Chemically Modified Hammerhead Ribozymes with Improved Catalytic Rates

Alex B. Burgin, Jr., Carolyn Gonzalez, Jasenka Matulic-Adamic, Alexander M. Karpeisky, Nassim Usman, James A. McSwiggen, and Leonid Beigelman*

Ribozyme Pharmaceuticals Inc., 2950 Wilderness Place, Boulder, Colorado 80301

Received May 28, 1996; Revised Manuscript Received August 9, 1996[⊗]

ABSTRACT: A site-specific chemical modification strategy has been employed to elucidate structure−function relationships at the only phylogenetically nonconserved position within the core of the hammerhead ribozyme (N7). Four different base substitutions at position 7 resulted in increased catalytic rates. A pyridin-4-one base substitution increased the rate of the chemical step up to 12-fold. These results are the first examples of chemical modifications within a catalytic RNA that enhance the rate of the chemical step. Four base substitutions resulted in decreased catalytic rates. The results do not correlate with proposed hydrogen bond interactions (Pley et al., 1994; Scott et al., 1995). This study demonstrates the utility of using unnatural nucleotide analogs—rather than mutagenesis with the four standard nucleotides alone—to elucidate structure−function relationships of small RNAs.

Ribozymes are RNA molecules with enzymatic activity that catalyze a variety of reactions including cleaving themselves or other RNA molecules (Cech, 1992; Symons, 1994). Because of their high sequence specificity and relatively small size (30−40 nt), *trans*-acting hammerhead ribozymes show promise as therapeutic agents to downregulate a given RNA species in the background of cellular RNA (Usman & Stinchcomb, 1996). Ribozyme activity is dependent on formation of a precise, three-dimensional folded structure between the ribozyme and its substrate RNA (Harris et al., 1994; Latham & Cech, 1989; Uhlenbeck, 1995). Despite a number of mutagenesis, phylogenetic, and chemical modification studies [see Usman et al. (1996) for review] and the availability of crystal structures for two hammerhead ribozyme−inhibitor complexes (Pley et al., 1994; Scott et al., 1995), the relationship between ribozyme structure and catalytic mechanism is not understood.

One of the most poorly understood positions within the ribozyme core is N7 (see Figure 1). Previous mutagenesis studies (Ruffner et al., 1990) have demonstrated that the four natural bases have relatively little effect on ribozyme activity when substituted at position 7. In addition, phylogenetic analyses demonstrated that position 7 is nonconserved; U is most common; however, natural hammerhead isolates containing A and C at this position have also been characterized (Forster & Symons, 1987). These results argue that no hydrogen bond donors or acceptors on the base at position 7 are critical for activity. Despite this argument, it has been proposed that two hydrogen bond contacts (see Figure 7A and Discussion) can form when uridine is present at position 7 (Pley et al., 1994; Scott et al., 1995). In addition, we have recently shown that an abasic substitution at position 7 results in a >50-fold decrease in activity (Beigelman et al., 1995a). This significant drop in activity may result from the removal of a critical hydrogen bond interaction and/or removal of some other function of the base itself.

To better define the structure−function relationships at position 7, we substituted the four natural nucleotides with eight different pyrimidine analogs and analyzed their effects on the ribozyme−substrate complex. We report that, unlike the four natural bases, the modified pyrimidine analogs have a profound effect on the catalytic rate. Four different modifications increase the catalytic rate of the hammerhead ribozyme. In one case (pyridin-4-one), the catalytic rate is increased 12-fold relative to an unmodified (and already extremely active) hammerhead ribozyme. This represents the first example of improving the rate of the chemical step, for a hammerhead ribozyme, through chemical modification. Four modifications decrease the catalytic rate. In one case (6-aza-U), the rate is decreased ∼20-fold. Our results do not support the hypothesis that the formation or disruption of any potential hydrogen bond interaction is important for activity. Rather, a preliminary analysis suggests that the base at position 7 may be modulating sugar pucker and that this feature may be important for ribozyme activity.

EXPERIMENTAL PROCEDURES

Preparation of RNAs. The synthesis of RNA containing the pyridin-4-one ribofuranoside (Matulic-Adamic et al., 1996a), *C*-(*p*-aniline) ribofuranoside (Matulic-Adamic & Beigelman, 1996), *N*-3-methyluridine (Zemlicka, 1970), *C*-phenyl ribofuranoside (Matulic-Adamic et al., 1996b), pyridin-2-one (Matulic-Adamic et al., 1996a), 6-methyluridine (Beigelman et al., 1995b), and 6-azauridine (Beigelman et al., 1995b) modifications has been previously described. Pseudouridine was purchased from Yamasa Corp. (Chiba-Ken, Japan) and converted to the standard phosphoramidite as previously described for this compound (Agris et al., 1995). Standard phosphoramidites of uridine (U), cytosine (C), guanosine (G), and adenosine (A) were purchased from Pharmacia Biotech AB.

The oligoribonucleotides (2.5 μmol scale) were synthesized on polystyrene supports (ABI) on an Applied Biosystems 394 DNA/RNA synthesizer. Synthesis, deprotection, and analysis of oligoribonucleotides were performed as previously described (Wincott et al., 1995). Ribozymes were purified on 15% polyacrylamide, TBE (89 mM Tris−borate,

* Corresponding author. Email: LNB@RPI.com. Phone: 303-449-6500. FAX: 303-449-6995.

[⊗] Abstract published in *Advance ACS Abstracts,* October 15, 1996.

S0006-2960(96)01264-0 CCC: $12.00

2 mM EDTA),[1] and 7 M urea gels. The full-length RNA was identified by UV shadowing of the gel, passively eluted into 10 mM Tris-HCl (pH 8) and 1 mM EDTA, and then concentrated by ethanol precipitation. Final ribozyme concentrations were determined by UV absorbance using an extinction coefficient of 350 mM^{-1} cm^{-1} at 260 nM.

To verify the incorporation of unaltered pyridin-4-one ribonucleoside, aniline ribonucleoside, *N*-3-methyluridine, and *C*-phenyl ribonucleoside into the ribozyme, aliquots of the purified ribozymes were analyzed by nucleoside compositional analysis (Beigelman et al., 1995c). The presence and integrity of pyridin-2-one ribonucleoside, 6-methyluridine, pseudouridine, and 6-azauridine were confirmed in several different ribozyme backgrounds that had been synthesized, deprotected, and purified identically to the ribozymes used in this study. The modified ribozymes were digested to ribonucleosides by incubation of 0.3 A_{260} units of oligonucleotide with 10 units of P1 nuclease (Boehringer Mannheim) and 2 units of calf intestinal alkaline phosphatase (Boehringer Mannheim) in 30 mM NaOAc and 1 mM $ZnSO_4$, at pH 5.2 (total volume = 100 μL) overnight at 50 °C. The digested material was then injected directly onto a C18 column (Waters, Symmetry, ODS 4.6 × 250 mm), and the ribonucleosides were separated by a gradient of buffers A (50 mM potassium phosphate, pH 7.0) and B (95% aqueous MeOH); 0−90% B in A over 35 min. The retention times were compared with monomer standards that were independently characterized by NMR.

Cleavage Assays. Ribozymes and 5′-^{32}P-end-labeled substrates were heated separately in reaction buffer to 95 °C for 1 min, quenched on ice, and then equilibrated to the final reaction temperature (37 °C) prior to starting the reactions. Reactions were carried out in enzyme excess and were initiated by mixing equal volumes (20 μL) of substrate (final concentration ≤1 nM) and ribozyme (final concentration indicated). Aliquots (4 μL) were removed at various times (5 s to 2 h), quenched in 8 μL of formamide loading buffer (95% formamide, 20 mM EDTA), and loaded onto 15% polyacrylamide, TBE (89 mM Tris−borate, 2 mM EDTA), and 7 M urea gels. Experiments presented in Figures 2 and 3 were performed in 50 mM MES, pH 6.5, and 10 mM $MgCl_2$. The pH of some reactions (Figure 5A) was varied by substituting different sulfonate buffers (MES, pH 5.5−7; HEPES, pH 7−8). Because of the different catalytic and binding properties of the ribozymes tested in Figure 4, different reaction conditions were required to ensure that the assay reflected changes in k_2; these conditions are indicated within the legend. The fraction of substrate and product present at each time point was determined by quantitation of scanned images using a Molecular Dynamics PhosphorImager. Ribozyme cleavage rates were obtained from plots of the fraction of substrate remaining *vs* time using a nonlinear, least-squares fit to a double exponential curve (KaleidaGraph, Synergy Software, Reading, PA). The initial, fast portion of the curve represented 80−90% of the total reaction; thus the observed cleavage rates (k_{obs}) were taken from the rate constant for the first exponential.

[1] Abbreviations: D_2O, deuterated water; E, ribozymes; EDTA, ethylenediaminetetraacetic acid, disodium dihydrate; ES, ribozyme−substrate-bound complex; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt; $J_{1'2'}$, proton spin coupling constant between H-1′ and H-2′; MES, 4-morpholineethanesulfonic acid, sodium salt; S, substrate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

Determination of Ribozyme−Substrate Binding Constant (K_D). Equilibrium disassociation constants (K_D) were determined using a modification of methods described previously (Pyle et al., 1990). Ribozymes at a final concentration of 0.001, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, 1, and 10 nM were annealed at 95 °C for 1 min with 0.3 nM 5′-^{32}P-end-labeled substrate, containing a single 2′-deoxynucleotide at the site of cleavage, in binding buffer (50 mM Tris−acetate, pH 6.5, 10 mM magnesium acetate, 5% sucrose). The Tris−acetate buffer is necessary to prevent loss of buffering capacity during the electrophoresis run. We have performed previous kinetics assays to demonstrate that replacement of the standard reaction buffer (50 mM MES, pH 6.5, 10 mM $MgCl_2$) with binding buffer has no effect on the rate of cleavage under both saturating and subsaturating conditions. Reactions (20 μL) were equilibrated at 37 °C for 15 min and immediately loaded onto a 12% acrylamide (19:1), 50 mM Tris−acetate (pH 6.5), and 10 mM $Mg(OAc)_2$ gel equilibrated at 37 °C running at 10 W constant power. The gel (19 × 28 × 0.5 cm) was run at constant temperature (37 °C), and the buffer was continuously recirculated to maintain constant pH for 6 h at 15 W. The gel was dried, and the fraction of substrate bound to ribozyme was determined by quantitation of scanned images using a Molecular Dynamics PhosphorImager. K_D values were obtained from plots of the fraction of substrate bound versus ribozyme concentration using a nonlinear, least-squares fit (KaleidaGraph, Synergy Software, Reading, PA) to the equation:

$$\text{fraction bound} = \frac{(K_D + R_0 + S_0) - \sqrt{(K_D + R_0 + S_0)^2 - 4R_0S_0}}{2S_0}$$

where R_0 and S_0 are the total ribozyme and substrate concentrations at each point. The full quadratic equation was used, in this case, because the total ribozyme concentration was not in excess over substrate concentration at all points on the curve. A simpler binding equation, fraction bound = $R_0/(K_D + R_0)$, is only valid when $R_0 \gg S_0$ and gave 3-fold higher estimates of K_D when used on the data in Figure 4.

RESULTS

Base Modifications at Position N7. To define the structural features critical for activity at position 7, we initially chose to substitute pyrimidine analogs at this position because these analogs retain the closest steric relationship to uracil, the most common base found at this position among natural isolates. (We have defined uracil as the wild-type or control base at position 7.) The three other standard bases (A, C, G), and eight base modifications, were tested at position 7 in the background of the all-RNA hammerhead ribozyme, RPI.1435 (see Figure 1). Figure 2 shows the cleavage rates for these modified ribozymes expressed as a fraction of the control ribozyme cleavage rate (the control rate was 0.52 min^{-1}). The rate of substrate cleavage was measured under single-turnover conditions, at a concentration (100 nM) that is ∼10-fold above the apparent K_M for the control ribozyme. Under these reaction conditions, the observed cleavage rate should approximate the pseudo-first-order rate constant for the conversion of enzyme-bound substrate into product (k_2,

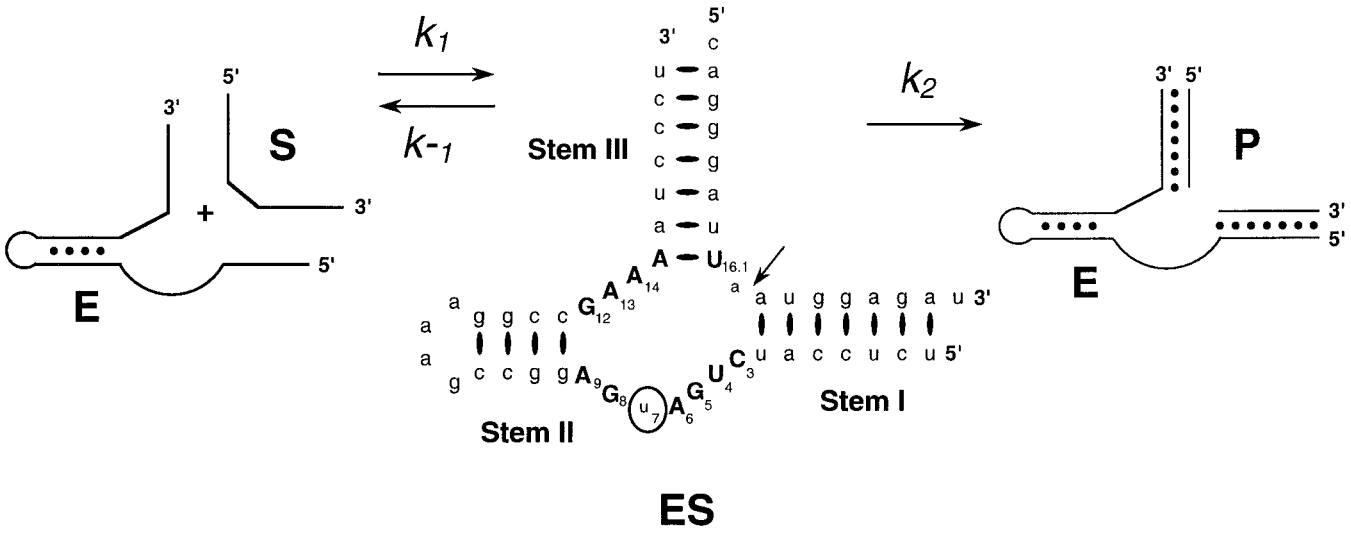

FIGURE 1: Simplified kinetic scheme of a ribozyme-catalyzed reaction. Free ribozyme (E) and substrate (S) are diagramed on the left, while ribozyme and cleaved product (P) are diagramed on the right. The center panel shows the ribozyme used in this study (RPI.1435) bound to its substrate (ES). Phylogenetically conserved bases are shown in bold. The nucleotide numbering scheme follows that proposed by Hertel et al. (1992). Position 7 is circled; the arrow indicates the site of ribozyme-catalyzed cleavage. k_1 represents the ribozyme−substrate association rate, k_{-1} represents the ribozyme−substrate dissociation rate, and k_2 represents the rate of chemical cleavage.

Figure 1). Reactions were also performed at 250 nM to verify that the reactions performed at a ribozyme concentration of 100 nM were near saturation; only small changes in rate were observed between these two reaction conditions (data not shown; except see Figure 3B below).

The modifications shown in Figure 2 have a profound effect on the observed cleavage rate. Four different base substitutions—*C*-phenyl, aniline, *N*-3-methyluracil, and pyridin-4-one—show reproducible increases in the rate of substrate cleavage relative to RPI.1435 (uracil at position 7, U7). A cytosine-substituted ribozyme was also slightly faster; however, this small increase was not consistently reproducible (data not shown). Two modifications show reductions (>10×) in ribozyme activity. At the extremes, the pyridin-4-one substitution (4P7) gave an ∼10-fold enhancement in rate under these conditions, whereas the 6-aza-U substitution gave a 17-fold decrease in rate.

All four standard nucleotides yield very similar cleavage rates when tested at position 7 (Figure 2). This result is in agreement with previous reports indicating that all four standard bases can be placed at position 7 without significant effects on cleavage rates (Ruffner et al., 1990) and that position 7 is phylogenetically nonconserved (Forster & Symons, 1987). The similarity in cleavage rates for the four standard bases makes even more surprising the wide range of cleavage rates seen for the modified nucleotides. Because of the significant increase in the rate of the pyridin-4-one substituted ribozyme, we have carried out a number of experiments to determine which step or steps in the kinetic pathway (Figure 1) are altered by this substitution.

Analysis of Ribozyme Containing Pyridin-4-one. Figure 3A shows the data used to derive the observed rate of cleavage (k_{obs}) for the wild-type (U7) and pyridin-4-one substituted (4P7) ribozymes in the single-turnover reactions described above (Figure 2). The fraction of substrate remaining uncleaved as a function of time was fit to a double exponential curve. k_{obs} values were derived from the slope of the first exponential. In theory, these data should follow a single exponential decay; however, ∼10−20% of the ribozyme−substrate complexes decay at a significantly slower rate. This slower population likely reflects ribozyme−substrate complexes in alternative (inactive) conformations. A major complication in the kinetic characterization of the ribozyme cleavage reaction is the propensity of the ribozyme and substrate to adopt multiple alternate conformations; however, the modifications presented in Figure 2 were kinetically homogeneous; the relative percentages of "fast" or "slow" components of the curve remained unchanged, only the rates of decay changed. The rates were always determined from the initial fast component of the curve fits.

To determine a more accurate value of k_2 (*i.e.*, k_{obs} at infinite ribozyme concentration) and to determine an apparent K_M for the reaction, the rate of substrate cleavage was determined as a function of ribozyme concentration for both U7 and 4P7 ribozymes. The results are presented in Figure 3B. The 4P7 ribozyme shows a 12-fold increase in k_2 relative to the U7 ribozyme (0.94 *vs* 12 min^{-1}). Measured rates of ∼10 min^{-1} are close to the upper limit that can be measured manually because of the very short time points required to generate reliable curve fits (5−15 s). However, when rate constants were determined at lower pH or magnesium concentrations (see Figure 5 below), the 4P7 ribozyme reproducibly provided an order of magnitude faster rate than the U7 ribozyme. These results provide confidence in the values presented in Figure 3B.

The results presented in Figure 3B also show an 8.5-fold increase in the apparent K_M for the 4P7 ribozyme (15 *vs* 130 nM). This increase in the apparent K_M does not necessarily reflect a decrease in the ability of ribozyme to bind substrate since K_M depends on the elemental rate constants for cleavage of substrate (k_2) as well as substrate binding (k_1) and dissociation (k_{-1}) according to the equation $K_M = (k_2 + k_{-1})/k_1$ (Fersht, 1985). It is therefore possible that the increase in K_M seen for the 4P7 ribozyme simply reflects the observed higher cleavage rate, rather than any changes in substrate affinity. To test this possibility, the equilibrium binding constant (K_D) between ribozyme and a noncleavable substrate analog was measured using a gel mobility shift assay. The results (Figure 4) demonstrate that

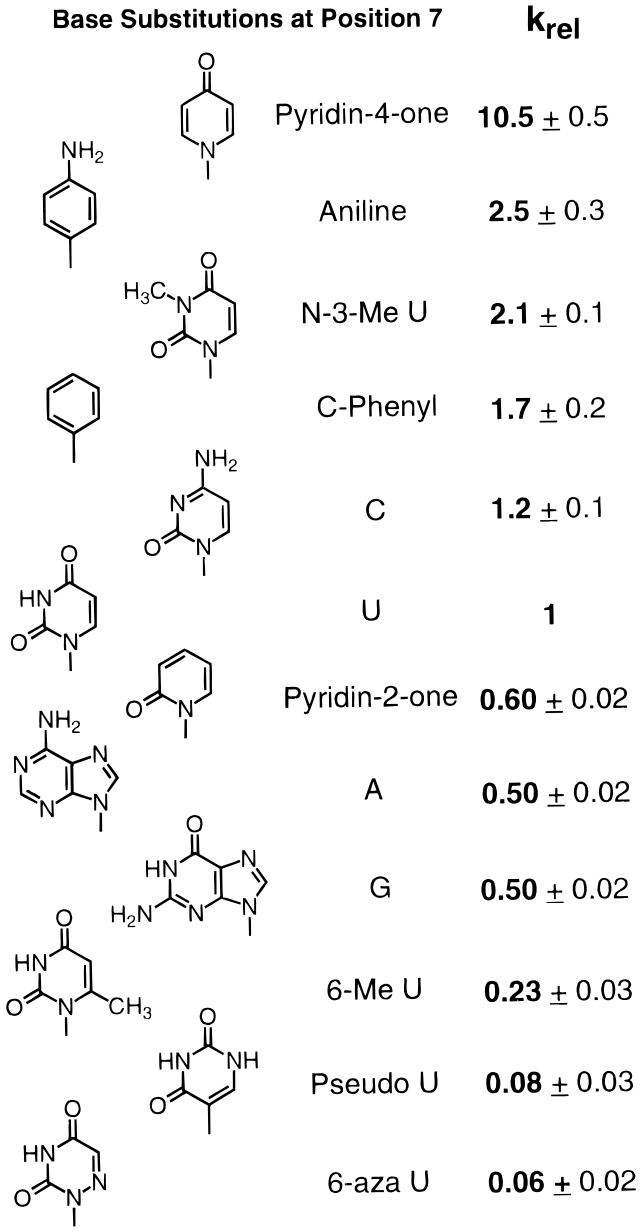

FIGURE 2: Ribozyme activity from position 7 base substitutions. The bases substituted at position 7 in ribozyme RPI.1435 are listed. The observed rates relative to control ribozyme (uracil at position 7) are indicated. The observed rate of cleavage (k_{obs}) under single-turnover conditions for the control ribozyme was 0.52 ± 0.02 min^{-1}. The errors indicate the standard error derived from the curve fit of the actual data.

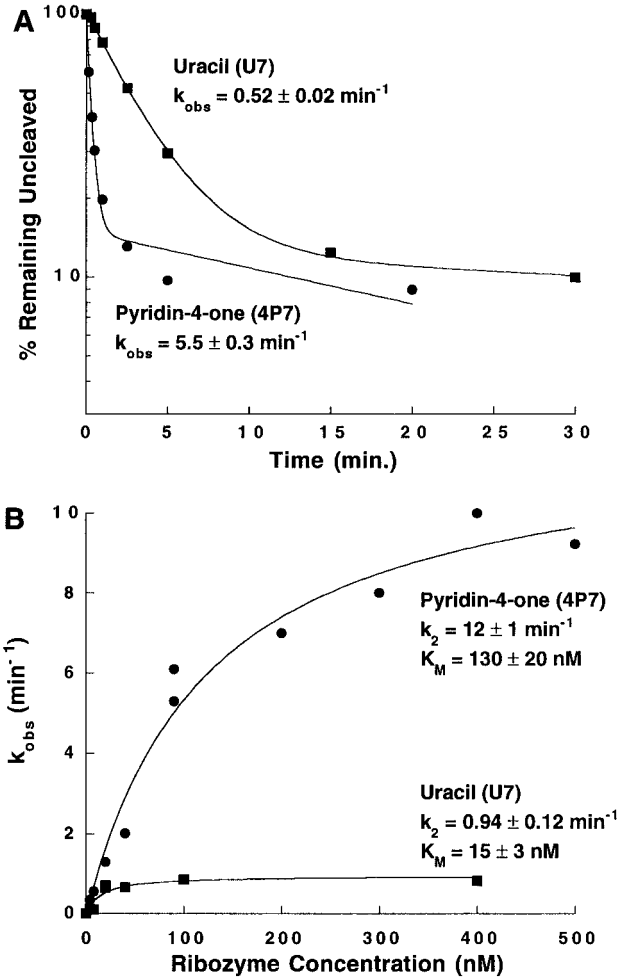

FIGURE 3: Single-turnover kinetics of the pyridin-4-one substituted ribozyme. (A) The fraction of substrate remaining uncleaved as a function of time is shown for ribozymes containing pyridin-4-one (circles) or uracil (squares) at position 7. The derived k_{obs} values for both ribozymes are indicated. (B) The rate of cleavage under single-turnover conditions as a function of ribozyme concentration is plotted for ribozymes containing pyridin-4-one (circles) or uracil (squares) at position 7. The derived k_2 and K_M values for both ribozymes are indicated.

K_D is unaltered by the 4P7 modification. The U7 and 4P7 ribozymes have nearly identical K_D values (0.07 and 0.05 nM, respectively) and are in close agreement with the values estimated from nearest neighbor free energy rules [$K_D = 0.1$ nM (Freier et al., 1986)]. These low values for K_D ($=k_{-1}/k_1$), and the 100−1000-fold higher values for K_M, demonstrate that the numerator in the K_M term is dominated by a cleavage rate that is much faster than substrate dissociation ($k_2 \gg k_{-1}$). Thus an increase in k_2, as seen in the 4P7 modification, would be expected to produce the observed large increase in K_M. In addition, pulse−chase experiments confirm that the rate of product dissociation is at least 10-fold slower than the U7 ribozyme cleavage rate (data not shown). Since $k_2 \gg k_{-1}$, the rate of substrate dissociation (k_{-1}) can be ignored in the K_M equation, and k_1 for both U7 and 4P7 ribozymes can be determined ($k_1 = k_2/K_M$). The values presented in Table 1 show that k_1 is unchanged, within experimental error, by the pyridin-4-one modification (62 *vs* 92 μM^{-1} min^{-1}). Finally, the value of k_{-1} for the U7 and 4P7 ribozymes can be determined since both K_D and k_1 values are available ($k_{-1} = K_D k_1$); k_{-1} is also unchanged by the 4P7 modification (0.3 s^{-1}).

The results presented in Figure 3B argue that the 4P7 substitution results in a greater than 10-fold increase in k_2. If the single-turnover reactions are reflecting changes in k_2, then the observed rate should show a log-linear dependence upon pH. Dahm et al. have previously demonstrated that the log of the rate of the chemical step (k_2) of hammerhead cleavage in Mg^{2+} increases linearly with pH (slope = 1) (Dahm et al., 1993). We therefore measured the rate of substrate cleavage for the U7 and 4P7 ribozymes as a function of pH. Figure 5A shows that, for both ribozymes, log(k_{obs}) is dependent upon pH with a slope of approximately 1 (0.91 and 0.86, respectively). In addition, it has been previously demonstrated that k_2 is dependent upon magnesium concentrations (Dahm et al., 1993), and as expected, the k_{obs} for both ribozymes shows the same, log-linear dependency upon magnesium concentration over the range

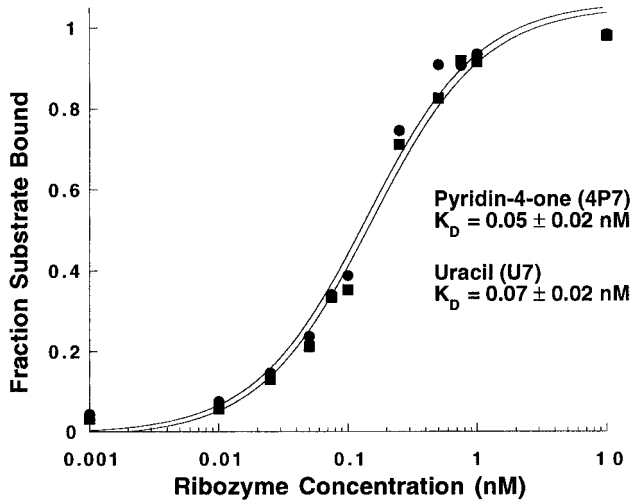

FIGURE 4: K_D determination of the pyridin-4-one substituted ribozyme. The fraction of noncleavable substrate bound to ribozyme as a function of ribozyme concentration is plotted for ribozymes containing pyridin-4-one (circles) or uracil (squares) at position 7. The substrate contains a single 2′-deoxy residue at the site of cleavage. The derived K_D values are indicated.

Table 1: Elemental Rate Constants for RPI.1435 (U7) and RPI.1435 Containing Pyridin-4-one Substitution at Position 7 (4P7)[a]

	U7	4P7
k_2 (min^{-1})	0.94 ± 0.12	12 ± 1
k_1 ($\mu M^{-1} min^{-1}$)	62 + 15	92 ± 16
k_{-1} (s^{-1})	0.3 ± 0.1	0.3 ± 0.1

[a]The k_2 values were obtained from Figure 3B. Values for k_1 and k_{-1} were calculated from the measured values for k_2, K_M and K_D as described in the text.

from 1 to 20 mM (Figure 5B). Taken together, these results provide confidence that the single-turnover reaction conditions are measuring the rate of the chemical step, k_2.

Effect of Pyridin-4-one in Multiple Ribozyme Backgrounds. Although all hammerhead ribozymes share a common "core" sequence (see Figure 1), the variable sequences in stems I, II, and III can influence activity. These "background" effects can be profound (Fedor & Uhlenbeck, 1990). It is therefore important to demonstrate that the rate enhancement supplied by the pyridin-4-one substitution is not unique to the RPI.1435 ribozyme background. We therefore synthesized two ribozymes (Figure 6) that have been extensively characterized, HH8 (Fedor & Uhlenbeck, 1992) and HH16 (Hertel et al., 1994), with and without the pyridin-4-one substitution at position 7, and measured the rate of cleavage of the complementary substrate under the previously described single-turnover conditions (Hertel et al., 1994). The results in Figure 6 show that in both cases the 4P7 modification confers enhanced activity over the corresponding U7 ribozyme. The observed rates of the unmodified ribozymes are similar to published values (Fedor & Uhlenbeck, 1992; Hertel et al., 1994). Two other, less well characterized, ribozymes also have been tested with the 4P7 modification, and they exhibit 2.5−10-fold enhancements in activity (data not shown).

In another test, the helical connections within the RPI.1435 ribozyme have been changed so that 5′- and 3′-ends of the ribozyme are located at the ends of stems I and II, respectively, instead of at the ends of stems I and III. In this format, the absolute rate of cleavage of the unsubstituted

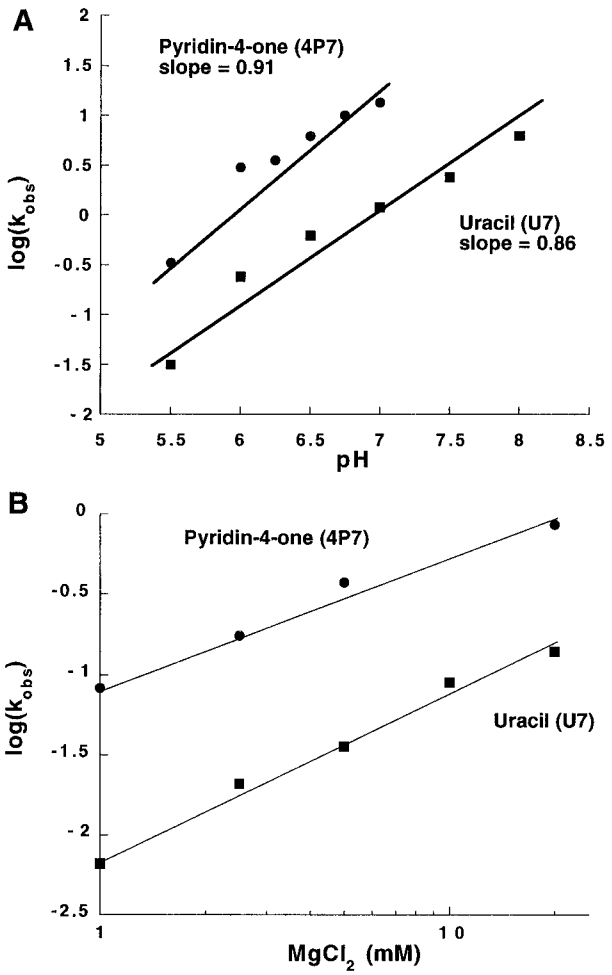

FIGURE 5: Rate of cleavage as a function of pH and magnesium concentrations. (A) The log of the observed rate of cleavage under single-turnover conditions (2.5 μM ribozyme, <1 nM substrate, 10 mM $MgCl_2$, 50 mM appropriate sulfonate buffer, 37 °C) as a function of pH is plotted for ribozymes containing pyridin-4-one (circles) or uracil (squares) at position 7. The slopes of the resulting lines are indicated. (B) The log of the observed rate of cleavage under single-turnover conditions (250 nM ribozyme, <1 nM substrate, 50 mM MES, pH 5.5, 37 °C) as a function of magnesium chloride concentration is plotted for ribozymes containing pyridin-4-one (circles) or uracil (squares) at position 7.

ribozyme is slower (0.1 *vs* 0.5 min^{-1}); however, the same ~10-fold rate enhancement is observed when pyridin-4-one is substituted at position 7. The results demonstrate that although the magnitude of the rate enhancement varies between the different ribozymes, the modification always results in a substantial, reproducible increase in the observed cleavage rate.

DISCUSSION

We have identified four modified bases, phenyl, *N*-3-methyluracil, aniline, and pyridin-4-one, that increase the catalytic activity when placed at position 7 in the hammerhead ribozyme (RPI.1435). To our knowledge, this is the first example of increasing the rate of the chemical step of a ribozyme by chemical modification. It has been previously demonstrated that it is possible to increase the rate of hammerhead catalysis by increasing the rate of ribozyme substrate association (10−20-fold enhancement; Herschlag et al., 1994; Tsuchihashi et al., 1993) or by increasing the rate of ribozyme product dissociation (10−

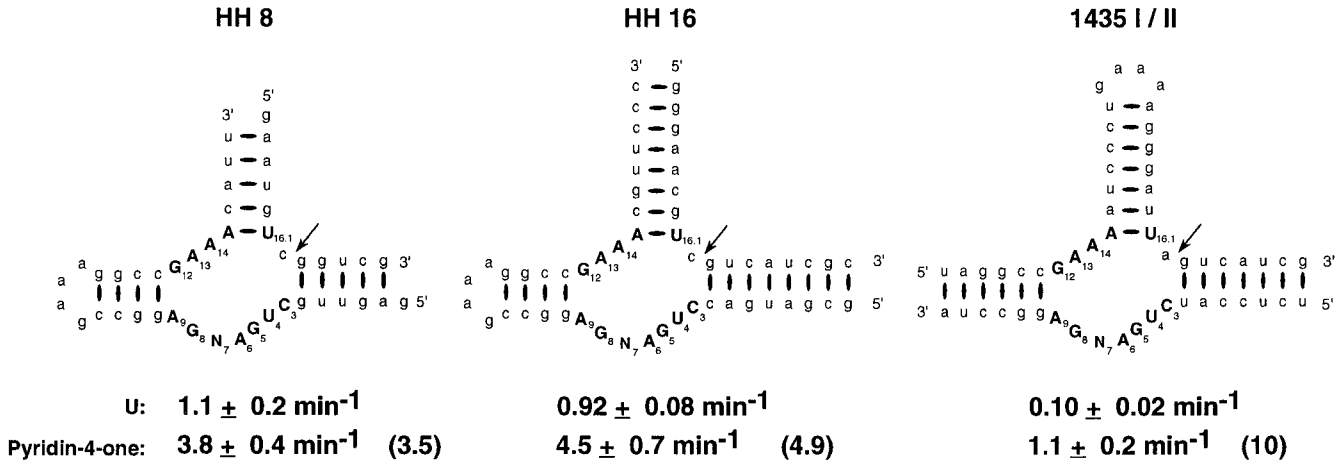

FIGURE 6: Effect of pyridin-4-one modification in multiple ribozyme backgrounds. Ribozyme and substrate sequences are indicated; capital letters indicate phylogenetically conserved positions (see Figure 1). The asterisk (*) indicates the sites of ribozyme-catalyzed cleavage. N indicates position 7; the rates (k_{obs}, min^{-1}) under single-turnover conditions when N is uracil or pyridin-4-one are indicated at the right. The relative enhancement due to the pyridin-4-one modification is indicated in parentheses. Reactions were performed under various conditions to ensure that the rate reflects the rate of the catalytic step (k_2). RPI.1435 I/II: 1 μM ribozyme, <1 nM substrate, 50 mM MES, pH 6.5, 10 mM $MgCl_2$, 37 °C. HH8: 1 μM ribozyme, <1 nM substrate, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 25 °C. HH16: 1 μM ribozyme, <1 nM substrate, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 25 °C. All reactions were performed at multiple ribozyme concentrations (0.5 and 5 μM) to ensure that the reactions were performed at saturating concentrations of ribozyme.

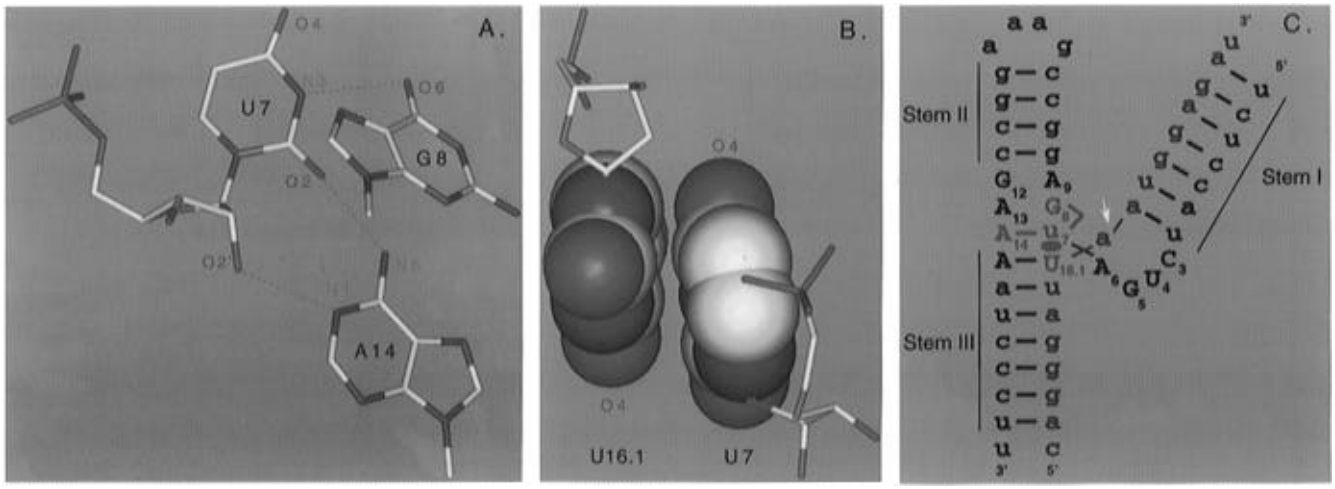

FIGURE 7: Structure−function relationships at position 7 based on available crystal structures (Pley et al., 1994; Scott et al., 1995). (A) A schematic diagram of the proposed hydrogen bond interactions between the (i) N3 hydrogen of U7 and O6 of position G8, (ii) N6 hydrogen of position A14 and O2 of position U7, and (iii) O2′ hydrogen of U7 and N1 of position A14. Carbon atoms are shown in yellow, oxygen in red, nitrogen in blue, and phosphorus in green. Individual hydrogen atoms are not diagramed. (B) CPK rendering of the bases (uracil) at positions 7 and 16.1, indicating the likelihood that these two bases stack upon one another within the crystal lattice. (C) A schematic diagram of RPI.1435 complexed with its substrate (see Figure 1) that shows the structural architecture of the ribozyme/substrate as seen in the crystal structure (Pley et al., 1994; Scott et al., 1995). The substrate RNA is shown in blue, with the exception that U16.1 is shown in red. The substrate phosphodiester cleaved is indicated by a yellow arrow. Position U7 and the three nucleotides that are proposed to interact with U7 (G8, A14, and U16.1) are shown in red.

100-fold enhancement; Shimayama et al., 1995; Hendry & McCall, 1995). We have demonstrated, however, that the increased activity of hammerhead ribozyme RPI.1435 substituted with pyridin-4-one does not result from changes in rates of substrate association (k_1) or dissociation (k_{-1}) but from a 12-fold enhancement in the rate of the chemical step (k_2).

Although it is clear that the base analogs listed in Figure 2 have both positive and negative effects on the rate of decay (k_2) of the ribozyme/substrate (ES) complex, it is not clear how these substitutions modify the structure of this complex. Previous mutagenesis studies (Ruffner et al., 1990) and the fact that position 7 is phylogenetically nonconserved (Forster & Symons, 1987) strongly argue that no particular functional group present on the base is important for activity. However, two different crystal structures of the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995) suggest the presence of a hydrogen bond to this nucleotide: the O2 atom of U7 and the N6 proton of A14, and the N3 proton of U7 and the O6 atom of G8 (see Figure 7A). Our data suggest that these proposed hydrogen bond interactions are not required for maintaining catalytic activity. First, if the presence of the 2-keto function at position 7 is important for activity, then one would not expect the pseudo-U substitution to result in a 13-fold decrease in activity and the pyridin-4-one or aniline modifications to result in increases in activity. Conversely, if the absence of the 2-keto function is important for activity, then one would not expect the *N*-3-methyl-U substitution to result in an increase in activity. Second, if the presence of the N3 proton is important for activity, then one would not expect the 6-methyluracil, pseudo-U, or 6-azauracil substitutions to result in decreased activity. In fact, the four "up"

modifications (pyridin-4-one, aniline, *N*-3-methyl-U, and phenyl) share the common feature of lacking a proton donor at position N3. However, the pyridin-2-one substitution also lacks a proton donor at position N3, but this modification results in a decrease in cleavage rate.

It is likely that the pyrimidine analogs are not just simply providing or removing potential hydrogen bond interactions but are affecting several interrelated structural features. On the basis of both three-dimensional crystal structure (Scott et al., 1995) and photo-cross-linking data (Laugaa et al., 1995), it has been proposed that the base at position 7 stacks upon uracil at position 16.1 (see Figure 7B). The pyrimidine analogs certainly change the stereoelectronic features of the base, and it is therefore reasonable to propose that the four modified bases that result in increased cleavage rates may provide more favorable stacking interactions with U16.1. Consistent with this notion, the reduced activity of the 6-aza-U and 6-Me-U substitutions may result from an alteration of the *syn*−*anti* conformation around the glycosidic bond (Beigelman et al., 1995b), which would be expected to interfere with stacking interactions. In addition, we and others have demonstrated that removing the base entirely (substituting with 1-deoxy-D-ribofuranose) reduces activity (Beigelman et al., 1994; Fu et al., 1994). However, the four natural bases also have very different stereoelectronic features, yet these bases do not affect activity.

In addition to modulating stacking ability and *syn*−*anti* conformation about the glycosidic bond, it is also known that base modifications can affect sugar pucker. As a preliminary analysis to test the possibility that the base analogs are modifying the sugar conformation at position 7, we measured the $J_{1'2'}$ proton spin coupling constant of the free nucleoside for each of the eight pyrimidine analogs, uridine, and cytidine. The $J_{1'2'}$ coupling constant is sensitive to geometry about the $C_{1'}-C_{2'}$ bond and is therefore a crude measurement of sugar pucker (Davies, 1978). A large $J_{1'2'}$ constant reflects a more "Southern" ($C_{2'}$-*endo*-$C_{3'}$-*exo*) conformation; a small $J_{1'2'}$ constant reflects a more "Northern" ($C_{3'}$-*endo*-$C_{2'}$-*exo*) conformation (Altona & Sundaralingam, 1972). When the $J_{1'2'}$ coupling constant of the free ribonucleoside was plotted as a function of ribozyme activity (k_{obs} under single turnover conditions), a correlation was observed (Figure 8). A larger $J_{1'2'}$ proton spin coupling constant of the free ribonucleoside correlates with faster rates of cleavage when the corresponding nucleotide was substituted at position 7 in the ribozyme. In addition to being sensitive to the geometry about the $C_{1'}-C_{2'}$ bond, the $J_{1'2'}$ constant is also sensitive to different elemental substituents present on $C_{1'}$ and $C_{2'}$. It is therefore necessary to analyze the C-nucleosides (carbon at position 1) independent of the N-nucleosides (nitrogen at position 1) (Davies, 1978; Thibaudeau et al., 1994). However, among both classes of molecules, the same correlation between $J_{1'2'}$ and activity is observed. Supporting the conclusion that sugar pucker is modulating activity at position 7, Heidenreich et al. observed a small increase in k_2 when 2′-amino-2′-deoxyuridine was present at position 7 instead of 2′-fluoro-2′-deoxyuridine (Heidenreich et al., 1994); one would expect the 2′-amino modification to increase the $J_{1'2'}$ coupling constant of the ribonucleoside relative to the 2′-fluoro modification (Guschlbauer & Jankowski, 1980).

The current diffraction patterns of the available ribozyme−inhibitor crystals do not provide the resolution needed to characterize the sugar conformation at position 7. However, inspection of the architecture of the ribozyme−inhibitor complex (see Figure 7C) does emphasize the central location of position 7 and suggests that sugar conformation could affect activity. Position 7 can be viewed as the "linker" between domain 2 and the proposed catalytic domain [nucleotides C3, U4, G5, and A6 (Scott et al., 1995)]. Sugar pucker changes the distance between adjacent phosphodiester bonds and the position of the base relative to the phosphodiester backbone. The sugar conformation at position 7 could therefore change the positioning of the catalytic domain relative to domain 2 and the cleavage site.

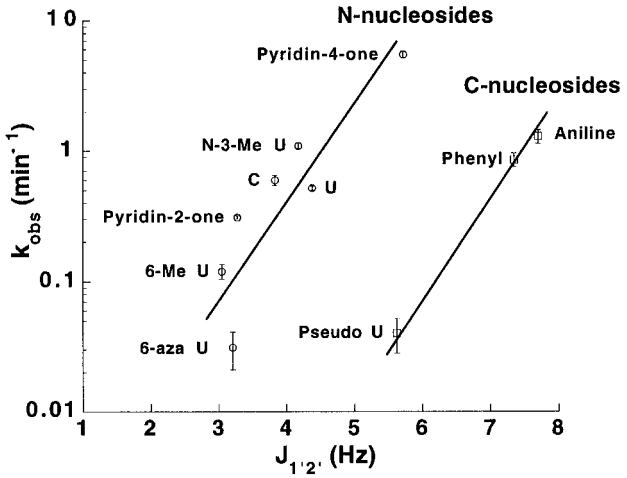

FIGURE 8: Correlation between the $J_{1'2'}$ proton spin coupling constant of the free ribonucleoside and activity when the corresponding ribonucelotide is present at position 7 in RPI.1435. The observed rates of cleavage (also presented in Figure 2) are plotted on a logarithmic scale. The $J_{1'2'}$ constant (expressed in hertz) of the free ribonucleoside was derived from ^{1}H NMR spectra, recorded on a Varian Gemini 400 spectrometer operating at 400.075 MHz. D_2O was used as a solvent; the internal standard was 3-(trimethylsilyl)propionic acid (sodium salt).

These preliminary results suggest that sugar pucker may be one factor modulating activity at position 7. We are in the process of attempting to separate and define the importance of stacking ability, *syn*−*anti* base conformation, and sugar pucker at this position by synthesizing and testing additional base and sugar modifications; however, it is important to note that many of these factors are interrelated. For example, changes in sugar pucker also change the orientation of the base relative to the sugar and phosphodiester backbone (Saenger, 1984). Changes in sugar pucker may therefore lead to a more favorable stacking conformation and thereby lead to increased ribozyme activity.

Whatever the underlying mechanisms, this study demonstrates the utility of using chemically modified base analogs, rather than simple mutagenesis, to elucidate structure−function relationships in small RNA molecules. The pyrimidine analogs used in this study do not introduce any novel

[2] An increase in k_2 could potentially lead to a decrease in substrate specificity. For example, if k_2 is fast relative to substrate dissociation (k_{-1}), every substrate molecule that binds will be cleaved. Naturally, the same scenario is true if the ribozyme binds a mismatched substrate. Therefore, to be an effective therapeutic, k_2 should be fast, but not as fast as k_{-1} for a mismatched substrate. Thus, it is possible that improved catalytic rates can only be achieved with a concomitant (and undesirable) reduction in target specificity. This seems unlikely since it is also possible to modulate k_{-1} by chemical modification and/or by shortening the lengths of the ribozyme binding arms.

functional groups, yet these analogs modulate activity over a 200-fold range. In contrast, the four standard bases showed less than 2-fold variations in activity. Clearly, the identity of the base at position 7 is an important determinant activity, despite the fact that this position is phylogenetically nonconserved. It has been proposed that one of the reasons why RNA catalysts are relatively uncommon and one of the driving forces in the transition from an "RNA world" to a "protein world" is that RNA lacks the diversity of functional groups needed to be an efficient catalyst (Orgel, 1986; Westheimer, 1986). Our results emphasize that RNA nucleotides not only lack diversity of functional groups but also lack structural diversity. For example, because the four standard nucleotides have a defined range of sugar conformations, limited stacking ability, or restricted *syn*−*anti* base conformation, they do not result in dramatic differences in catalytic rates when present individually at position 7. By comparison, the pyrimidine analogs used in this study do not introduce any unique functional groups that are likely to improve catalysis directly, yet they have significant effects on the catalytic rate, presumably because they can more easily adopt different conformations or provide improved interactions with other nucleotides. Within this framework, it is not surprising that many naturally occurring structural RNAs are highly modified posttranscriptionally. These arguments also suggest that both mutagenesis studies and *in vitro* selection of small RNAs could benefit from additional nucleotides that not only provide unique functional groups but also possess more structural diversity.

In conclusion, because of the increase in the rate of the chemical step (k_2), the pyridin-4-one substitution will likely be useful for improving the efficacy of exogenously delivered ribozyme therapeutics.[2] This chemical modification increases the rate of the chemical step but does not alter the affinity of the ribozyme for its substrate. In addition, the modification can be placed in different ribozyme backgrounds, making it applicable to a variety of different targets. Finally, our initial studies have shown that the pyridin-4-one substitution is resistant to nuclease degradation in human sera (data not shown), thus making the modification attractive for use in ribozyme stabilization as well as rate enhancement.

ACKNOWLEDGMENT

We thank Fran Wincott and Tony DiRenzo for oligonucleotide synthesis. We are indebted to Olke Uhlenbeck and Tom Cech for critical review of an early version of the manuscript.

REFERENCES

Agris, P. F., Malkiewics, A., Kraszewski, A., Everett, K., Nawrot, B., Sochacka, E., Jankowska, J., & Guenther, R. (1995) *Biochimie 77*, 125−134.

Altona, C., & Sundaralingam, M. (1972) *J. Am. Chem. Soc. 94*, 8205−8212.

Beigelman, Karpeisky, & Usman (1994) *Bioorg. Med. Chem. Lett. 4*, 1715−1720.

Beigelman, L., Karpeisky, A., Matulic-Adamic, J., Gonzalez, C., & Usman, N. (1995a) *Nucleosides Nucleotides 14*, 907−910.

Beigelman, L., Karpeisky, A., & Usman, N. (1995b) *Nucleosides Nucleotides 14*, 895−899.

Beigelman, L., McSwiggen, J. A., Draper, K. G., Gonzalez, C., Jensen, K., Karpeisky, A. M., Modak, A. S., Matulic-Adamic, J., DiRenzo, A. B., Haeberli, P., Sweedler, D., Tracz, D., Grimm, S., Wincott, F. E., Thackray, V. G., & Usman, N. (1995c) *J. Biol. Chem. 270*, 25702−25708.

Cech, T. R. (1992) *Curr. Opin. Struct. Biol. 2*, 605−609.

Dahm, S. C., Derrick, W. B., & Uhlenbeck, O. C. (1993) *Biochemistry 32*, 13040−13045.

Davies, D. B. (1978) in *Progress in NMR Spectroscopy*, pp 135−225, Pergamon Press Ltd., Great Britain.

Fedor, M. J., & Uhlenbeck, O. C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1668−1672.

Fedor, M. J., & Uhlenbeck, O. C. (1992) *Biochemistry 31*, 12042−12054.

Fersht, A. (1985) *Enzyme Structure and Mechanism*, 2nd ed., W. H. Freeman and Company, New York.

Forster, A. C., & Symons, R. H. (1987) *Cell 50*, 9−16.

Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T., & Turner, D. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 9373−9377.

Fu, D.-j., Benseler, F., & McLaughlin, L. W. (1994) *J. Am. Chem. Soc. 116*, 4591−4598.

Guschlbauer, W., & Jankowski, K. (1980) *Nucleic Acids Res. 8*, 1421−1433.

Harris, M. E., Nolan, J. M., Malhotra, A., Brown, J. W., Harvey, S. C., & Pace, N. R. (1994) *EMBO J. 13*, 3953−3963.

Heidenreich, O., Benseler, F., Fahrenholz, A., & Eckstein, F. (1994) *J. Biol. Chem. 269*, 2131−2138.

Hendry, P., & McCall, M. J. (1995) *Nucleic Acids Res. 23*, 3928−3936.

Herschlag, D., Khosla, M., Tsuchihashi, Z., & Karpel, R. L. (1994) *EMBO J. 13*, 2913−2924.

Hertel, K. J., Pardi, A., Uhlenbeck, O. C., Koizumi, M., Ohtsuka, E., Uesugi, S., Cedergren, R., Eckstein, F., Gerlach, W. L., Hodgson, R., & Symons, R. H. (1992) *Nucleic Acids Res. 20*, 252.

Hertel, K. J., Herschlag, D., & Uhlenbeck, O. C. (1994) *Biochemistry 33*, 3374−3385.

Latham, J. A., & Cech, T. R. (1989) *Science 245*, 276−282.

Laugaa, P., Woisard, A., Fourrey, J.-L., & Favre, A. (1995) *C. R. Acad. Sci., Paris 318*, 307−313.

Matulic-Adamic, J., & Beigelman, L. (1996) *Tetrahedron Lett.* (in press).

Matulic-Adamic, J., Gonzalez, C., Usman, N., & Beigelman, L. (1996a) *Bioorg. Med. Chem. Lett.*, 373−378.

Matulic-Adamic, J., Beigelman, L., Portmann, S., Egli, M., & Usman, N. (1996b) *J. Org. Chem. 61*, 3909−3911.

Orgel, L. E. (1986) *J. Theor. Biol. 123*, 127−149.

Pley, H. W., Flaherty, K. M., & McKay, D. B. (1994) *Nature 372*, 68−74.

Pyle, A. M., McSwiggen, J. A., & Cech, T. R. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8187−8191.

Ruffner, D., Stormo, G., & Uhlenbeck, O. (1990) *Biochemistry 29*, 10695−10702.

Saenger, W. (1984) *Principles of Nucleic Acid Structure*, 2nd ed., Springer-Verlag, New York.

Schimayama, T., Nishikawa, S., & Taira, K. (1995) *FEBS Lett. 368*, 304−306.

Scott, W. G., Finch, J. T., & Klug, A. (1995) *Cell 81*, 991−1002.

Symons, R. H. (1994) *Curr. Opin. Struct. Biol. 4*, 322−330.

Thibaudeau, C., Plavec, J., Watanabe, K. A., & Chattopadhyaya, J. (1994) *J. Chem. Soc., Chem. Commun.*, 537−540.

Tsuchihashi, Z., Khosla, M., & Herschlag, D. (1993) *Science 262*, 99−102.

Uhlenbeck, O. (1995) *Nat. Struct. Biol. 2*, 610−614.

Usman, N., & Stinchcomb, D. T. (1996) in *Nucleic Acids and Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Springer-Verlag, Berlin and Heidelberg (in press).

Usman, N., Beigelman, L., & McSwiggen, J. A. (1996) *Curr. Opin. Struct. Biol. 6* (4), 527−533.

Westheimer, F. H. (1986) *Nature 319*, 534−536.

Wincott, F., DiRenzo, A., Shaffer, C., Grimm, S., Tracz, D., Workman, C., Sweedler, D., Gonzalez, C., Scaringe, S., & Usman, N. (1995) *Nucleic Acids Res. 23*, 2677−2684.

Zemlicka, J. (1970) *Collect. Czech. Chem. Commun. 35*, 3572−3583.

BI961264U