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Caged RNA: photo-control of a ribozyme reaction

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ABSTRACT

We report here the first photo-chemical control of a ribozyme reaction by the site-specific modification of the 2′-hydroxyl nucleophile in the hammerhead system with a caging functionality. Rapid laser photolysis of the *O*-(2-nitrobenzyl) caging group initiates an efficient and accurate hammerhead-catalyzed cleavage of substrate RNA under native conditions. RNAs in which reactive functionalities or recognition elements are caged in this manner will be useful tools to probe RNA reactivity and dynamics.

INTRODUCTION

In addition to being a passive carrier of genetic information, RNA is involved in the regulation of both transcription and translation, and plays a key role in the splicing of pre-mRNA in eukaryotes (1). The study of many RNA systems is complicated by the dynamic nature of RNA secondary and tertiary structures, the transient nature of RNA·RNA or RNA·protein complexes, and in some cases the chemical reactivity of the RNA itself. One useful approach to the study of discrete RNA (and DNA) structures has been to limit available conformations through site-specific intra-strand cross-linking (2–6). We have developed a complementary approach which allows the isolation of specific RNA structures or complexes through the transient blocking or 'caging' of RNA functional groups involved in the transition between two different states. The caging of cofactors or reactive substrates with photo-labile groups has proven useful in a wide variety of investigations ranging from mechanistic enzymology to cell biology (7–9). In the context of an RNA molecule, a caging approach may be used to block either chemical reactivity of the RNA or formation of secondary or tertiary structure. The caged RNA system can be studied both before and after photolysis, thus permitting characterization of the two states and the transition between them. Here we report the first example of the caging of an RNA molecule, through the site-specific modification of a single 2′ hydroxyl functionality, and the demonstration of this caging in a well-defined system: the hammerhead ribozyme (10).

Our initial interest has been in blocking chemical reactivity associated with an RNA functionality and for this purpose we targeted the 2′ hydroxyl group, since specific RNA 2′ hydroxyls act as nucleophiles in a number of biologically important transesterifications (10–15). The hammerhead system was chosen for our preliminary studies because it has been the subject of intense investigation culminating in high resolution X-ray structures of both model and native hammerhead·substrate complexes (16–18).

The hammerhead ribozyme is a site-specific RNA endonuclease derived from self-cleaving plant viroid and satellite RNAs (10). The ribozyme·substrate complex contains three base-paired stems and a central core region of 13 conserved nucleotides which form the catalytic site (Fig. 1A). Cleavage of the substrate RNA (RNA **1** in Fig. 1A) is magnesium dependent and occurs through intramolecular attack of a 2′ hydroxyl on the adjacent phosphodiester (Fig. 1B). As part of efforts to understand the hammerhead system, many studies have involved the use of altered reaction conditions or mutant ribozymes or substrates. For example, since the ribozyme-mediated cleavage is dependent on the presence of a 2′ hydroxyl 5′ to the site of cleavage, replacement of this functionality with hydrogen or -OMe substituents has allowed the study of catalytically inactive ribozyme·substrate complexes (16,17). Modification of the this single 2′ hydroxyl of the hammerhead with a photo-dissociable group has allowed us to cage this reactive functionality, thus permitting photo-control of the hammerhead mediated reaction (Fig. 1C).

MATERIALS AND METHODS

Synthesis of 2′-*O*-(2-nitrobenzyl)adenosine phosphoramidite

Chemical reagents were purchased from Aldrich Chemical Company, Inc. Silica gel (0.03–0.07 mm) for flash chromatography was purchased from Acros Inc. Solvents were distilled as follows: tetrahydrofuran from Na/benzophenone; pyridine, dichloromethane and triethylamine from CaH_2. ^1H NMR spectra were collected on a Varian Gemini-200 instrument (200 MHz) and ^{31}P NMR on a Varian Gemini-300 instrument (300 MHz). All FAB-HRMS analyses were performed on a VG ZAB-SE machine (Medical Science Mass Spectrometry Facility, University of Toronto). HPLC was performed using a Waters 501 HPLC pump and an analytical C18 column (3.9 mm × 300 mm; Bondapak) with detection at 254 nm on a Waters 486 detector.

2′-*O*-(2-nitrobenzyl)adenosine (1)

Adenosine (1.0 g, 3.75 mmol; evaporated three times from dry pyridine) was dissolved in 34 ml of hot DMF. To this solution was added NaH (225 mg, 60% in oil, washed three times with hexanes) as a suspension in 4 ml of DMF. The resulting solution was stirred at 0°C for 45 min after which 2-nitrobenzylbromide (1.21 g, 5.6 mmol) in 2 ml of DMF was added. The reaction mixture was then stirred at room temperature under nitrogen for 5 h after which it was poured into 375 ml of ice-cold H_2O and stirred overnight. The resulting yellow precipitate was collected

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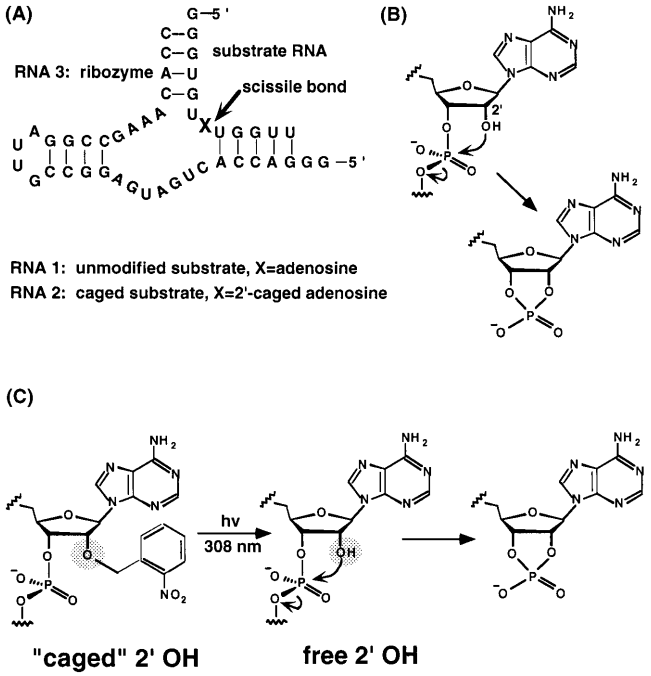

Figure 1. (**A**) Secondary structure of a canonical hammerhead ribozyme·substrate complex showing the scissile phosphodiester linkage. (**B**) Intramolecular transesterification catalyzed by the hammerhead ribozyme. (**C**) Photolysis of a 2′-caged hammerhead substrate to initiate the hammerhead cleavage reaction.

by vacuum filtration and concentrated *in vacuo*. The ^{1}H NMR (200 MHz) of the crude product was consistent with the literature (19). The yield was assumed to be 100% for the next step in the preparation.

2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyladenosine (2)

Trimethylsilylchloride (3.8 ml, 30 mmol) was added to a suspension of 2′-*O*-(2-nitrobenzyl)adenosine (1.5 g, 3.73 mmol) in 20 ml pyridine under nitrogen. The mixture was then stirred for 30 min at room temperature after which time benzoyl chloride (2.4 ml, 20.6 mmol) was added and the reaction stirred for another 2.5 h. The resulting mixture was then cooled to 0°C, H_2O (4 ml) was added, the reaction was stirred for 5 min, NH_4OH (33%, 8 ml) was added and stirring continued for an additional 30 min. The mixture was concentrated *in vacuo* and subjected to silica gel flash chromatography in 5% MeOH/CH_2Cl_2 (R_f = 0.3) to yield 647 mg of 2′-*O*-(2-nitrobenzoyl)-*N*^{6}-benzoyladenosine (43% from adenosine).

^{1}H NMR ($CDCl_3$, 200 MHz): δ(p.p.m.) 8.72 (s, 1H, H8), 8.12 (s, 1H, H2), 8.04 (d, 2H, NO_2-ArH), 7.85–7.82 (m, 1H, NO_2-ArH), 7.61–7.28 (m, 6H, ArH), 6.00 (d, 1H, 1′H), 5.09–4.91 (m 2H, methylene), 4.71 (s, 1H, 3′H), 4.65 (d, 1H, 2′H), 4.21 (s, 1H, 4′H), 3.87 (dd, 2H, 5′H). The 2′ position of the nitrobenzyl was confirmed by an NOED experiment.

FAB-HRMS: calculated for $C_{24}H_{22}N_6O_7$ (MH^+), 507.1628; observed, 507.1612.

5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyl adenosine (3)

2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyladenosine (455 mg, 1.13 mmol; evaporated three times from dry pyridine), DMAP (dimethylaminopyridine, 6 mg, 0.05 mol%) and DMTCl (para-dimethoxytrityl chloride; 355 mg, 1.1 mmol) were dissolved in 2 ml of dry pyridine. The reaction mixture was stirred at room temperature for 5 h under nitrogen. The resulting mixture was concentrated *in vacuo* and subjected to silica gel flash chromatography in neat EtOAc (R_f = 0.75) to yield 562 mg (74%) of 5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyladenosine.

^{1}H NMR($CDCl_3$, 200 MHz): δ(p.p.m.) 8.61 (s, 1H, H8), 8.16 (s, 1H, H2), 7.97 (d, 2H, NO_2-ArH), 7.83 (d, 1H, NO_2-ArH), 7.51–7.14 (m, 15H, ArH), 6.76 (m, 4H, DMT-ArH), 6.18 (d, 1H, 1′H), 5.05 (dd, 2H, methylene), 4.27 (d, 1H, 4′H), 3.71 (s, 6H, methoxy), 3.64–3.35 (m, 2H, 5′H).

FAB-HRMS: calculated for $C_{45}H_{40}N_6O_9$ (MH^+), 809.29347; observed, 809.2913.

5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyl adenosine-3′-*O*-(2-cyanoethyl-*N*,*N*-diisopropylamino) phosphoramidite (4)

5′-*O*-(4,4′-dimethoxytrityl)-2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyl-adenosine (520 mg, 0.6 mmol) was dissolved in dry distilled THF (3 ml) under nitrogen in flame-dried glassware. Then Et(iPr)$_2$N (0.71 ml, 4 mmol) and 2-cyanoethyl-*N*,*N*-diisopropylaminochloro-phosphite (0.29 ml, 1.3 mmol) were added and the reaction was allowed to proceed for 6 h. The resulting mixture was poured into EtOAc (200 ml), washed three times with 5% $NaHCO_3$ (100 ml), dried over $MgSO_4$, concentrated *in vacuo* and subjected to silica gel flash chromatography in 80% EtOAc/hexanes (R_f = 0.5–0.6, both diastereomers) to yield 622 mg (92%) of 5′-*O*-(4,4′-dimethoxy-trityl)-2′-*O*-(2-nitrobenzyl)-*N*^{6}-benzoyladenosine-3′-*O*-(2-cyano-ethyl-*N*,*N*-diisopropylamino) phosphoramidite.

^{1}H NMR ($CDCl_3$, 200 MHz): δ(p.p.m.) 9.55 (bs, 2H, NH_i, NH_{ii}), 8.61 (d, 2H, $H8_i$, $H8_{ii}$), 8.23 (d, 2H, NO_2-ArH_i, NO_2-ArH_{ii}), 7.94 (s, 2H, $2H_i$, $2H_{ii}$), 7.94 (d, 4H, NO_2-ArH_i, NO_2-ArH_{ii}), 7.64 (d, 2H, NO_2-ArH_i, NO_2-ArH_{ii}), 7.51–7.12 (m, 30H, ArH_i, ArH_{ii}), 6.80–6.74 (m, 8H, DMT-ArH_i, DMT-ArH_{ii}), 6.26 (m, 2H, $1'H_i$, $1'H_{ii}$), 5.26–4.90 [m, 4H, $methylene_{(i)}$, $methylene_{(ii)}$], 4.69 (m, 2H, $3'H_i$, $3'H_{ii}$), 4.46 (m, 2H, $2'H_i$, $2'H_{ii}$), 4.39 (m, 2H, $4'H_i$, $4'H_{ii}$), 4.09–3.99 (m, 4H, $5'H_i$, $5'H_{ii}$), 3.78–3.31 [m, 8H, ethylene, $cyanoethyl_{(i)}$, $cyanoethyl_{(ii)}$], 3.70 [d, 12H, $methoxy_{(i)}$, $methoxy_{(ii)}$], 2.49–2.33 [m, 4H, methine, $iPr_{(i)}$, $iPr_{(ii)}$], 1.29–0.91 [m, 24H, methyl, $iPr_{(i)}$, $iPr_{(ii)}$].

^{31}P NMR ($CDCl_3$, 300 MHz): δ, 151.27 p.p.m., δ, 150.94 p.p.m. (85% H_3PO_4 in H_2O as external standard).

FAB-HRMS: calculated for $C_{54}H_{57}N_8O_{10}P$ (MH^+), 1009.4013; observed, 1009.4019.

Photolysis of caged adenosine

Nucleoside model studies. A 200 µl solution of 2′-*O*-(2-nitrobenzyl)-adenosine (32 µM) in 20 mM Tris, pH 8.0 in a pyrex reaction vessel was irradiated with 100 pulses (308 nm; 300 mJ/pulse; full beam: 1 × 3 cm) from a Lambda Physik EMG 201 MSC excimer laser. The reaction mixture was directly injected onto a C-18 reverse-phase HPLC column (Waters) and was analyzed using an elution gradient from 0.05 M TEAA (triethylammonium acetate) to 1:1 0.1 M triethylammonium acetate/acetonitrile (16 min; ml/min). The chromatogram was monitored at 254 nm, peaks were identified by comparison with authentic standards, and conversion yields determined by integration of the peaks. Irradiation at 300 mJ/pulse

resulted in 100% conversion, while irradiation at 45 mJ/pulse resulted in 80–85% conversion.

Oligonucleotide synthesis and characterization. All oligonucleotide synthesis was carried out on an Applied Biosystems 392 DNA/RNA Synthesizer. DNA phosphoramidites and 2′-TBDMS protected RNA phosphoramidites and 500 Å controlled pore glass resin for the synthesis of DNA and unmodified RNA substrate **1** were from CPG Inc. The 2′-Fpmp RNA phosphoramidites and controlled pore glass resin used in the synthesis of caged RNA **2** were from Cruachem. Standard DNA and RNA synthesis cycles were used in all cases except that the standard 1 µmol RNA synthesis cycle was modified to give a coupling time of 15 min. All phosphoramidites were 0.1 M in acetonitrile. All synthetic oligonucleotides were purified by denaturing 20% (19:1) PAGE and stored in double distilled water at –20°C. Oligonucleotides were quantified based on UV absorption at 260 nm (Perkin-Elmer Lambda 2).

T7 transcription. The hammerhead ribozyme, **3**: 5′-GGGACCAC-UGAUGAGGCCGUUAGGCC GAAACACC-3′ was synthesized by *in vitro* transcription with T7 RNA polymerase from synthetic DNA templates. Transcription reactions (1 ml) contained 40 mM Tris, pH 7.5, 5 mM DTT, 1 µM spermidine, 3 mM nucleoside triphosphates (Pharmacia), 0.01% Triton X-100, 0.2 µM DNA template (from a template stock solution which contained 10 mM Tris, pH 7.5, 10 mM $MgCl_2$ and 2 µM DNA template containing the T7 promoter region), 2 U T7 RNA polymerase (Promega) and 20 mM $MgCl_2$. The transcriptions were performed at 37°C for 3 h after which time the reaction mixture was extracted with phenol/chloroform/isoamyl alcohol and chloroform and then ethanol precipitated. The crude product was then purified by denaturing 20% (19:1) PAGE.

Automated synthesis. The unmodified hammerhead substrate RNA **1:** 5′-GGGUGUAUGGUU-3′ was synthesized using phosphoramidites with 2′-*O*-*tert*-butyldimethylsilyl protecting groups on a 1 µmol scale. The RNA was cleaved from the resin and deprotected by treatment with saturated NH_3/MeOH (1 ml) at 55°C for 20 h. The supernatant was recovered and lyophilized to dryness. Deprotection of the 2′ hydroxyls was carried out by treatment with tetrabutylammonium fluoride (600 µl, 0.1 M in THF) at room temperature for 24 h. The oligonucleotide solution was added to 100 ml of 1 M triethylammonium bicarbonate, loaded onto a C-18 cartridge (Waters/Millipore), washed with 20 ml of 20 mM triethylammonium bicarbonate, eluted with 30% CH_3CN/0.1 M triethylammonium bicarbonate, and lyophilized to dryness. The resulting crude product was then purified by denaturing 20% (19:1) PAGE.

The modified hammerhead substrate RNA **2:** 5′-GGGUG-UA*****UGGUU-3′ (**A*** represents 2′ caged adenosine) was synthesized using the 2′-*O*-(2-nitrobenzyl)adenosine phosphoramidite and 2′-Fpmp phosphoramidites on a 1 µmol scale. The RNA was cleaved from the resin and deprotected by treatment with saturated NH_3/MeOH (1 ml) at 55°C for 20 h. The supernatant was recovered and lyophilized to dryness. The 2′-Fpmp groups were removed by treatment with 500 µl NaOAc (pH 3.25) for 40 h at room temperature after which the mixture was neutralized with 500 µl Tris buffer (3.15 M, pH 9). The mixture was ethanol precipitated, the residue lyophilized to dryness and the crude product purified by denaturing 20% (19:1) PAGE. The recovered RNA consisted of an 85:15 mixture of RNAs **2** and **1** and was subjected to C-18 reverse-phase HPLC to yield pure RNA **2** (gradient elution from 9:1 0.05 M triethylammonium acetate/acetonitrile to 7:3 0.05 M triethylammonium acetate/acetonitrile over 25 min; 1 ml/min). Yields of unmodified RNA **1** and modified RNA **2** were 20% as determined by UV absorption at 260 nm.

Nucleoside composition analysis. Enzymatic RNA digests (37°C, 8 h) of modified and unmodified RNAs were performed in 60 µl reactions containing RNA substrate (5 nmol), 0.2 mM $ZnCl_2$, 16 mM $MgCl_2$, 250 mM Tris, pH 6.0, 0.2 U snake venom phosphodiesterase (Pharmacia) and 4 U calf-intestinal alkaline phosphatase (Boehringer-Mannheim). Following digestion, samples were injected onto a reverse-phase C-18 HPLC column (Waters) with a gradient elution from 0.05 M triethylammonium acetate to 1:1 0.1 M triethylammonium acetate/acetonitrile (16 min; 1 ml/min). Peaks corresponding to U, G, A and the modified nucleoside 2′-*O*-(2-nitrobenzyl)adenosine were identified by co-injection of nucleoside standards with the chromatogram monitored at 254 nm.

RNA photolysis. RNAs (5 nmol) were photolyzed in a pyrex reaction vessel essentially as described above (308 nm; 140 pulses; 250 mJ/pulse), enzymatically digested, and analyzed by reverse-phase HPLC as described above. Hammerhead·substrate solutions were cooled to 0°C before flashing and were immediately transferred to a 30°C bath following photolysis.

Hammerhead ribozyme reactions

5′-End-labeling. Typical labeling reactions (20 µl) contained: 10 pmol RNA, 70 mM Tris pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 U T4 polynucleotide kinase (New England Biolabs) and 10 µl [γ-^{32}P]ATP (3000 Ci/mmol, 5 mCi/ml, NEN). Reactions were carried out at 37°C for 10 min, extracted with phenol/chloroform/isoamyl alcohol and chloroform, and then ethanol precipitated.

Catalytic ribozyme cleavage reaction. Excess substrate cleavage reactions contained 40 nM ribozyme **3**, 100 nM substrate (RNA **1** or **2**), 50 mM Tris pH 6.0 and 10 mM $MgCl_2$ (or 10 mM EDTA for control reactions) in a reaction volume of 20 µl. Ribozyme and substrate solutions in reaction buffer were heated to 90–95°C for 1 min and then allowed to cool to 22°C over 15 min after which the substrate solution was brought to 10 mM in $MgCl_2$ (or 10 mM in EDTA). The cleavage reaction was initiated by adding ribozyme to the substrate and then flashed, if required, as described above (308 nm; 140 pulses; 250 mJ/pulse). After 2 h, the reaction was stopped by the addition of 20 µl of stop solution (0.05% bromophenol blue, 0.05% xylene cyanole, 8 M urea and 100 mM EDTA) and heated to 90–95°C for 1 min before loading onto 20% (19:1) polyacrylamide denaturing sequencing gels for quantification. Gels were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software version 3.22.

Saturating ribozyme kinetics. Hammerhead cleavage reactions were performed under saturating ribozyme conditions at pH 7.5. The cleavage reactions contained 7500 nM ribozyme **3**, 10 nM substrate (RNA **1** or **2**), 50 mM Tris, pH 7.5, and 10 mM $MgCl_2$ in a total volume of 100 µl. Separate solutions of ribozyme and substrate were prepared, heated to 90–95°C for 1 min and allowed to cool over 5 min to 30°C. Then both solutions were brought to 10 mM in $MgCl_2$ and 50 mM in Tris, pH 7.5, and combined to initiate the reaction. Reactions containing the modified hammerhead substrate were photolyzed as described above (308 nm; 140 pulses; 250 mJ/pulse). Aliquots, from 0 to

60 min, taken from the reactions were combined with an equal volume of stop solution (0.05% bromophenol blue, 0.05% xylene cyanole, 8 M urea and 100 mM EDTA) and heated to 90–95°C for 1 min before loading onto 20% (19:1) polyacrylamide denaturing sequencing gels for quantification. Gels were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software version 3.22.

Measurement of equilibrium dissociation constants for ribozyme–RNA complexes. Equilibrium dissociation constants (K_d values) for ribozyme–RNA complexes were determined as reported elsewhere (20). Binding experiments were carried out under native conditions (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5% sucrose, 0.02% bromophenol blue, 0.02% xylene cyanole) comparing the caged RNA **2** with an RNA containing a single deoxy-adenosine residue 5′ to the cleavage site. Binding reactions were allowed to equilibrate for 15 h at room temperature and resolved on 15% native polyacrylamide gels (19:1; 13 × 22 × 0.15 cm) in 50 mM Tris acetate, pH 7.5/10 mM magnesium acetate buffer (pre-run for 2 h at 6 W followed by buffer exchange and then run at 6 W for 6 h at 4°C). Gels were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software version 3.22. K_d values were determined from Scatchard analysis of the binding data.

RESULTS AND DISCUSSION

We are interested in using caged RNAs as an approach to the study of dynamic or reactive RNA systems and chose to demonstrate the feasability of RNA caging by transiently blocking the reactivity of a single 2′ hydroxyl in the hammerhead ribozyme system. This is analagous to the approach of Noren and co-workers who recently caged a specific serine residue in *T.litoralis* Vent polymerase for studies on the mechanism of protein splicing (21). For our studies, we chose the 2′-nitro-benzyl group as the caging functionality because its photo-chemistry had been well characterized and because it can be removed, by irradiation at 308 nm, under conditions which will not damage nucleic acids or proteins.

The residue 5′ to the cleavage site in the hammerhead substrate can be a C, U or A residue. We chose to synthesize a 2′ caged adenosine for incorporation into a hammerhead substrate because of applications of this monomer to studies of Group II self-splicing introns and pre-mRNA splicing. We therefore synthesized the nucleoside 2′-nitro-benzyladenosine (19,22,23) and studied its reactivity under a variety of conditions. Upon photolysis at 308 nm with an excimer laser, the 2′-nitrobenzyladenosine was quickly, cleanly and quantitatively converted to adenosine as monitored by C-18 reverse-phase HPLC (data not shown) with the yield of uncaged adenosine independent of pH in the range of 6–8. We next checked the compatability of the 2′-nitrobenzyl functionality with standard RNA synthesis conditions. While the 2′-nitrobenzyl ether was stable to most conditions of RNA synthesis, it was partially removed by fluoride under the conditions used in deprotection of 2′-silyl protected RNAs (~20% cleavage observed). Thus the 2′-*tert*-butyldimethylsilyl (TBDMS) group commonly employed in RNA synthesis was incompatible with the introduction of the caged monomer into RNA. As an alternative, we decided to use the acid labile [1-(2-fluorophenyl)-4-methoxypiperidin-1-yl (Fpmp)] group as a 2′ protecting functionality (24). The 2′-Fpmp group is quantitatively removed upon treatment with mild aqueous acid over 30–40 h, conditions under which the nitrobenzyl ether is stable, with RNA synthesis yields comparable with those obtained using the 2′-TBDMS group. In order to incorporate the caged monomer into RNA, the 2′-modified adenosine was elaborated into a 5′-dimethoxytrityl-3′-phosphoramidite by standard procedures. This monomer was used, along with 2′-Fpmp phosphoramidites, in the solid phase synthesis of the RNA **2**: 5′-GGGUGU**A***UGGUU-3′ (a modified version of a well-characterized hammerhead substrate in which **A*** represents a caged nucleotide 5′ to the cleavage site (25). Deprotection of the crude product with methanolic ammonia followed by aqueous acid afforded an RNA in which a single residue was modified at the 2′ position with the desired caging functionality. The unmodified control oligonucleotide substrate **1**: 5′-GGGUGU-**A**UGGUU-3′ was synthesized by standard solid phase synthesis procedures and the ribozyme **3** was synthesized by T7 transcription from a synthetic DNA template (26). HPLC nucleoside composition analysis of the purified caged RNA, **2**, following enzymatic digestion with snake venom phosphodiesterase and calf intestinal alkaline phosphatase, showed the presence of a single nitrobenzyl modified adenosine residue. Upon photolysis of **2** at 308 nm, the caged adenosine was cleanly and quantitatively converted to adenosine with no changes in the composition of the oligonucleotide as monitored by denaturing PAGE and nucleoside composition analysis (Fig. 2).

As expected, incubation of the control hammerhead substrate **1** with catalytic amounts of ribozyme **3** led to site-specific cleavage of **1** (Fig. 3, lane 2) with kinetics similar to those reported for related substrates (25). Under identical conditions, the caged RNA substrate **2** was not cleaved (Fig. 3, lane 5). Following photolysis, however, the uncaged RNA was site-specifically cleaved in a magnesium-dependent reaction to the same extent as the unmodified substrate **1** (Fig. 3, lane 6; typically, 70–80% cleavage of either unmodified or uncaged substrate was observed).

In order to more fully characterize the caged RNA substrate **2**, we compared it with the unmodified RNA substrate **1** in the presence of saturating ribozyme. Under these conditions, following photolysis, the uncaged substrate was cleaved as quickly as the unmodified RNA substrate (k_{obs} = 0.20 ± 0.02/min for RNA **1** and 0.22 ± 0.02/min for RNA **2**; Fig. 4).

We were interested in measuring the effect, if any, of the caging functionality on the stability of the ribozyme–substrate complex. We therefore performed equilibrium binding studies measuring the dissociation constants for interaction of the ribozyme **3** with the caged RNA **2** and a modified version of RNA **1** containing 2′-deoxy-adenosine 5′ to the cleavage site (20). The K_ds for both of the ribozyme complexes were measured to be 220 nM, suggesting that the caging functionality does not appreciably disrupt the ribozyme–substrate complex.

Together, these results demonstrate that the reactive 2′ hydroxyl functionality in a hammerhead substrate can be caged and that an efficient and accurate hammerhead reaction is initiated upon photolysis of the caged substrate. Furthermore, neither the photolysis conditions nor the nitroso-aldehyde product of photolysis adversely affect the course of the reaction even under saturating conditions. Finally, the presence of the caging functionality does not disrupt the ribozyme–substrate complex as evidenced by equilibrium binding studies.

Caged hammerhead ribozyme substrates should permit Laue diffraction studies (27,28) of the native hammerhead system which may help address mechanistic issues raised by comparison of several X-ray structures with biochemical data (29,30). Large

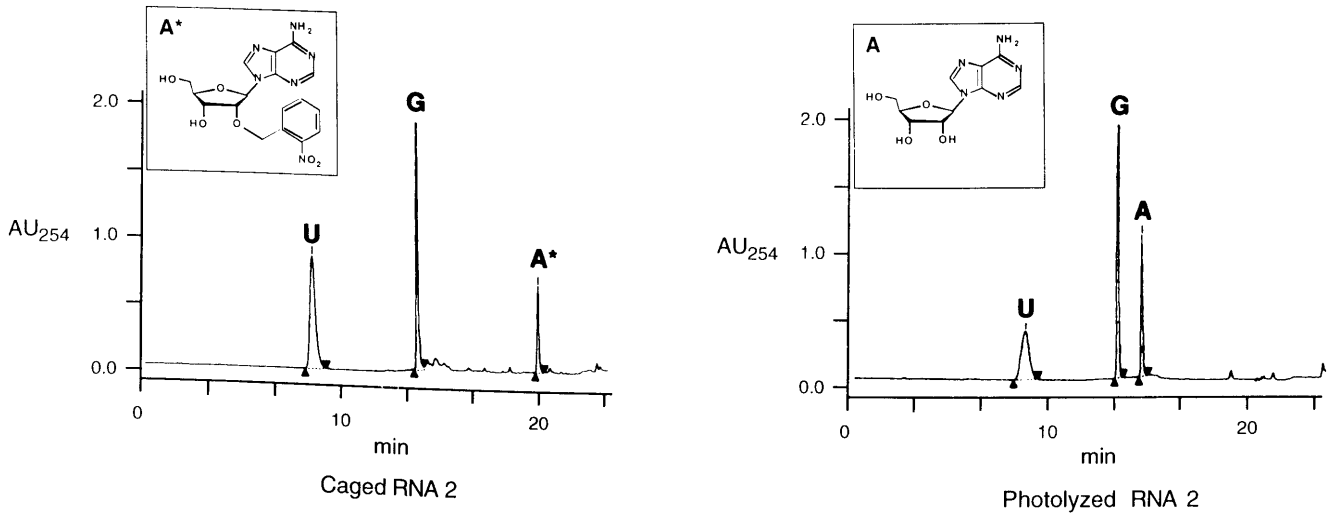

Figure 2. HPLC nucleoside composition analysis following enzymatic digestion: (**A**) RNA **2** containing a single 2′-caged adenosine residue; (**B**) RNA **2** following photolysis at 308 nm showing complete conversion of 2′-caged adenosine to adenosine.

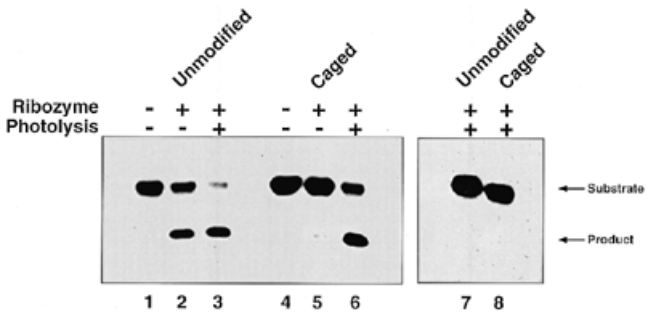

Figure 3. Denaturing PAGE analysis of ^{32}P-end-labeled unmodified (RNA **1**) and caged (RNA **2**) substrates and 7 nt product of ribozyme-mediated cleavage under conditions of catalytic ribozyme. Lane 1, unmodified substrate; lane 2, unmodified substrate and ribozyme, t = 120 min; lane 3, unmodified substrate and ribozyme, photolyzed, t = 120 min; lane 4, caged substrate; lane 5, caged substrate and ribozyme, t = 120 min; lane 6, caged substrate and ribozyme, photolyzed, t = 120 min; lane 7, unmodified substrate and ribozyme in the absence of magnesium, t = 120 min; lane 8, caged substrate and ribozyme, photolyzed, in the absence of magnesium, t = 120 min.

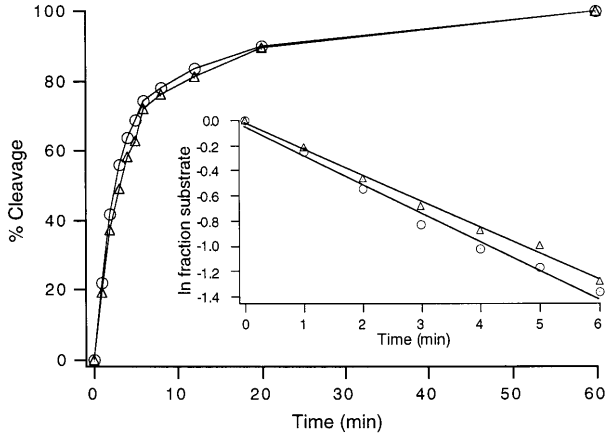

Figure 4. Kinetics of hammerhead catalyzed cleavage of RNA **1** and RNA **2**, following photolysis with an excimer laser, under conditions of saturating ribozyme: normalized percent cleavage as a function of time for RNA **1** (Δ) and RNA **2** (○); plots of ln fraction substrate present versus time give k_{obs} values of 0.20 ± 0.02/min for RNA **1** and 0.22 ± 0.02/min for RNA **2**.

RNAs containing caged 2′ hydroxyls, synthesized by a combination of chemical synthesis and enzymatic ligation (31–33), will permit the 'pre-chemistry' study of a variety of systems where this group acts as a chemically reactive functionality (10–15). This approach should be particularly applicable to studies of pre-mRNA splicing since the spliceosome does not exist independent of its RNA substrate. Because the 2′ hydroxyl of RNA plays important structural roles in the assembly of complex RNA structures in such structural motifs as 'ribose zippers' (34) large RNAs containing caged 2′ functionalities will be useful in the study of kinetic intermediates in the folding of complex RNAs (35,36).

Finally, the hydrogen bond donor/acceptors of the nucleic acid bases are the principal determinants governing most nucleic acid–nucleic acid and protein–nucleic acid interactions through both Watson–Crick and non-Watson–Crick hydrogen bonding patterns (37). Caging of these base functionalities will allow the disruption of specific recognition events allowing characterization of the formation of and transition between different nucleic acid complexes.

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