been examined for some RNAs,[2] tertiary folding and unfolding transition states have yet to be investigated. Studying these transition states is important because it can help to pinpoint where on a pathway different regions of structure assemble (or denature).[3,4] Here we describe experiments designed to investigate the tertiary unfolding transition state of a tRNA. Our data are most consistent with early unfolding of the D/T-loop interface, possibly to reduce the high negative charge density in this region of tertiary structure.

Our approach is based on experiments by Clarke and Fersht who demonstrated that engineered disulfide cross-links bridging units of secondary structure that associate in the native state provide a means to probe protein folding and unfolding pathways.[4] Briefly, a disulfide bond linking structural regions that unfold prior to (or during) the rate-determining step (RDS), will destabilize the transition state and decrease the rate of unfolding.[4] Conversely, a disulfide bond tethering regions that unfold after the RDS should stabilize the transition and native states to similar extents leaving the unfolding rates unaffected.

Unmodified yeast tRNA[Phe] was selected for study because its structure is known[5] and thiol-modified tRNAs can be synthesized easily in high yield.[6] The first tRNA we studied possesses a cross-link between residues 1 and 72 at the terminus of the amino acid acceptor stem (site **I**; Figure 1). This is not a tertiary interaction and no effect on unfolding kinetics is expected. A second tRNA we examined contains a cross-link where the D-stem folds onto the variable loop, proximal to the base triple interactions, and it spans the 2′-hydroxyl groups of C11 and C25 (site **II**, Figure 1).

The last tRNA we investigated has a cross-link bridging the 2′-hydroxyl positions of U16 and C60, at the interface of the D- and T-loops (site **III**, Figure 1). Several conserved tertiary hydrogen bonds that stabilize the characteristic L-shape of tRNAs are present in this region of tertiary structure.

In solution, unmodified yeast tRNA[Phe] exists in a rapid Mg^{2+}-dependent equilibrium between secondary and tertiary structure.[7] Tertiary unfolding rates were measured below the melting temperature of the cloverleaf (generally between ca. 20–35 °C) by observing the hyperchromicity changes that occur upon addition of excess EDTA to folded tRNA solutions using a stopped-flow instrument as previously described.[7] Eyring plots were constructed to extract values of $\Delta G_{F-\ddagger}$.[2a,8]

Unfolding kinetics of $\mathbf{I_{XL}}$ and $\mathbf{I_{SH}}$ are monophasic and consistent with the expectation described above, values of $\Delta G_{F-\ddagger}$ for $\mathbf{I_{XL}}$ and $\mathbf{I_{SH}}$ are within error. $\mathbf{II_{XL}}$ and $\mathbf{II_{SH}}$ also exhibit monophasic kinetics and Eyring plots for these sequences provide indistinguishable values of $\Delta G_{F-\ddagger}$. These results suggest that the region of tertiary structure in the vicinity of C11 and C25 unfolds after the RDS. However, melting studies of $\mathbf{II_{XL}}$ and $\mathbf{II_{SH}}$,[6b] suggest that the disulfide bond in $\mathbf{II_{XL}}$ imparts a minimal stability increase to tertiary structure relative to the cloverleaf secondary structure. Hence, this cross-link is not useful to probe tertiary unfolding.

$\mathbf{III_{SH}}$ unfolds with monophasic kinetics and $\Delta G_{F-\ddagger}^{SH} = 13.30 \pm 0.05$ kcal/mol. In contrast, the unfolding kinetics of $\mathbf{III_{XL}}$ are biphasic in which the first phase is associated with ca. 90% of the hyperchromic change.[9] The amplitude of the first phase increases slightly with temperature, as expected for tertiary unfolding,[7] while the amplitude of the second phase does not. Furthermore, the amplitude of the first phase, measured as a function of temperature, is within error of predicted absorbance values from UV thermal denaturation curves measured in the presence and absence of Mg^{2+}.[6b] We propose that $\mathbf{III_{XL}}$ first unfolds to a cloverleaf which then undergoes a minor conformational rearrangement. This rearrangement may involve a torsional rotation about the disulfide bond (analogous to *cis/trans*-proline isomerization observed in protein folding).[3a] Such a rotation presumably would be accompanied by a slight change in base stacking which could produce a small absorbance increase, like that observed in the second phase of $\mathbf{III_{XL}}$ unfolding.

The $\Delta G_{F-\ddagger}$ obtained from analysis of an Eyring plot constructed using rates from the tertiary unfolding phase of the $\mathbf{III_{XL}}$ kinetic data is 14.10 ± 0.04 kcal/mol. The difference between the values of $\Delta G_{F-\ddagger}$ for reduced and oxidized site **III** ($\Delta\Delta G_{F-\ddagger}^{XL-SH}$) suggests that the unfolding transition state of $\mathbf{III_{XL}}$ is destabilized by 0.80 ± 0.06 kcal/mol relative to $\mathbf{III_{SH}}$. Further interpretation of this $\Delta\Delta G_{F-\ddagger}^{XL-SH}$ requires establishing the difference in free energy between the cloverleaf secondary structure and native tertiary structure, both reduced and oxidized ($\Delta\Delta G_{F-C}^{XL-SH} = \Delta G_{F-C}^{XL} - \Delta G_{F-C}^{SH}$). This value cannot be obtained through melting studies because the melting transitions of secondary and tertiary structure overlap.[6b] Therefore, thiol–disulfide exchange experiments[10] were conducted to measure $\Delta\Delta G_{F-C}^{XL-SH}$ for **III**. Equilibrium constants K_1 and K_2 can be obtained by equilibration of a thiol-modified tRNA in redox buffer, and as seen in Figure 2, $K_1/K_2 = K_3/K_4 = \Delta\Delta G_{F-C}^{XL-SH} = 0.87 \pm 0.1$ kcal/mol, the difference in free energy between the dithiol and disulfide forms of the tRNA tertiary and secondary structures.[10]

A difference free energy diagram for tertiary unfolding of **III** combining the kinetic and thiol–disulfide exchange data is shown in Figure 3. This analysis indicates the majority of the stabilization afforded by the disulfide bond (0.80 ± 0.06 kcal/mol) is lost before or during the tertiary unfolding transition state. The small amount of stabilization energy that remains may either result from some unfolding of the D/T-loop interface after the RDS or from
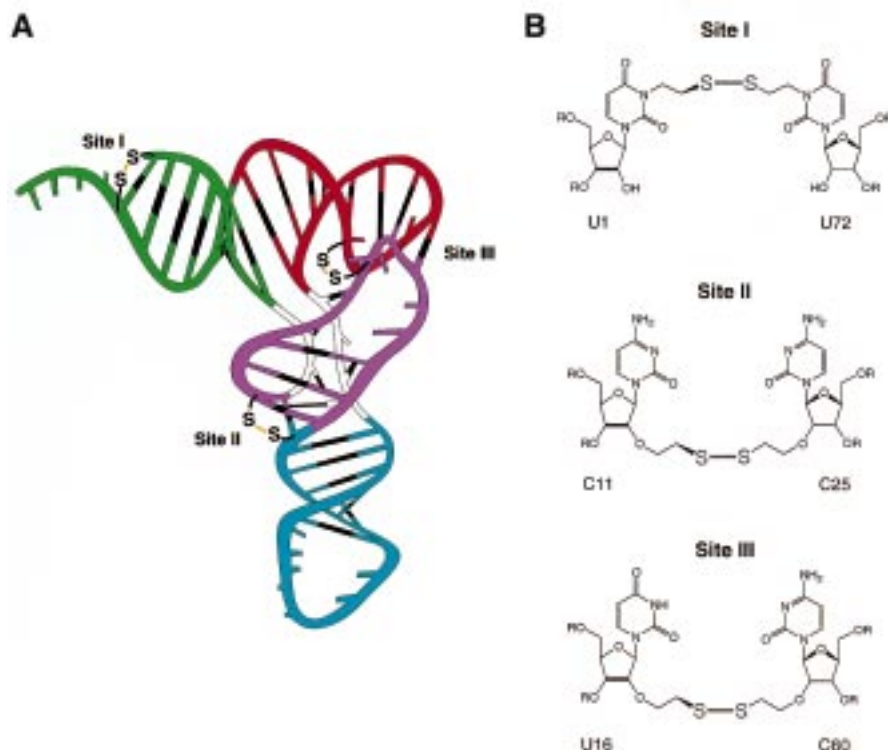
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(9) Unfolding of $\mathbf{III_{XL}}$ does not occur over the temperature range employed to study $\mathbf{III_{SH}}$. At the temperatures where $\mathbf{III_{XL}}$ begins to unfold, the rate of unfolding of $\mathbf{III_{SH}}$ is >1000 s^{-1}, and rates this fast cannot be measured in our stopped flow apparatus.[8]

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Figure 1. Model of yeast tRNA^{Phe} showing location (A) and (B) structures of cross-links. Footprinting experiments indicate that the alkylthiol linkers do not alter either the secondary or tertiary structure of these tRNAs.[6] Green, acceptor stem; purple, D-stem and loop; blue, anticodon stem and loop; red, T-stem and loop. Note that to form I_{XL}, G1 and C72 are replaced with N3-thioethyluridine.
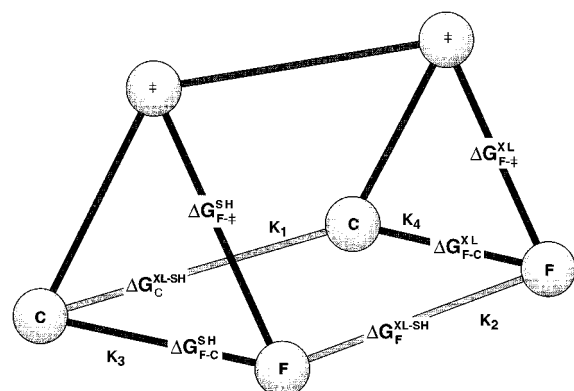

Figure 2. Thermodynamic cycle relating disulfide bond formation to folding. Superscript designates oxidation state of thiol pair, where XL is disulfide and SH is dithiol. Subscript designates conformational state of tRNA; F (native folded tertiary structure), ‡ (transition state), or C (cloverleaf secondary structure).

Figure 3. (Left) Difference free energy diagram for **III**. The stability of the disulfide cross-linked and corresponding dithiol molecule are compared to any state, A, using F as the reference.[3,4] (Right) Limiting case where regions tethered by a disulfide bond unfold prior to or during RDS (solid line, dithiol tRNA; dashed line, disulfide tRNA).

stabilization of the cloverleaf ground-state by the disulfide bond.[4] Given the magnitude of the data, it is difficult to distinguish between these possibilities.

Our data indicate that the D-loop/T-loop interface unfolds before or during the major RDS.[11,12] We propose that this region unfolds early to relieve unfavorable electrostatic interactions that arise when the D- and T-loops associate. This hypothesis is supported by two lines of evidence. First, within native tertiary structure, the phosphates on T-loop residues U59 and C60 have the highest electrostatic potential, and the C60 phosphate has the second lowest counterion accessibility.[13] Second, equilibrium studies of tertiary structure formation as a function of Mg^{2+} suggest that the D/T-loop interface in unmodified yeast tRNA^{Phe} forms when $[Mg^{2+}] \geq 3$ mM.[7] The relatively high $[Mg^{2+}]$ needed to fold this region of structure relative to other regions of the molecule is consistent with the high negative charge density that must be neutralized to allow close packing of the D- and T-loops.[7,14,15]

Because tRNA tertiary structure is conserved, it is possible that early unfolding of the D/T-loop junction is a common feature among tRNAs.[16] In addition, loop−loop interactions such as those at the D/T-loop junction occur in other RNAs.[1] It is therefore possible that comparable electrostatic effects occur in these systems.

Supporting Information Available: Procedures for the kinetic measurements and data analysis along with representative data and Eyring plots for III_{SH} and III_{XL} (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) The first tertiary interaction in native yeast tRNA^{Phe} that becomes accessible to carbodiimide in melting experiments is in the D/T-loop interface. Rhodes, D.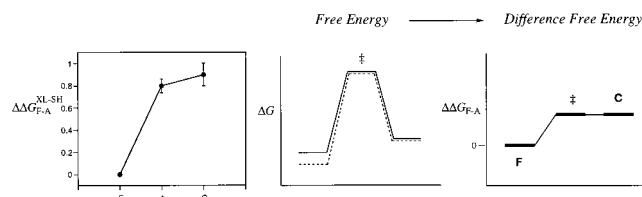 *J. Mol. Biol.* **1975**, *94*, 449−460.