Conformationally restricted nucleotides as a probe of structure–function relationships in RNA

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ABSTRACT

We introduce the use of commercially available locked nucleic acids (LNAs) as a functional probe in RNA. LNA nucleotides contain a covalent linkage that restricts the pseudorotation phase of the ribose to C3'-endo (A-form). Introduction of an LNA at a single site thus allows the role of ribose structure and dynamics in RNA function to be assessed. We apply LNA probing at multiple sites to analyze self-cleavage in the lead-dependent ribozyme (leadzyme), thermodynamic stability in the UUCG tetraloop, and the kinetics of recognition of U1A protein by U1 snRNA hairpin II. In the leadzyme, locking a single guanosine residue into the C3'-endo pucker increases the catalytic rate by a factor of 20, despite the fact that X-ray crystallographic and NMR structures of the leadzyme ground state reported a C2'-endo conformation at this site. These results strongly suggest that a conformational change at this position is critical for catalytic function. Functional insights obtained in all three systems demonstrate the highly general applicability of LNA probing in analysis of the role of ribose orientation in RNA structure, dynamics, and function.

Keywords: locked nucleic acid; lead-dependent ribozyme; RNA–protein recognition; sugar pucker; thermodynamics

INTRODUCTION

As with all biological macromolecules, the function of RNA is dependent on its three-dimensional structure. The conformation of a nucleotide residue within an RNA or DNA molecule is defined by the six backbone torsion angles α , β , γ , δ , ϵ , and ζ , the glycosidic torsion angle χ , and the furanose sugar pucker phase P and amplitude Φ (Saenger 1984). The pucker phase P varies between 0° and 360° and defines the nucleotide's position on the pseudorotation cycle for the five-membered ribose ring (Altona and Sundaralingam 1972). The sugar pucker phase is generally confined to two distinct regions of the 360° pseudorotation cycle, C3'-endo ("North"), roughly corresponding to a range of 0° to 36° and observed in A-form helices, and C2'-endo ("South") corresponding to a range of 144° to 180° and observed in B-form helices (Fig. 1; Altona and Sundaralingam 1972; Saenger 1984). Nonhelical regions

may contain nucleotides of either conformation or display an equilibrium between the two states.

Conformational flexibility in RNA is essential for processes including protein–RNA recognition and RNA catalysis (Perez-Canadillas and Varani 2001; Treiber and Williamson 2001; Fedor and Williamson 2005). However, it often proves difficult to probe directly the functional significance of a particular RNA conformational change or dynamic region. Our group has recently introduced a specific isotope-labeling scheme that allows the facile NMR analysis of dynamics within the ribose ring of RNA, which are hypothesized to most often represent C3'-endo/C2'-endo interconversions (Johnson et al. 2006). Preliminary application of this method to the lead-dependent ribozyme, or leadzyme, has shown a variety of dynamic features at ribose rings within the active site (M. Sumita, J.E. Johnson, and C.G. Hoogstraten, unpubl.; data not shown). Thus, we are interested in developing methods to assess the functional significance of C3'-endo/C2'-endo conformational changes. Historically, such probing has been attempted by taking advantage of conformational preferences of nucleotides with various substitutions at the 2'-carbon, but these efforts have been handicapped by the relatively modest magnitude of such preferences, representing a maximum of ~ 0.8

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FIGURE 1. Conformationally locked nucleotides. (*Top*) Edge-on views of C3'-endo and C2'-endo nucleic acid ribose pucker conformations. (*Bottom*) The introduction of a covalent linkage between C2' and C4' in locked nucleic acid (LNA) nucleotides. This linkage is geometrically incompatible with the C2'-endo pucker and thus locks the nucleotide into the C3'-endo conformation. *Upper panel*, structure redrawn from Altona and Sundaralingam (1972).

kcal/mol at 298 K for the most commonly used nucleotides (Uesugi et al. 1979; Guschlbauer and Jankowski 1980).

A recently introduced nucleotide modification of great interest for therapeutic and diagnostic applications of oligonucleotides is locked nucleic acid (LNA), which features a methylene linkage between the 2'-oxygen and 4'-carbon to completely restrict the nucleotide to the 3'-endo conformation (Fig. 1; Koshkin et al. 1998a,b; Obika et al. 1998). The properties and applications of LNAs and related molecules have been reviewed (Braasch and Corey 2001; Vester and Wengel 2004; Karkare and Bhatnagar 2006; Kaur et al. 2007). In brief, by significantly reducing the entropy cost of the transition from the single-stranded state to an A-form helix, even a small number of LNA nucleotides introduced into an oligomer can dramatically increase the stability of RNA double helices, DNA-RNA hybrids, and triple helices via preorganization effects (Kool 1997; Kierzek et al. 2005). Extensive applications of LNA-containing oligonucleotides to antisense, siRNA, and related technologies have been reported (Karkare and Bhatnagar 2006).

In this article, we report the novel application of LNAs as probes of functional relationships in folded RNA molecules. We measure the effects of individual substituted LNA nucleotides on catalytic function in the leadzyme, the folding thermodynamics of the stable UUCG tetraloop, and the kinetics of recognition by the spliceosomal U1A protein of its target sequence in the U1 small nuclear RNA (snRNA). The results give precise insight into site-specific conformational and energetic properties in the probed systems.

RESULTS

Conformational restriction and RNA catalysis

The small, catalytic lead-dependent ribozyme consists of an A-form RNA duplex with an asymmetric internal loop that contains the active site for self-cleavage (Fig. 2). Catalysis occurs in the presence of lead ions, which promote a two-

step cleavage reaction of the phosphodiester linkage between residues C6 and G7 (Pan and Uhlenbeck 1992; Pan et al. 1994). A unique feature of this ribozyme is its strong selectivity for divalent lead compared to all other metal ions tested. Thus, mechanistic analysis provides the opportunity for insight into the construction of a highly specific functional metal-ion site by RNA. Although the structure of the leadzyme has been analyzed by both NMR (Hoogstraten et al. 1998; Legault et al. 1998) and X-ray crystallography (Wedekind and McKay 1999, 2003), the resulting structures have neither displayed the in-line attack geometry necessary for self-cleavage nor provided a satisfactory explanation of the biochemical data for the system (Pan et al. 1994; Chartrand et al. 1997; Legault et al. 1998). These observations can be explained if the active-site internal loop of the leadzyme must undergo a transient fluctuation from the observed ground-state structure ES to an active conformation ES* prior to the chemical step of the reaction (Fig. 2, bottom). Thus, determining the features of the conformational change that creates ES* is a critical issue in delimiting the mechanism and metal specificity of this model ribozyme. In the current study, we analyzed the cleavage kinetics of three leadzyme constructs, each containing a

FIGURE 2. The lead-dependent ribozyme. (*Top*) Secondary structure of the leadzyme construct used in this work (LZ2), including the protonated noncanonical C6-A25 wobble base pair and the dynamic A8-G24 interaction. Sugar pucker and glycosidic angle parameters from X-ray (Wedekind and McKay 2003) and NMR (Hoogstraten et al. 1998) structures, along with the self-cleavage rates of 8BrG substitutions relative to wild type (k_{rel}) (Yajima et al. 2007), are indicated for nucleotides G7, G9, and G24. For G7, the crystallographic and NMR structures differ for both sugar pucker and glycosidic angle. For G9, the glycosidic angle takes on multiple conformations in the crystal structure due to packing constraints. The site of lead-dependent self-cleavage is indicated with an arrow. (*Bottom*) Sketch of the postulated transition from the ground-state, inactive form of the leadzyme (ES) to a low-population active conformation of unknown structure (ES*).

single LNA-G substitution at G7, G9, or G24. Previously reported measurements of 2'-deoxy variants at these positions yielded k_{rel} (2'-H) values varying from 0.20 for dG24 to 0.76 for dG9 (Table 1; Chartrand et al. 1997). Thus, the loss of the 2'-OH hydrogen bond donor at these positions leads to a modest to moderate loss of function, providing an important control for LNA substitutions.

A unimolecular leadzyme construct, termed LZ2, was used for all LNA kinetics experiments (Fig. 2). Representative time courses of cleavage at pH 7.0 and 50 μM Pb^{2+} are shown in Figure 3, and measured values of k_{obs} under these conditions are reported in Table 1. The self-cleavage rate of wild-type LZ2 was found to be $0.93 \pm 0.15 \text{ min}^{-1}$, in agreement with the initial results of the Uhlenbeck group (Pan and Uhlenbeck 1992; Pan et al. 1994). Cleavage rates in the wild-type leadzyme are generally seen to increase with increasing concentration of Pb^{2+} to a maximum in the neighborhood of 1 min^{-1} and then decrease as $[\text{Pb}^{2+}]$ is further increased due to the formation of lead polyhydroxide species at the pH used (Pan et al. 1994; Yajima et al. 2007). Consistent with these earlier observations, we find a maximum rate of leadzyme activity at $\sim 50 \mu\text{M}$ Pb^{2+} for all variants (Fig. 4). The decline in activity at $>50 \mu\text{M}$ Pb^{2+} precludes an analysis of saturation (Michaelis-Menten) kinetics, but k_{obs} values at 50 μM Pb^{2+} report well on the initial slopes of the k_{obs} versus $[\text{Pb}^{2+}]$ curves (i.e., $k_{cat} / K_M [\text{Pb}^{2+}]$) (Fig. 4, inset).

It is apparent from the data that conformational restriction at site 7 has only modest effects on catalysis, consistent with the varying conformations observed in structures for this nucleotide (Fig. 2; Table 1) and the relative insensitivity of this site to nucleotide base modifications (Pan et al. 1994; Chartrand et al. 1997; Yajima et al. 2007). By contrast, LNA modification at G24 induces a moderate loss of reactivity, decreasing self-cleavage by >15 -fold relative to wild type. This observation is unexpected given that a C3'-endo conformation is consistently observed at this position (22 of 25 calculated NMR structures and all crystallographic structures). Although the attenuation of activity is modest relative to the fivefold decrease upon 2'-deoxy substitution (Chartrand et al. 1997), this observation may suggest that the ability to escape the A-form ground state at this site, an ability suppressed by the LNA motif, is advantageous for catalysis. This possibility is strengthened by the observation that substitution at this position with 8-bromoguanosine (8BrG), which strongly disfavors the *anti* glycosidic conformation and concomitantly prefers a C2'-endo pucker, results in a fourfold enhancement of catalytic rate (Yajima et al. 2007). Taken together, these results suggest the existence of a conformational change at this position that favors self-cleavage.

TABLE 1. Kinetic data for LNA-modified leadzyme sequences at pH 7, 50 μM Pb^{2+}

	Pucker phase (crystal) ^a	Pucker phase (NMR) ^b	k_{rel} (2'-H) ^c	k_{obs} (LNA) (min^{-1})	k_{rel} (LNA) ^d
G7	C2'-endo	C3'-endo	0.50 ± 0.04	0.42 ± 0.07	0.45 ± 0.10
G9	C2'-endo	C2'-endo	0.76 ± 0.12	19.1 ± 1.7^e	20.4 ± 3.78^e
G24	C3'-endo	C3'-endo	0.20 ± 0.01	0.059 ± 0.003	0.063 ± 0.011

^aFrom Wedekind and McKay (2003), PDB codes 1NUJ and 1NUV.

^bFrom Hoogstraten et al. (1998), PDB code 1LDZ. Reported puckers are from direct J-coupling measurements for G7 and G9 and from calculated structures for G24.

^cReported by Chartrand et al. (1997); relative to $k_{obs}^{wt} = 0.74 \pm 0.04 \text{ min}^{-1}$.

^dThis work; relative to $k_{obs}^{wt} = 0.93 \pm 0.15 \text{ min}^{-1}$.

^eThese values should be considered as approximate lower bounds due to the limitations of manual pipetting experiments (see the text).

At site G9, NMR and crystallographic structures agree on a C2'-endo conformation in the ground state.¹ Nevertheless, restriction of G9 to the C3'-endo conformation using LNA modification leads to a dramatic increase in the measured rate of self-cleavage to 19.1 min^{-1} , an increase over the wild-type rate of more than 20-fold. (Due to the limitations of kinetic measurements using manual pipetting, this value is best considered as a lower bound on the rate of self-cleavage; our confidence in its accuracy, however, is increased by the linearity of the k_{obs} versus $[\text{Pb}^{2+}]$ curve for this variant between 10 μM and 50 μM Pb^{2+} ; Fig. 4, inset). To our knowledge, this value is the highest catalytic rate observed in any leadzyme variant under any conditions. The relevance of the observed reaction to the mechanism of the wild-type leadzyme was verified by the absence of measurable activity in 10 mM Mg^{2+} in the absence of Pb^{2+} (data not shown). The rate versus $[\text{Pb}^{2+}]$ plots in Figure 4 reemphasize the dramatic increase in cleavage rate for the LNA-G9 variant under all conditions tested. Significantly, this increase in rate was achieved by preventing the formation of the ground-state structure determined experimentally by two different techniques. This result implies that an inversion of the pseudorotation state of G9 from C2'-endo to C3'-endo is an energetically significant factor in the conformational change postulated to generate the active state of the leadzyme (Fig. 2). We propose that locking this ribose in the active (C3'-endo) conformation greatly increases the catalytic rate by substantially increasing the population of the active conformer. In combination with previous results (see Discussion), the LNA substitution analysis reported here strongly supports and further refines the model that a dramatic rearrangement of active-site structure in the leadzyme is necessary to generate a catalytically competent conformation.

¹It should be noted, however, that Boisbouvier et al. (2000), in an initial assessment of the potential for cross-correlated NMR relaxation techniques to determine sugar pucker conformation, found these parameters for G9 to cluster with those in C3'-endo nucleotides.

FIGURE 3. Reaction profiles for leadzyme self-cleavage. Progress curves for wild type (Fig. 2) and LNA-G variants in 15 mM MOPS (pH 7.0) and 50 μ M Pb^{2+} are shown. Data points were calculated as the average of two runs with error bars representing the difference between duplicate determinations averaged over a single time course.

Conformational restriction and tetraloop folding thermodynamics

The UUCG tetraloop (Fig. 5) is a well-studied RNA hairpin with unusually high stability that has been the subject of detailed calculational and thermodynamic studies (Groebe and Uhlenbeck 1988; Tuerk et al. 1988; Sakata et al. 1990; Antao and Tinoco 1992; Miller and Kollman 1997; Williams and Hall 1999, 2000; Williams et al. 2001; Ma et al. 2006). The well-defined molecular structure has been determined by both NMR (Cheong et al. 1990; Varani et al. 1991) and X-ray crystallography (Ennifar et al. 2000). In this tetraloop, U5 and G8 have C3'-endo sugar puckers and U6 and C7 are observed in C2'-endo conformations. Structural stabilization is provided by hydrogen bonding interactions between U5 (*anti*) and G8 (*syn*), as well as between C7 and the U6 phosphate group. C2'-endo nucleotides can span a longer backbone distance (Saenger 1984) and thus allow the formation of a loop structure from the two nucleotides spanning the U5–G8 base pair. Preorganization of the *syn* conformation at site G8 via 8BrG substitution has been observed to enhance thermodynamic stability relative to the unmodified hairpin (Proctor et al. 2004).

In order to investigate the thermodynamic importance of sugar conformation in the structured UUCG tetraloop, we introduced LNA nucleotides at the U6 and G8 positions, which are observed to have C2'-endo and C3'-endo conformations, respectively. Because the LNA–uridine phosphoramidite is not commercially available, LNA–thymidine was used for modification

at U6. Thymidine was used as a control for the effects of the loss of the 2'-hydroxyl. At site 8, 2'-O-methyl guanosine (Gm) was used as a parallel control for the LNA–guanosine modification.

UV melting curves of UUCG tetraloop analogs were performed at pH 7.0 in low salt buffer (10 mM Na^+) to favor the formation of the hairpin form over an RNA duplex. The two-state unfolding transitions occurred between 50°C and 85°C and were independent of RNA concentration between 5 μ M and 100 μ M, demonstrating unimolecular folding of the hairpin structure. Nondenaturing polyacrylamide gel electrophoresis did not reveal any evidence for duplex formation for any of the UUCG tetraloop analogs (data not shown). The curves were well fit by a two-state model in terms of melting temperature (T_m), ΔH° , ΔS° , and ΔG°_{37} . Thermodynamic parameters obtained from the resulting fits are listed in Table 2.

For nucleotide U6 (UUCG), the uridine base is exterior to the loop and has no important interactions with other groups (Cheong et al. 1990; Ennifar et al. 2000). At this site, the ΔG°_{37} values for UUCG (wild type) and UTCG are identical within error, consistent with the uridine to thymidine modification (including the deletion of the 2'-hydroxyl) having no significant effect on hairpin stability. Our data for UTCG are very similar to previously reported results for a deoxyuridine substitution at this position (Williams and Hall 1999; Williams et al. 2001). The 2'-hydroxyl at position 6 clearly plays no important role in the structure or stability of the tetraloop.

In contrast, an tetraloop with an LNA substitution at position 6 to yield U(LNA-T)CG is ~ 2 kcal/mol less stable than the UTCG analog at 37°C, with a decrease in T_m of almost 10°C. In structures of the wild-type tetraloop, this residue has a C2'-endo conformation, accounting for the

FIGURE 4. Metal dependence of leadzyme analog activity. Self-cleavage rates for LZ2 wild type and LNA substitutions as a function of added $[Pb^{2+}]$. Curves are cubic spline fits to guide the eye. (Inset) Expansion of the data with linear interpolation for $[Pb^{2+}]$ through 50 μ M. At this scale, the fit for LNA-G9 rapidly moves off the top of the plot.

FIGURE 5. The UUCG tetraloop. (*Left*) The secondary structure of the construct used in this work is shown, with nucleotides individually substituted with LNA drawn in bold and labeled with pucker phase and glycosidic conformation. (*Right*) X-ray crystallographic conformation, pdb code 1F7Y (Ennifar et al. 2000). Three-dimensional figure drawn with PyMOL (Delano 2002).

drastic decline in tetraloop stability when a C3'-endo conformation is enforced. Additionally, the enthalpy change decreases in magnitude from -44.1 kcal/mol (UTCG) to -32.0 kcal/mol (U[LNA-T]CG), suggesting that the base pair between U5 and G8 may be disrupted due to the rigid C3'-endo sugar pucker orientation of LNA-T6. This observation is consistent with an NMR study of a UUCG tetraloop with a modification at position 6 to 2'-deoxy-2'-fluoro-uridine, which prefers a C3'-endo conformation (Guschlbauer and Jankowski 1980). The 2'-fluoro substituted tetraloop displayed broadening and loss of intensity of the loop imino proton resonances, suggesting disruption of hydrogen bonding and a more dynamic loop structure (Williams et al. 2001). Thus, the results of LNA substitution at position 6 are consistent with the known structure and properties of the tetraloop and indicate that the sugar pucker for this nucleotide is a critical determinant of tetraloop stability.

Residue G8 has a C3'-endo sugar orientation and no close hydrogen-bonding contacts involving the 2'-hydroxyl group. Nevertheless, a deoxy substitution at this position showed a moderate decrease in loop stability ($\Delta T_m = -4.1^\circ\text{C}$, $\Delta\Delta G^\circ = +0.5$ kcal/mol at 55°C) (Williams et al. 2001). Ennifar et al. (2000) suggested that the thermodynamics at this position were dominated by a requirement for a C3'-endo ribose. Since a 2'-O-methyl nucleotide slightly favors the C3'-endo ribose, this model would predict a negligible effect or slightly increased stability for Gm at position 8, and a potential increase in stability for LNA-8 due to preorganization of the tetraloop structure. In contrast, the present data show a substantial decrease in loop stability upon 2'-O-methyl substitution that is still more severe in the LNA modification. These data suggest that, rather than the thermodynamics of G8 substitutions being driven by ribose

conformational preferences, the 2'-hydroxyl at position 8 plays a heretofore unrecognized role in the tetraloop structure. Interestingly, a molecular dynamics simulation study of the tetraloop showed the development of a hydrogen bond between the 2'-OH at G8 and the phosphate backbone (Miller and Kollman 1997). Therefore, the destabilizing effect of modified RNAs at the G8 position may in fact be attributable to the deletion of the 2'-OH hydrogen bond donor. In any case, our results at this site underline the importance of analyzing 2'-deoxy and/or 2'-O-methyl nucleotides in parallel with an LNA substitution to control for any effects of chemical modification at the 2' position that are independent of ribose conformation.

Conformational restriction and RNA-protein recognition

The interaction between the N-terminal RNA recognition motif (RRM) of the A protein of the U1 small ribonucleoprotein particle (U1A) and its RNA target in the U1 snRNA, U1 hairpin II (U1hpII), is one of the best-characterized RNA-protein interactions (Fig. 6). This very high affinity complex ($K_D \sim 30$ pM) has been examined structurally, biochemically, and kinetically (Scherly et al. 1989, 1990; Lutz-Freyermuth et al. 1990; Hall and Stump 1992; Hall 1994; Oubridge et al. 1994; Kranz and Hall 1998, 1999; Katsamba et al. 2001, 2002a; Law et al. 2005, 2006b; Showalter and Hall 2005). The basis for this tight interaction is an induced fit between regions of the protein and residues 1-7 of the unstructured 10-nucleotide (nt) loop, resulting in an intricate network of RNA-protein contacts (Hall 1994; Oubridge et al. 1994; Kranz and Hall 1998, 1999; Katsamba et al. 2001; Pitici et al. 2002; Law et al. 2006a; Kormos et al. 2007). A high-resolution structure of the U1A-U1hpII complex has been solved (Oubridge et al. 1994), but the structure of the U1hpII RNA alone has not been reported.

In this work, we used LNA substitution to probe the relevance of sugar pucker conformation to RNA-protein binding for two nucleotides, C5 and A6, within the U1hpII terminal loop (Fig. 6). In the co-crystal structure of the complex, residue C5 took on a C2'-endo conformation, whereas A6 was C3'-endo (Oubridge et al. 1994). We introduced LNA individually at C5 and A6 in the form of

TABLE 2. Thermodynamics of UUCG tetraloop RNA analogs

RNA	ΔH° (kcal/mol)	ΔS° (cal/mol·K)	ΔG°_{37} (kcal/mol)	ΔG°_{55} (kcal/mol)	T_m ($^\circ\text{C}$)
UUCG (wt)	-43.5 ± 2.7	-125.5 ± 7.9	-4.6 ± 0.3	-2.3 ± 0.2	73.4
UTCG	-44.1 ± 3.1	-127.1 ± 8.7	-4.7 ± 0.4	-2.4 ± 0.2	74.1
U(LNA-T)CG	-32.0 ± 2.8	-94.8 ± 8.6	-2.6 ± 0.2	-0.9 ± 0.1	64.5
UUCGm	-33.9 ± 2.4	-98.6 ± 7.3	-3.3 ± 0.2	-1.5 ± 0.1	70.3
UUC(LNA-G)	-41.6 ± 2.4	-123.3 ± 7.5	-3.3 ± 0.2	-1.1 ± 0.1	64.1

FIGURE 6. Structure of U1 snRNA hairpin II in complex with U1A protein. The secondary structure of the hairpin used for the SPR analysis is shown at *left*. Nucleotides U-5 to G15 are identical to the wild-type hairpin. The seven highly conserved nucleotides of the terminal loop, which are well structured in the complex, are outlined in red. The two nucleotides probed in this work are indicated along with their sugar pucker phases as observed in the cocrystal structure. (*Right*) The crystal structure of the protein-RNA complex. The coordinates are taken from Oubridge et al. (1994), pdb code 1URN, with the U1A protein in grayscale. In the numbering convention used, C5 and A6 are the fifth and sixth nucleotides of the 10-nt RNA terminal loop, corresponding to residues 10 and 11 in the numbering of Oubridge et al. (1994). Drawn with PyMOL (Delano 2002).

LNA- m^5C5 (LNA-5-methyl cytidine) and LNA-A6, respectively. To control for the effects of the loss of the 2'-OH functional group as well as of the additional methyl group on the cytosine base, we compared the results to m^5dC5 (5-methyl-deoxycytidine) and Am6 (2'-*O*-methyl adenosine) substituted species, respectively, as well as to the wild-type U1hpII (Katsamba et al. 2001). The effects of these substitutions on protein-RNA recognition were analyzed by surface plasmon resonance (SPR), which allows a simultaneous determination of on and off rates for the complex and the binding equilibrium constant K_D (Myszka 1999; Katsamba et al. 2001, 2002b).

We first examined the ability of the U1A N-terminal RRM to bind to wild-type U1hpII, m^5dC5 U1hpII, and LNA- m^5C5 U1hpII (Fig. 7; Table 3). Although the effect of the 5-methyl-dC substitution versus wild type was negligible and not statistically significant, locking the C5 ribose in the 3'-endo conformation was very unfavorable for binding, significantly reducing the association rate as well as increasing the dissociation rate, resulting in an overall loss in affinity of over three orders of magnitude. This result is consistent with the requirement for a C2'-endo ribose at position C5 to enable binding. Alternatively, the possibility

that the interference with binding arises from an introduced RNA-protein steric clash cannot be fully ruled out with the present data (see Discussion).

Next, we examined the effects on binding of Am6 and LNA-A6 substitutions. The 2'-*O*-methyl A6 showed no change in binding kinetics or affinity, indicating that the 2'-hydroxyl hydrogen bond donor at this position is unnecessary for protein recognition. Conformational restriction at position A6 also had very minor effects. LNA-A6 showed statistically significant but small changes in association and dissociation rates, resulting in a very modest (three-fold) loss in affinity. In other words, preorganization of this nucleotide into the bound conformation observed in the cocrystal structure does not improve the affinity or kinetics of RNA-protein recognition, despite the expectation that such preorganization might increase affinity by reducing the entropic penalty upon binding (Kool 1997). A formal possibility to explain this effect would be that C2'-endo and C3'-endo conformations at this position are equally compatible with protein binding, but the tightly structured nature of this region of the cocrystal structure would seem to render such a possibility unlikely. Instead, we propose that nucleotide A6 is already fully C3'-endo in the free form of the RNA hairpin. Since SPR data are acquired by using a prestructured hairpin, restriction to the C3'-endo conformation using LNA would be expected to be reflected solely in the folding of the isolated hairpin, rather than in the docking process. At this site, therefore, LNA substitution may be seen as a probe of the properties of the free form of the RNA by indicating a preorganization of the wild-type U1hpII hairpin into a C3'-endo conformation at A6 in the unbound state. The minor negative effects of substitution at this site may arise from effects of the LNA on the surrounding RNA structure or from the need for flexibility during the induced fit process.

DISCUSSION

In this work, we have investigated the utility of conformational restriction of nucleotide sugar pucker using LNAs as a probe of RNA structure and function. The three systems considered represent three distinct types of conformational change: the fluctuation of an inactive ground-state structure of the leadzyme to a low-population active conformation of unknown structure, the unfolding of a well-defined structure in the UUCG tetraloop, and a rearrangement of the U1 snRNA hairpin II from a free conformation of unknown structure and dynamics to a protein-bound form with a known structure. In each of these cases, conformational restriction has allowed a distinct insight into the structure and conformational dynamics of the respective system. At sites U6 of the UUCG tetraloop and C5 of U1hpII, both of which are C2'-endo in the available crystal structures, imposition of the C3'-endo conformation using LNA causes a drastic decrease in thermodynamic stability

FIGURE 7. Sensorgrams showing kinetic analyses of the U1A N-terminal RRM with U1hpII and mutant RNAs. Protein concentrations injected are as indicated. The scales across each row are consistent, except for the LNA- m^5C5 plot (*upper right*) for which the scale was adapted to better show the binding curves of this much weaker interaction. Two different wild-type RNA surfaces were used, to match the surface densities of the C5 and A6 coated RNAs (*top and bottom left*, respectively). The two wild-type surfaces showed no significant differences in k_a , k_d , or K_D , and the results from all six wild-type runs were averaged. Black lines represent triplicate injections performed in random order over an indicated RNA surface. A 3-min association was followed by a 5-min dissociation phase. Red lines represent the global fit of data sets using CLAMP (Myszka and Morton 1998). Kinetic parameters obtained from the experiments are given in Table 3.

and protein binding, respectively. These results validate the LNA-substitution method using these well-understood systems and also demonstrate the thermodynamic importance of the observed ribose conformations at these sites. At G7 of the leadzyme and A6 of U1hpII, by contrast, only minor effects are observed, implying that conformational changes at these sites are not critical for the formation of the active state of the leadzyme or for protein recognition by U1hpII. The surprising loss of thermodynamic stability upon LNA substitution at G8 of the UUCG tetraloop, despite the C3' structure at this site, may arise from a previously unrecognized structural interaction of the 2'-OH group, although this requires further investigation. If correct, this analysis suggests a reinterpretation of some previous results in the UUCG system. The results at this site also emphasize the importance of the use of appropriate controls for LNA experiments. Finally, the 20-fold increase in catalytic rate in the leadzyme upon LNA substitution at G9 provides a precise mapping of a conformational change

at this site that appears to be a key part of the generation of the active state of the ribozyme.

For the leadzyme, a pronounced discrepancy between determined structures and chemical and biochemical data has become evident, largely because structural studies, whether NMR or crystallographic, generally report on the most populated (ground-state) form of the molecule (ES). In contrast, the gain or loss of function upon mutation or base substitution reflects the structural requirements of the active conformation, ES* (cf. Fig. 2). Thus, an understanding of this ribozyme's catalytic mechanism and metal-ion specificity requires a knowledge of the features of the ES* state. The results in this article move this effort forward by demonstrating that the ribose ring of G9, and possibly also of G24, invert pseudorotation phase upon activation.

Using mutagenesis and base-substitution data as input for the modeling program MC-SYM (Major et al. 1991; Gautheret et al. 1993), Lemieux et al. (1998) derived a structural model for the leadzyme that, since it is based on

TABLE 3. Kinetic values for the interaction of U1A RRM1 with wild-type, 2'-modified, and LNA RNAs

RNA target	k_a ($M^{-1}sec^{-1}$)	Fold slower (wt/mut)	k_d (sec^{-1})	Fold faster (mut/wt)	K_D (M)	Fold weaker (mut/wt)
U1hpII wt	$(1.5 \pm 0.1) \times 10^7$	1	$(4.2 \pm 0.5) \times 10^{-4}$	1	$(2.9 \pm 0.4) \times 10^{-11}$	1
m^5dC5	$(1.45 \pm 0.07) \times 10^7$	1	$(7 \pm 2) \times 10^{-4}$	2	$(5 \pm 1) \times 10^{-11}$	2
LNA- m^5C5	$(1.1 \pm 0.5) \times 10^6$	13 ^a	$(5.8 \pm 0.2) \times 10^{-2}$	140 ^a	$(9 \pm 4) \times 10^{-8}$	3000
Am6	$(1.1 \pm 0.2) \times 10^7$	1	$(3.1 \pm 0.1) \times 10^{-4}$	1	$(3.1 \pm 0.6) \times 10^{-11}$	1
LNA-A6	$(7 \pm 1) \times 10^6$	2 ^a	$(5.8 \pm 0.2) \times 10^{-4}$	1.4 ^a	$(10 \pm 2) \times 10^{-11}$	3 ^a

^aBoth the C5 LNA and the A6 LNA showed statistically significant changes (t -test $P < 0.05$) in the association and dissociation rates compared to the wild-type RNA. In the case of C5 LNA, these changes were considerable, resulting in an over three orders of magnitude loss in affinity. In the case of A6 LNA these changes were statistically significant but minor, resulting in a threefold loss in affinity.

functional data, may well resemble the active conformation in many respects (Lemieux et al. coordinates are available at <http://www-lbit.iro.umontreal.ca/structures/leadzyme.pdb>). The potential relevance of this model is supported by its ability to correctly predict the results of three distinct 8BrG substitutions, a feat accomplished by neither the NMR nor crystallographic ground-state structures (Yajima et al. 2007). The MC-SYM model further contains a C3'-endo ribose at position G9, consistent with the LNA substitution results reported above. Correlations of NMR spin-relaxation studies with biochemical data have also allowed insight, suggesting that the A25–C6 protonated base pair observed in the NMR structure is broken in ES* (Hoogstraten et al. 2000), and crystallographic analysis under varying metal-ion conditions has begun to reveal the conformational requirements for in-line attack (Wedekind and McKay 1999, 2003). Nevertheless, additional probes are of great interest in order to further constrain the active conformation. Bevilacqua and coworkers took a major step in this direction by using 8BrG substitutions to perturb the glycosidic angle (Proctor et al. 2003, 2004; Yajima et al. 2007). In this work, we use LNA substitution in a similar fashion to probe the functional relevance of various pucker modes of the ribose moiety. The remarkable 20-fold increase in catalytic rate upon forcing G9 away from its observed ground-state conformation strongly supports both a C3'-endo conformation in ES* and an opposing C2'-endo structure in ES, consistent with both crystallographic and NMR J-coupling analyses. Interestingly, 8BrG substitution at G24 gave k_{obs} values of up to $\sim 9 \text{ min}^{-1}$ at pH 6 and elevated Pb^{2+} concentrations, which would extrapolate to the approximate k_{obs} of LNA-G9 at pH 7 assuming log-linear dependence of rate on pH. Whether this implies that the two substitutions shift the equilibrium between ES and ES* to the same extent or in the same structural manner is a matter for future investigation. We suggest that the incorporation of data including 8BrG and LNA substitutions could enhance the accuracy and relevance of computational models of the active state of the leadzyme, as well as providing novel insight into other and possibly less well understood ribozymes.

LNA nucleotides have previously been incorporated into helical regions and/or recognition arms of the 10–23 catalytic DNA and the hammerhead ribozyme in an attempt to improve the targeting of cellular and viral RNA sequences, resulting in sometimes dramatic effects upon structural stability and target affinity (Vester et al. 2002; Christiansen et al. 2007; Donini et al. 2007). The purpose of the LNA-ribozyme substitutions reported here is quite different, as we intend to probe the catalytic mechanism rather than to enhance hybridization affinity. Thus, the two techniques employing LNA substitutions with catalytic nucleic acids, namely, insertions within double-helical regions for biotechnology and related applications and substitutions within nonhelical regions as a mechanistic probe, are fully complementary.

With any mutation or modification, the primary limitation is the possibility of functional effects arising from features of the probe other than the intended perturbation. In the case of LNAs, this possibility arises either due to the loss of hydrogen-bonding groups on the 2'-hydroxyl or to the steric bulk of the bridging methylene group. A partial control for these effects is the parallel analysis of 2'-deoxy and/or 2'-O-methyl modifications, as demonstrated in this work by the case of G8 in the UUCG tetraloop. In cases for which either of these modifications is poorly tolerated, the usefulness of LNA probing is reduced. In the ideal case, it would be possible to perform a complementary experiment by locking the ribose into the C2'-endo conformation. Such data would allow a more unambiguous interpretation of loss-of-function effects from one or the other variant, as for site C5 of U1hpII in the present study. Nucleosides of various forms that accomplish this have been synthesized (Choi et al. 2003; Maier et al. 2004), but are not yet generally available.

To sum up, the ability to conformationally lock individual nucleotides is a useful addition to the arsenal of techniques for probing the nature and importance of structural and dynamic features in RNA systems. In this article, we have demonstrated insights in cases including ribozyme catalysis, folding thermodynamics, and RNA–protein recognition, including a 20-fold increase in leadzyme catalysis upon a single modification that provides a strong constraint on conformational aspects of the catalytic mechanism. We have used well-understood systems in order to validate the method by comparison to existing structures and other data, but we believe that the approach may also be very useful in newer systems with less existing information.

MATERIALS AND METHODS

Reagents and RNA synthesis

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich at the highest purity available. Urea and EDTA were from Invitrogen, acrylamide, bis-acrylamide, and yeast tRNA were from Roche, acetylated bovine serum albumin was from New England Biolabs, surfactant P20 was from GE Healthcare, and boric acid was from Bio-Rad. All LNA phosphoramidites used in RNA synthesis were from Glen Research.

The 30-nt lead-dependent ribozymes were chemically synthesized with a 6-fluorescein tag at the 5' end. Wild-type oligonucleotides from Dharmacon, Inc. were deprotected according to the manufacturer's protocol. LNA-leadzyme constructs synthesized by the W.M. Keck Foundation Biotechnology Resource laboratory at Yale University were deprotected using tetrabutylammonium fluoride (TBAF) solution in tetrahydrofuran (THF), 6 h at room temperature. UUCG tetraloop RNA analogs and 5'-biotin labeled U1 snRNA analogs were obtained from the W. M. Keck Facility and deprotected as above. Oligomer sequences, along with their shorthand representations, are as follows:

Leadzyme (LZ2)

wt	5'-Fl-GCGACCG ₇ AG ₉ CCAGCGAAAGUUGG G ₂₄ AGUCCG-3'
LNA-G7	5'-Fl-GCGACCG(LNA-G) ₇ AGCCAGCGAAAGU UGGGAGUCCG-3'
LNA-G9	5'-Fl-GCGACCGA(LNA-G) ₉ CCAGCGAAAGU UGGGAGUCCG-3'
LNA-G24	5'-Fl-GCGACCGAGCCAGCGAAAGUUG G(LNA-G) ₂₄ AGUCCG-3'

UUCG tetraloop

UUCG	5'-GGACUUCG ₈ GUCC-3'
UTCG	5'-GGACU ₆ CGGUCC-3'
U(LNA-T)CG	5'-GGACU(LNA-T) ₆ CGGUCC-3'
UUCGm	5'-GGACUUCGm ₈ GUCC-3'
UUC(LNA-G)	5'-GGACUUC(LNA-G) ₈ GUCC-3'

U1 snRNA hairpin II (U1hpII)

wt	5'-Bi-AGCUUAUCCAUUGC ₅ A ₆ CUC CGGAUAAGCU-3'
m ⁵ dC5	5'-Bi-AGCUUAUCCAUUG(m ⁵ dC) ₅ ACUCCGGAUAAGCU-3'
LNA-m ⁵ C5	5'-Bi-AGCUUAUCCAUUG(LNA-m ⁵ C) ₅ ACUCCGGAUAAGCU-3'
Am6	5'-Bi-AGCUUAUCCAUUGCAm ₆ CUCCGGAUAAGCU-3'
LNA-A6	5'-Bi-AGCUUAUCCAUUGC(LNA-A) ₆ CUCCGGAUAAGCU-3'

where LNA indicates a locked nucleic acid with a methylene linkage between 2'-O and 4'-C, Gm is 2'-O-methyl guanosine, Am is 2'-O-methyl adenosine, m⁵dC is 2'-deoxy-5-methyl cytidine, Fl is fluoroscein, and Bi is biotin. In some cases, the choice of the particular modification used was dictated by the current availability of the corresponding phosphoramidite. Deprotected RNAs were desalted on Sephadex G-25 (Sigma-Aldrich) and then dialyzed repeatedly against RNase-free, double-deionized water using Amicon Ultra 15 centrifugal filtration units. Following dialysis, RNAs were analyzed for purity on a 14.5% denaturing polyacrylamide analytical gel, lyophilized to dryness and stored at -20°C.

Leadzyme catalytic assays

RNA concentrations were determined using a single-stranded extinction coefficient of $2.95 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm for all variants. Cleavage reactions contained 5 μM RNA dialyzed against 15 mM MOPS (pH 7.0) and were performed at $27^\circ\text{C} \pm 1^\circ\text{C}$. The RNA was renatured by heating to 85°C for 2 min followed by cooling to 22°C and incubation at 27°C for 15 min. Cleavage reactions were initiated by addition of freshly prepared Pb(OAc)₂ at 10 μM , 25 μM , 50 μM , 100 μM , 250 μM , or 500 μM . For each time point, 35 μL aliquots were removed, quenched in an equal volume of 9 M urea/50 mM EDTA, and stored at -20°C. The unreacted substrate and product RNAs were separated on 14.5% denaturing polyacrylamide gels. Bands were detected with a FluorS MAX MultiImager with excitation at 494 nm and measurement of emission at 518 nm and analyzed with Quantity One software

(BioRad). Bands showing significant pixel saturation were removed from the analysis. Values for substrate and product bands were corrected for background and reported as percent of substrate remaining, $[S]/([S] + [P])$. Reactions proceeded to >90% completion in all cases. Time points representing the average of two runs were fit to a single exponential ($y_0 + A \cdot e^{-kx}$) using Igor Pro 5.04B to yield reported k_{obs} values. Error estimates for rate determinations were generated from the covariance matrices as reported by the fitting software.

Ultraviolet melting analysis

The absorbance versus temperature curves of UUCG tetraloop analogs were monitored on a Cary 300 Bio UV/Vis spectrophotometer equipped with an electrothermal temperature controller using 0.1- and 0.2-cm path length cuvettes (volumes of 350 and 700 μL , respectively). The buffer used for this experiment contained 10 mM sodium cacodylate and 0.5 mM EDTA (pH 7.0). The total RNA concentration of each sample was determined from the UV absorbance values (260 nm) at 95°C using single-stranded extinction coefficients of $111,600 \text{ M}^{-1} \text{ cm}^{-1}$ for UUCG, UUC (LNA-G), and UUCGm, and $94,800 \text{ M}^{-1} \text{ cm}^{-1}$ for UTCG and U(LNA-T)CG. These RNA extinction coefficients were calculated using the nearest neighbor algorithm (Cantor and Tinoco 1965) using an extinction coefficient of $12.2 \text{ M}^{-1} \text{ cm}^{-1} \times 10^{-3}$ at pH 7.0 for guanosine, LNA-G, and Gm and $8.7 \text{ M}^{-1} \text{ cm}^{-1} \times 10^{-3}$ at pH 7.0 for thymine and LNA-T (Gray et al. 1995). The melting data were collected from 20°C to 95°C at 280 nm with a heating rate of $0.5^\circ\text{C}/\text{min}$. The thermodynamic parameters were calculated by fitting to a standard two-state model using Meltwin 3.5 (McDowell and Turner 1996). Reported parameters are the average of results at six different RNA concentrations between 5 μM and 100 μM , each recorded in triplicate.

Surface plasmon resonance of RNA-U1A binding

Binding experiments were performed on a BIACORE 2000 instrument (GE Healthcare) as described with small modifications (Katsamba et al. 2002b; Law et al. 2006b). RNA was diluted to a final concentration of 0.1 μM in HBS buffer (10 mM HEPES at pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20) followed by heating at 80°C for 10 min and cooling to room temperature for 60 min to allow annealing of the stem. The sample was then diluted (50-fold for wild type, m⁵dC5, and LNA-m⁵C5 and 1000-fold for Am6 and LNA-A6) in $2\times$ running buffer to 10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 5% glycerol, 125 $\mu\text{g mL}^{-1}$ yeast tRNA, 62.5 $\mu\text{g mL}^{-1}$ acetylated bovine serum albumin, 1 mM dithiothreitol, 0.005% surfactant P20 and injected over a commercial streptavidin-coated sensor chip (SA chip, GE Healthcare) surface at $10 \mu\text{L min}^{-1}$ at 20°C . For the C5 experiment, three comparable density surfaces were generated on one chip (wild-type U1hpII, m⁵dC5, LNA-m⁵C5), with the fourth flow cell left blank to allow background signal subtraction. A separate chip was generated for the A6 analysis, containing wild-type U1hpII, Am6, LNA-A6, and an unmodified surface. RNA densities (including the wild-type reference channel) coated on the C5 chip were slightly higher than on the A6 surfaces to compensate for the weak binding of C5 LNA. Recombinant, C-terminally hexahistidine-tagged U1A protein (amino acids 1–101) (Law et al. 2006b)

was serially diluted in running buffer to the concentrations indicated in Figure 7 and injected at 20°C at a flow rate of 50 $\mu\text{L min}^{-1}$ for 3 min (association phase). Disruption of any complex that remained bound after a 5-min dissociation was achieved using a 1-min injection of 2 M NaCl at 20 $\mu\text{L min}^{-1}$. Samples with different concentrations of protein were injected in random order, and every injection was performed in triplicate within each experiment. All experiments were done at least three times. To subtract any background noise from each data set, all samples were also run over an unmodified sensor chip surface and random injections of running buffer were performed throughout every experiment ("double referencing") (Myszka 1999). Data were processed using Scrubber and analyzed using CLAMP XP (Myszka and Morton 1998) (developed by the Biomolecular Interaction Facility at the University of Utah, www.cores.utah.edu/interaction). The data were fit globally using a simple 1:1 Langmuir interaction model with a correction for mass transport (Myszka and Morton 1998). The results for all mutants were compared to the wild-type RNA using the Student's *t*-test. Equal or unequal variance of the samples was determined using the *F*-test. Equilibrium binding constants were calculated for each individual experiment, and these values were used to compute the averages and standard error of the means reported in Table 3.

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