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Cobalt(III)Hexaammine-Dependent Photocrosslinks in the Hairpin Ribozyme

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Abstract

We have utilized the hairpin ribozyme, an RNA enzyme whose structure has been solved by high-resolution methods, to develop a new tool for mapping nucleobase-stacking interactions and potential metal-binding sites in RNA molecules. This tool involves the photoactivation of a specifically bound cobalt(III)hexaammine molecule at wavelengths corresponding to excitation of the metal ion complex only; no base excitation is involved. The photoexcitation initiates a process which strongly promotes the formation of a novel covalent bond or crosslink between one base (termed the “first base”), which is close in space to the excited cobalt(III)hexaammine complex, and another base upon which the first base is closely stacked. These crosslinked species can be isolated and sequenced; their activities can be analyzed to ensure that the crosslinked structures represent an active conformation of the molecule. We have shown that, as in electron transfer in DNA, several criteria must be met to result in the successful formation of these crosslinks. These include the appropriate oxidation potential of the first donor base, the stacking and close interaction of the two donor bases involved in the crosslink, and the binding of a specific cobalt(III)hexaammine molecule to the first donor base. Additionally, we have determined that this crosslinking is pH-sensitive, although the cause of this sensitivity remains unknown. This tool has proven useful in the past for the analysis of the hairpin ribozyme folded structure, and has been applied to identifying potential metal-binding sites on the hairpin and extended hammerhead ribozymes.

Keywords

Hairpin ribozyme; cobalt(III)hexaammine; photocrosslinking

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1. Introduction

1.1 The hairpin ribozyme, its structure and function

Small self-cleaving ribozymes, enzymes composed entirely of RNA, comprise one family of catalytic RNA molecules. Examples of self-cleaving ribozymes include the hammerhead, hepatitis delta virus, *Neurospora* VS, glmS and the hairpin (reviewed in [1]). The latter is a well-studied RNA molecule that is isolated from the negative strand of the satellite RNA of the tobacco ringspot virus [2, 3]. In this paper and in related work, it is comprised of a long segment (RzA), a short segment (RLB) and the SV5 substrate (Figure 1a). It is capable of catalyzing a reversible, site-specific phosphodiester-bond cleavage reaction of its substrate in *cis* or *trans*. In order to catalyze the cleavage, it has been proposed that the 2'-hydroxyl group at A1 must attack the g+1 phosphorus [4] (labeling of this ribozyme follows the convention of capital letter designating ribozyme and lower-case letter designating substrate strand). Other groups on the ribozyme participate directly in catalysis [5, 6], including G8 and A38. In the folded, active structure, these groups take precise positions that allow them to participate in the cleavage of the substrate.

The crystal structure of this molecule coordinated with calcium ions has been solved [5, 7] and has proved to be close to the active folded structure [8]; the structure shows two domains (labeled A and B in Figure 1a) which undergo a major conformational change, where they “dock”, or come into close association with each other, in the presence of bound substrate and cations [9–11]. The structures of the separated domains have been solved independently by NMR [12, 13]. This docked structure allows for close interactions to form between the two ribozyme segments and the substrate, which locates the probable catalytic bases (G8 and A38) at the scissile phosphodiester linkage [14]. The folded structure can cleave the bound substrate at a specific location, marked by the arrow in Figure 1a. Its catalytic activity can be readily assessed with biochemical assays; the kinetics and thermodynamics of the probable acid-base catalysis used to cleave its substrate have been studied in detail [14–26] [reviewed in [27–30]]. This cleavage reaction can be mediated by various cations, including magnesium(II) ions (typically 12 mM) and high concentrations (> 1 M) of monovalent ions [31].

1.2 Cobalt(III)hexaammine and its effects on the structure and function of the hairpin ribozyme

Interestingly, $\text{Co}(\text{NH}_3)_6^{3+}$ was also found to mediate activity in the hairpin ribozyme [17, 32–36]. $\text{Co}(\text{NH}_3)_6^{3+}$ is an exchange-inert [$k_{\text{ex}} = \sim 10^{-11} \text{ s}^{-1}$ [37]] orange-colored trivalent metal ion complex with octahedral symmetry. It features a central cobalt(III) ion with a d_6 , low-spin electron configuration surrounded by six ammine ligands [38]. $\text{Co}(\text{NH}_3)_6^{3+}$ only interacts with the RNA molecule through outer-sphere binding – there is no direct interaction of the cobalt(III) ion with any portion of the ribozyme. Its ionic radius (3.989 Å) is very similar to that of hexahydrated magnesium(II) ($\text{Mg}(\text{H}_2\text{O})_6^{2+}$) (4.038 Å), although the charge (3+) is higher than the 2+ charge on magnesium(II) ions. Both have octahedral geometry and preference for binding to the major groove of RNA [39]. $\text{Co}(\text{NH}_3)_6^{3+}$ is therefore a useful probe for functional binding of fully hydrated magnesium(II) ions in an outer-sphere interaction [6, 40] (for a recent review, see [41]).

1.3 Crosslinking studies in the hairpin ribozyme

Upon irradiation with 312 or 365 nm light in the presence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$, three novel covalent bonds were formed in the hairpin ribozyme bound to the SV5 substrate [14, 36]. To identify the bases involved in the formation of the new covalent crosslink, sequence assays involving alkali or T1RNase were performed and the resultant films could be read to determine the location of the crosslinks (see 2.4 and also [19]). The first crosslink appeared

to have been formed between the end of the substrate strand and the end of one of the other strands; it gave no clear cutoff when sequenced, regardless of which portion of the ribozyme/substrate complex was labeled ([36]; J.E.H. and J.M.B., 365 nm irradiation data not shown). The second crosslink, located between nucleobases G8 and a-1 [14], was consistent with the spatial relationships of these two nucleotides in the crystal structure of the hairpin ribozyme, which shows a close stacking interaction between these two bases (Figure 1b) [5, 6]. The third crosslink was formed between nucleobases u+2 and G36 [36]. This last crosslink is not supported by the crystal structure, which shows G36 bulged out into solvent, ~12 Å from u+2 (Figure 1b). Each of these last two crosslinks involved catalytic nucleobases in the RNA that had become coordinately bound to substrate nucleobases around the active site.

1.4 Activity of the crosslinked species

Both major crosslinked species (G8/a-1 and u+2/G36), when isolated, reconstituted in a complex and allowed to cleave in the presence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ or 12 mM Mg^{2+} , retained catalytic activity at pH 8 (albeit with a hundredfold decrease in rate from the non-crosslinked wild type, in the case of the 8/-1 crosslink, and an even larger decrease in rate for the +2/36 crosslink) [14, 36]. This suggests that these two stacking interactions (8 on -1 and +2 on 36) are representative of an active structure of the hairpin ribozyme when bound to the SV5 substrate. Additionally, the Watson-Crick face of the guanosine at position 8, which has been found to be at least partially responsible for the chemistry of the reaction [14, 21], did not appear to be involved in the crosslink, as any alteration of this portion of G8 would lead to obliteration of activity in the crosslinked species. Because the u+2/G36 crosslink is not consistent with the crystal structure, obtained in the presence of Ca^{2+} ions, this crosslink may reflect an active conformational variant of the hairpin ribozyme complex that is populated in cobalt(III)hexaammine as opposed to calcium ions [36].

1.5 Cobalt(III)hexaammine-mediated photocrosslinks in the hairpin ribozyme

We report herein the results of an in-depth crosslinking study of the hairpin ribozyme and selected variants performed in the presence of 1 mM cobalt(III)hexaammine. We elucidate key physicochemical properties of the bases participating in the crosslinks, including their oxidation potentials and specific functional groups. Additionally, we postulate a theoretical (albeit unproven) mechanism for photocrosslinking as mediated by cobalt(III)hexaammine. Our results indicate that the photochemistry of the cobalt(III)hexaammine molecule plays a crucial role in the formation of crosslinks in the hairpin ribozyme. This type of specific RNA crosslink where the metal ion complex mediates the photochemistry has not been observed in previous crosslinking studies in the absence of cobalt(III)hexaammine. Typically, the metal ion facilitates folding to a structure that is then amenable to the formation of a novel crosslink, but in the photocrosslinking procedure mediated by $\text{Co}(\text{NH}_3)_6^{3+}$, the metal ion complex actually appears to be absorbing the ultraviolet light and initiating the formation of the crosslink, as well as stabilizing the tertiary RNA structure that is capable of forming the crosslink. Through sequencing and determination of activity of the crosslinked molecules, we can obtain useful structural constraints such as the identification of distant base-stacking relationships and also may be able to map possible metal-binding sites on the ribozyme. This analysis can assist in the determination of active and significantly populated inactive structures of the hairpin ribozyme and, ultimately, other RNA molecules whose activity is supported by cobalt(III)hexaammine. If used in conjunction with results from other biochemical and biophysical studies, this procedure can be applied to the elucidation of structures of other RNAs that have not been analyzed by high-resolution structural determination methods or whose crystal structures may not represent the active conformation.

Materials and Methods

2.1 Preparation of RNA Oligonucleotides

RNA phosphoramidites were purchased from Glen Research, Inc. (Sterling, VA). RNA was synthesized using solid-phase phosphoramidite chemistry and deprotected as described previously [42]. Once fully deprotected, oligonucleotides were purified as described previously [19, 43, 44].

2.2 Ribozyme cleavage assays

Ribozyme segments (200 nM) were incubated with 20 nM of 5'-³²P-end-labeled substrate and 50 mM Tris-based buffer adjusted to the appropriate pH with acetic acid (at or below pH 7.5) or HCl (pH 8 or 9) and annealed for 10 minutes at 37 °C. After 2 minutes at room temperature, the reaction was initiated with the addition of the metal to the desired concentration (typically 12 mM Mg²⁺ or 1 mM Co(NH₃)₆³⁺). At specific timepoints in the 2-hour incubation, aliquots of the reaction were quenched with 8 volumes of 95% formamide. The cleavage products and uncleaved substrates were separated with denaturing polyacrylamide gel electrophoresis (DPAGE) and quantitated with a BioRad Molecular Imager FX system. The cleavage rates were calculated using Microcal Origin by non-linear regression analysis.

2.3 Photocrosslinking assays

Ribozyme segments (200 nM) were incubated at 37 °C for 5–10 minutes with 20 nM of 5'-³²P-end-labeled noncleavable substrate and 1 mM Co(NH₃)₆³⁺ and appropriate buffer (50 mM Tris adjusted to the appropriate pH with acetic acid or HCl (as above) in most experiments or, to assess the effects of buffer on the crosslinking, 50 mM sodium acetate (pH 5.0), 50 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.5–6.5), 50 mM (4-(2-hydroxyethyl)-1-piperazine)ethanesulfonic acid (HEPES) (pH 7.0–8.0), and 50 mM Tris (pH 8.5 and 9.0). If utilized, dithiothreitol (DTT) or ascorbic acid was added at this time to the desired concentration. In some experiments, metals other than Co(NH₃)₆³⁺ were used; in these cases, the metal (e.g., [Co(NH₃)₅Cl]²⁺, Co²⁺, Ca²⁺ or Mg²⁺) was added to the desired concentration instead of Co(NH₃)₆³⁺. Each sample was placed in one well of a 96-well tray. The tray was incubated at 37 °C for 5–10 minutes to allow for assembly and folding of the molecules (although 5 minutes' incubation for cleavable substrates was sufficient, 10 minutes was used with a noncleavable substrate to ensure full folding). The samples were shielded with a polystyrene filter, which absorbs light with wavelengths below ~300 nm, and irradiated for 5 minutes at a distance of ~40 mm with ultraviolet light from a hand-held source (312 nm model – VL-GM, IBI Inc.; 365 nm model – UVL-56, UVP). Following crosslinking, RNA was denatured with 95% formamide, and 0.1 mg/mL tRNA was added. The crosslinked molecules were separated on 20% DPAGE as above.

2.4 Mapping of the crosslinked species

5'-³²P-end-labeled substrate (87.5 nM) was incubated with 250 nM (each strand) pre-annealed ribozyme fragments, 50 mM Tris buffer adjusted to the appropriate pH with acetic acid or HCl as above, and 1 mM Co(NH₃)₆³⁺ for 5 minutes at 37 °C. These conditions have been shown to result in efficient assembly of the active ribozyme and quantitative binding of substrate [14]. If the experiments were to be performed with a labeled ribozyme strand, 87.5 nM 5'-³²P-end-labeled RzA or RLB (or the necessary variants) was incubated with 800 nM unlabeled substrate, 250 nM of the missing ribozyme strand, 50 mM Tris buffer as above, and 1 mM Co(NH₃)₆³⁺ at 37 °C for 5 minutes. The sample was then distributed evenly into 6 wells in a 96-well tray, screened by a polystyrene filter at a distance of ~40 mm and crosslinked for 5 minutes at the appropriate wavelength with a hand-held UV

transilluminator. The samples were collected and the reaction was quenched with 1 volume of 95% formamide, and 0.1 mg/mL tRNA was added. The crosslinked species were separated with DPAGE. The harvested crosslinked species were then sequenced as described [19].

2.5 Cleavage assays of the crosslinked species

Each crosslinked species (<5 nmol, 6000 dpm) was incubated for 2 hours at 37 °C with 200 nM of the missing ribozyme strand, 50 mM Tris-HCl (pH 8) and 0.1 mM aurintricarboxylic acid (ATA, to minimize nuclease activity), either in the presence or absence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$. An aliquot of each sample was removed and quenched with 8 volumes of 95% formamide, and loaded directly onto a denaturing 20% polyacrylamide gel.

Alternatively, the ribozyme strands and 5'- ^{32}P -end-labeled cleavable substrate were assembled under standard crosslinking conditions, incubated for 5 minutes at 37 °C, distributed into wells of a 96-well tray, and crosslinked as described above. The samples were then removed to eppendorf tubes and incubated at 37 °C for 2 hours. Aliquots of each sample were removed and quenched with 8 volumes of 95% formamide. The cleavage reactions were then analyzed with DPAGE as above.

2.6 Reversal studies of the crosslinked species

Each crosslinked species (<5 nmol, 6000 dpm) was incubated for 2 hours at 25 °C with 200 nM of the missing ribozyme strand, 50 mM Tris-HCl adjusted to the appropriate pH as described above, and 0.1 mM ATA (to minimize nuclease activity) in the presence or absence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ or 12 mM Mg^{2+} . After 2 hours, the samples were quenched with 1 volume of 95% formamide, and loaded directly onto a denaturing 20% polyacrylamide gel. Where appropriate, the crosslinks and substrate/crosslink reversal products were quantitated with a BioRad Molecular Imager FX system through analysis of the labeled substrate. Fractional reversal was calculated as

$$f = \frac{\text{uncrosslinked substrate}}{\text{uncrosslinked substrate} + \text{crosslinked substrate}}.$$

3. Results

3.1 Photochemical crosslinks are initiated by excitation at wavelengths corresponding to $\text{Co}(\text{NH}_3)_6^{3+}$, not nucleobase, absorption

The cobalt(III)hexaammine-dependent crosslinks described herein have unusual wavelength dependencies. Absorbance spectra of the bases indicate that none of the naturally occurring nucleotides absorb energy with wavelengths longer than 300 nm [45]. However, in this study, irradiation of the hairpin ribozyme and variants in the presence of cobalt(III)hexaammine was performed at 312 or 365 nm, well removed from the absorbance wavelengths of the nucleobases used herein. Excitation at 312 nm may involve partial excitation of the electrons of the guanine bases, as they have a slight absorption near 300 nm. This partial absorption may explain why excitation at 312 nm yielded higher amounts of crosslinked species than excitation at 365 nm, as shown in Figure 2. Additionally, some wavelength scatter is to be expected from the 312 nm lamp, which may involve light with wavelengths slightly below 300 nm to be transmitted to the sample. To eliminate most of the contaminating radiation, a polystyrene filter, which absorbs light with wavelengths less than ~300 nm, was used in the experiments. Irradiation with 365 nm light does not involve excitation of the electrons of any of the bases [45], yet photocrosslinking with 365 nm light

yields an observable population of crosslinked molecules (Figure 2). This indicates that photoirradiation of the nucleobases alone does not initiate the crosslinking reaction.

Cobalt(III)hexaammine, on the other hand, does absorb light with wavelengths around 365 nm. The molecular orbital diagram and absorbance spectrum of $\text{Co}(\text{NH}_3)_6^{3+}$ (the latter has a $\text{Co}(\text{NH}_3)_6^{3+}$ concentration of 10 mM at pH 5.75 and 7.5) are shown in Figure 3. As can be seen, there are two main transitions involved. One requires light with a wavelength between 380 and 540 nm, with a λ_{max} at 472 nm, and results in excitation of electrons between the non-bonding t_{2g} and the e_g^* orbitals. The second transition involves absorbance of light between 280 and 380 nm, with a λ_{max} at 338 nm, and is sufficient for excitation of a ground-state t_{1u} electron to a higher energy level (most likely e_g^*) [46]. While we were not able to excite our samples at 338 nm exactly (which is almost exactly halfway between the wavelengths of the lamps that were used) due to experimental constraints, the 312 and 365 nm lamps used in this study, with their experimental variability, most likely did emit some light near the 338 nm region. The absorbance spectrum of $\text{Co}(\text{NH}_3)_6^{3+}$ was unchanged in these regions when the pH of the solution was increased from 5.75 to 7.5 (as shown in Figure 3a).

This ability to absorb light at these longer wavelengths, unique to the metal ion complex, strongly suggested that excitation of the cobalt(III)hexaammine was directly involved in the formation of covalent bonds between nucleobases. Therefore, even allowing for some “wavelength scatter” from the lamps used, it is the $\text{Co}(\text{NH}_3)_6^{3+}$ molecule that is responsible for the absorption of the light and the concomitant formation of the crosslink. It is for this reason that we refer to this type of crosslink as a $\text{Co}(\text{NH}_3)_6^{3+}$ -dependent photocrosslink. Any new covalent bonds formed *via* irradiation with long-wavelength UV light in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$ were a result of both the direct photochemical participation of the metal ion complex and the presence of a structure in the RNA conducive to a novel crosslink, rather than merely the $\text{Co}(\text{NH}_3)_6^{3+}$ -mediated formation of a specific structure in the RNA whose nucleobases are then capable of initiating crosslinks when irradiated with ultraviolet light.

Previous photocrosslinking studies on the hairpin ribozyme bound to the SV5 substrate in the presence of 12 mM Mg^{2+} yielded only one crosslink between G21 and U42 upon irradiation with 254 nm light [43]. This photocrosslink is wavelength-dependent – irradiation with 312 nm light resulted in almost none of the G21/U42 crosslink, whereas irradiation with 254 nm light provided a significant amount of the crosslinked species [18, 43]. In addition, photocrosslinking in the presence of Co^{2+} or Ca^{2+} did not yield any crosslinks in the wild type ribozyme or any tested variants (Figure 4). These results indicate that the photocrosslinking procedure is dependent upon the presence of a covalent metal ion complex such as cobalt(III)hexaammine. To support this hypothesis, we have also studied formation of photoinduced crosslinks in the presence of $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$, another inorganic metal ion complex with photochemical properties similar to $\text{Co}(\text{NH}_3)_6^{3+}$. Crosslinks in this metal ion complex were observed and the pH dependence of the +2/36 crosslink did appear to be similar to that observed in $\text{Co}(\text{NH}_3)_6^{3+}$ (Figure 5). The dependence of the 8/–1 species was difficult to analyze due to its high yield overwhelming the analysis technique utilized; the error in the analysis precluded any useful quantitative conclusions. Qualitatively, the pH dependence of the 8/–1 crosslink in $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ does appear to be different from the dependence of the 8/–1 crosslink in cobalt(III)hexaammine (data not shown).

Formation of the photocrosslinks was inhibited by ascorbic acid and dithiothreitol, as shown in Figure 4. These results are consistent with a process that involved the formation of a radical that was not a diffusible free radical. (Note that ATA, a compound that forms a free radical and has a conjugated system of aromatic rings that could possibly absorb in the

visible range, was not used in any of the experiments involving formation of crosslinked species. Instead, this compound was used only for nuclease inhibition in crosslinking activity and crosslink reversal studies where the molecule had already been crosslinked.)

Most of the experiments described herein were performed in 50 mM Tris buffer adjusted to the appropriate pH with HCl or acetic acid (Figure 6). To rule out the possibility of buffer effects, initial experiments were performed using alternative buffer conditions such as MES and bis-Tris adjusted to the appropriate pH as above. None of these conditions altered the yield of crosslinked species beyond experimental error (see Figure S1).

3.2 A nucleobase is postulated to donate an electron to the $\text{Co}(\text{NH}_3)_6^{3+}$ excited state

It was originally thought that the removal of the endocyclic nitrogen at position 7 of G8 would lead to a decrease in crosslinking, since earlier studies have shown this portion of guanosines (along with the carboxyl group at position 6 of the same base and the phosphate group of a nearby ribose) to be important for the coordination of cobalt(III)hexaammine [47, 48] and, especially in the case of N7 of guanines, other metals including nickel and platinum metal complexes [49–51]. Therefore, loss of this nitrogen might have been expected to lead to the decrease in affinity of metal complex binding and a concomitant decrease in crosslinking intensity at this position. However, the 7-deazaguanosine 8 (Z8) variant formed a sequenced crosslink to the a–1 position (C.M.K. and J.M.B., data not shown) that was increased 30-fold in intensity compared to the wild type G8 ribozyme (Figure 7a). This was also observed for the 7-deazaguanosine 36 (Z36) variant, which maintained its +2/36 crosslink but to a much lesser extent (ca. 1.2-fold increase upon 312 nm excitation; ca. 7-fold increase upon 365 nm excitation). This indicates that other factors or favorable RNA-metal ion complex interactions more than compensate for the potential lowering of binding affinity to the cobalt(III)hexaammine complex due to the removal of the endocyclic nitrogen at position 7 of G8. The Z36 variant did exhibit a crosslink between G8 and u+2, although the yield was very low; this might have also decreased the intensity of the Z36/u+2 crosslink through some competitive process.

We postulate that electron transfer from one base involved in the crosslink (referred to as the first donor base herein) to the metal ion complex is required for the formation of the photocrosslinks; if this is indeed true, lowering the oxidation potential of this base theoretically should lead to an increase in crosslinking yield. This increase is qualitatively charted in Figure 7b, which provides an initial comparison of how the one-electron standard oxidation potential of the first donor base affected the yield of the 8/–1 crosslink. The replacement of nitrogen with carbon within the five-membered ring of this first donor base (as in the case of 7-deazaguanosine substituted for guanosine) results in a decreased oxidation potential (although it is important to remember that this N7/C7-H mutation could alter the local geometry slightly, which may contribute to changes in the crosslinking yield). The identities of the bases at positions 8 and, to a lesser extent, 36 appear to play important roles in increasing the yield of crosslinked RNA – the more energy necessary to remove an electron from the first donor base, the lower the yield of the crosslink.

The 8/–1 crosslink formed with the 7-deazaguanosine 8 (Z8) variant also appeared different chemically from the standard crosslink formed in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$. The crosslink formed between Z8 and a–1 was more readily reversed than the crosslink formed between G8 and a–1 – incubation of the separate crosslinked species over a period of time resulted in a noticeably decreased fractional population of the Z8/a–1 crosslinked species (where quantification of the uncrosslinked substrate band from crosslink reversal is compared to total population of crosslinked plus uncrosslinked substrate). Indeed, reversal to uncrosslinked substrate with the Z8 variant was present even at time 0 under all of the conditions tested, although the pH did appear to affect moderately the amount of crosslink

reversal after 2 hours. However, the (wild-type) G8/a-1 crosslink showed no reversal after 2 hours under any of the conditions tested (Figure 8). The results of one experiment of the Z8 reversal to uncrosslinked species, where the SV5 substrate is labeled and quantified, are graphed in Figure 8. Although the reversal was tested only once for each condition, the experiment was repeated several times in the absence of metal at pH 7 at different temperatures and in the presence of ascorbic acid or DTT (Figure S2). The results of these studies indicated that, under varying conditions, the Z8 8/-1 crosslink reversed readily to uncrosslinked species, supporting the results shown in Figure 8b and described in the text.

Additionally, although the Z8 variant was active for cleavage in 12 mM magnesium(II) and 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ in uncrosslinked form (for example, at pH 7 the Z8 variant exhibited rate of cleavage that was 0.14 times the rate of wild type in 12 mM magnesium(II) and 1.9 times the rate of wild type in 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$, as shown in Figure 9), the crosslinked species was not active in the presence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$, even upon prolonged incubation. This suggested that the normal G8 crosslink might involve N7, leaving the Watson-Crick face of the base available to participate in cleavage. We postulate that the loss of N7 coordination to the metal bound to the ribozyme at the 8 position leads to more flexibility when $\text{Co}(\text{NH}_3)_6^{3+}$ is present, thus increasing the rate of reaction, but detrimentally decreases the affinity of binding of magnesium(II) ions to the same position.

The Z36 variant was, like the Z8 variant, more active for cleavage of the substrate in uncrosslinked form than the wild type under the same conditions, but unlike the Z8 variant, the Z36/u+2 crosslinked species did retain its activity. This indicates that the structures of these variants are most likely very similar to the structure of the wild type hairpin ribozyme and that the specific electronic rearrangements that occur upon crosslinking in the Z36 variant do not affect the activity, unlike the Z8 rearrangements.

Replacement of the guanosine at position 8 with certain nucleobases other than 7-deazaguanosine, such as cysteine 8 (C8), purine 8 (pur8) and inosine 8 (I8), obliterated the 8/-1 crosslink (although the +2/36 crosslink is invariably maintained) (Figure 2 and Table 1), as shown by sequencing analysis. It is postulated that the difference in the crosslinking of these variants is due to a number of factors, including the base stacking, structure, metal-RNA interactions, and possibly the oxidation potential of the bases (which may explain why the inclusion of bases with higher oxidation potentials than guanosine at position 8 results in a g+1 crosslink). The results of the mutant crosslinking studies are summarized in Table 1. Mutation of u+2 to any of the other three naturally occurring nucleobases (a, c and g) did not have a deleterious effect on the yield of the +2/36 crosslink. However, when 7-deazaguanosine is present at position +2, the yield of the +2/36 crosslink with this +2 mutation decreased dramatically from the wild-type +2/36 crosslink involving u+2 (Figure 10). This may be due to the same factors that affected the yield of the crosslinks formed between SV5 -1 and the 8 variants as described above, or it may be due to a change in the electron transfer procedure when the oxidation potential of the other base involved in the formation of the novel crosslink is lower than that of the first donor base. The pH profiles of the G8/a-1 and z+2/G36 crosslinks formed in the z+2 variant are shown in Figure S3; the +2/36 crosslink pH profile appears similar to that of the wild-type, whereas the 8/-1 pH profile is noticeably different at higher pH values. The cause of this altered pH profile remains unclear. (Note that the higher values for crosslinking intensity in the z+2 variant as shown in Figure S3 as compared to the wild-type as shown in Figure 6 is an artifact of the quantification process and does not represent actual yield of crosslinked species.)

It is important to remember that the exact alignment of the donor and acceptor bases and the interactions of the bases with the $\text{Co}(\text{NH}_3)_6^{3+}$ molecule may not be conserved from one mutant to the next. Stacking of the bases is important for electron transfer, both in the actual

procedure and in the lowering of the oxidation potential of the donor base [52]; some of the loss or gain in crosslinking may have been due to a rearrangement, either gross or slight, of the area of interest. Therefore it is difficult to quantitatively determine the effect of oxidation potential alone on the intensity of the crosslinking. Native gel electrophoresis at pH 7.5 and hydroxyl-radical footprinting at pH 7.0 shows that, to the limits of the resolutions of these techniques, all variants at position 8 explored in this study, with the exception of cytosine, dock efficiently, including those that strongly inhibit the reaction [14]. This suggests that the loss of the 8/–1 crosslink of most of the position 8 variants studied was not completely due to the misfolding of the hairpin ribozyme but to other factors, such as slight alterations of the orientations of the bases with each other and/or with the $\text{Co}(\text{NH}_3)_6^{3+}$ molecule, solvent exposure, oxidation potentials of the bases involved and affinity to the $\text{Co}(\text{NH}_3)_6^{3+}$.

3.3 pH-dependence of crosslinking

1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ drove the cleavage reaction of the SV5 substrate with a rate between two and three times lower than the rate in 12 mM magnesium(II) at pH 7.5. There was no significant pH dependence of cleavage between pH ~5.5 and 9 in either magnesium(II) or $\text{Co}(\text{NH}_3)_6^{3+}$ at room temperature [3, 27]. In contrast, the yields of the different crosslinked species exhibited distinct pH profiles (Figure 6). For example, the yields of the two crosslinks obtained between u+2 and G36 and between a–1 and G8 in the wild type hairpin ribozyme had different pH profiles. As shown in Figure 6b, the 8/–1 crosslink had its highest intensity at pH 7.5–8, and then fell to a lower value at pH 9. However, formation of the +2/36 crosslink increased steadily above pH 7 and reached no discernable maximum at the highest pH probed.

These results are indicative of a pH-dependent process that causes the intensity of the crosslink to increase or decrease. This process is not completely understood. It was originally postulated that the pH dependence involved an alteration of the oxidation potential of the donor base through the changing of the protonation state of the N1 position of the donor. The presence of a hydrogen ion bound to an endocyclic secondary nitrogen may serve to act as a slight electron withdrawing group, leading to the possible increase in the oxidation potential of the ring structure. The oxidation potentials of electron donors are usually pH sensitive; as pH increases, oxidation potential tends to decrease, making the donor more susceptible to electron donation [38]. G8 and G36 have different hydrogen-bonding patterns and stacking interactions, both of which may affect the oxidation potential of the bases and therefore the pH-dependence of the yields of their respective photocrosslinks. However, as is shown in Figure 11, both Z8 and G8 have almost identical normalized pH profiles of crosslinking, even though 7-deazaguanosine has a higher N1 pK_a (11.2) than guanosine (9.4). (Figure S4 shows the pH profiles of the 8/–1 and +2/36 crosslinks of several tested mutants.)

It is also possible that the pH dependence of crosslinking stems, at least in part, from a characteristic of the $\text{Co}(\text{NH}_3)_6^{3+}$ reduction procedure. The hexaammine and chloropentaammine complexes of cobalt(III) are known to be electron acceptors of photoexcited species (for example, see [53] and [54]). We postulate that $\text{Co}(\text{NH}_3)_6^{3+}$ appears to be reduced to $\text{Co}(\text{NH}_3)_6^{2+}$ by the first donor base upon excitation with ultraviolet light, although due to the extremely small yield of crosslinked species, we could not validate this theory. If this reduction does take place as theorized, the $\text{Co}(\text{NH}_3)_6^{2+}$ product is not exchange-inert, especially under acidic conditions; when Co(III) is reduced to Co(II), the ammine ligands can be readily exchanged for water molecules [55]. If $\text{Co}(\text{NH}_3)_6^{2+}$ does undergo ligand dissociation to form hexahydrated Co(II) under acidic conditions, it is possible that the pH dependence is partially due to the absence of a specifically-bound $\text{Co}(\text{NH}_3)_6^{3+}$ molecule at that position. Ligand dissociation would involve the Co(II) metal exchanging its ammine ligands with water molecules in solution, thus removing $\text{Co}(\text{NH}_3)_6^{3+}$

from the vicinity of the ribozyme binding sites. As $\text{Co}(\text{NH}_3)_6^{3+}$ and hexahydrated $\text{Co}(\text{II})$ have varying physicochemical properties, this replacement would alter the photochemistry of the crosslinking at the binding sites.

3.4 Postulated model for cobalt(III)hexaammine-dependent photocrosslinking

The data we have obtained can be utilized to postulate a theoretical model of the mechanism of the $\text{Co}(\text{NH}_3)_6^{3+}$ -dependent photocrosslinking procedure. (Unfortunately, the yield of crosslinked species is in the femtomolar range, thus precluding conventional structural analysis or quantitative studies; therefore, we wish to stress that this is a postulated model only.) Irradiation with 365 nm and, to a slightly lesser extent, 312 nm light does not cause excitation of the nucleobases. Based on the molecular orbital diagram and the absorbance spectrum of $\text{Co}(\text{NH}_3)_6^{3+}$, it is likely that the inorganic metal ion complex is either entirely or primarily responsible for the absorption of 312 and 365 nm radiation. Irradiation of $\text{Co}(\text{NH}_3)_6^{3+}$ with light whose wavelength is between 280 and 380 nm results in excitation of a specific electron from the t_{1u} energy level to a higher energy level (most likely e_g^*). The excitation leaves a vacancy or an electron hole in the t_{1u} level of $\text{Co}(\text{NH}_3)_6^{3+}$. The vacancy provided by the excited electron becomes filled by an electron donated from a closely neighboring base (in this case, either G8 or G36), originating from an orbital with higher energy than the t_{1u} $\text{Co}(\text{NH}_3)_6^{3+}$ orbital. The excited $\text{Co}(\text{NH}_3)_6^{3+}$ is reduced by this first donor base, leaving the donor base with an electron hole. This yields a guanosine cation radical at either position 8 or position 36. What happens next is unclear, but it appears that the cation radical shares this lone electron or an electron pair (if the radical is reduced somehow) with the base upon which it is stacked. This electron sharing results in the formation of a novel covalent bond between these two bases, which can be resolved with DPAGE and sequenced by standard means. The proposed model is shown in Figure 12. (For further reference, see [52].) Because a specifically bound $\text{Co}(\text{NH}_3)_6^{3+}$ molecule is required, this process not only provides structural constraints *via* the creation of these new bonds, but also locates one or more specific metal-binding sites on the ribozyme.

4. Discussion

There are several useful biochemical and biophysical tools to assist in the determination of the folded tertiary structures of catalytically active RNA enzymes. Some of these, such as FRET (fluorescence resonance energy transfer [9]), Tb(III) cleavage assays [44] or backbone protection assays using hydroxyl radicals, are relatively rapid but frequently provide lower-resolution results. Others, such as NMR or x-ray crystallography, yield high-resolution structural results but are costly, require more material and are both time- and labor-intensive. Additionally, these probes do not guarantee that the result is indeed descriptive of the active structure.

Alternatively, photolabile bases such as 6-thiouridine can be introduced into the wild type background for the ready formation of novel crosslinks. Unfortunately, the resultant crosslinked molecules involve a significant rearrangement of the structures of the bases involved in these crosslinks [56]. If maintaining the structure of the base or bases involved in the crosslink is necessary for catalytic activity, the ability of this type of crosslink to elucidate the active structure of the molecule may be diminished. Demonstration of the catalytic activity of the crosslinked species is an important first step in proving that the crosslinked bases are stacked in the active structure or a very similar alternative fold that retains activity; using photolabile bases that become altered and potentially inactive upon crosslinking removes or hinders the ability to obtain this proof.

Other useful methods for obtaining information about the native fold of the RNA molecule include the use of photoaffinity reagents placed at site-specific positions throughout an RNA

structure, including azidophenacyl modifications. The method developed by our group involves introducing azidophenacyl groups at defined internal thiophosphate positions in short synthetic RNAs; these groups, when chemically treated, assembled and photoirradiated, react relatively indiscriminately with their surroundings to form covalent attachments. These crosslinked species can then be identified [18]. An alternative method involving azidophenacyl modifications also yields good results, but does have its drawbacks: Direct placements are confined to the 5' or 3' ends of the RNA; if incorporated within the RNA strand itself, a series of circularly permuted RNAs must be developed, which either alter the overall three-dimensional structure or limit the positions available for inclusion of these reagents (summarized in [57]).

Long-wavelength ultraviolet irradiation of the RNA in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$ as described herein yields more straightforward results. Utilizing $\text{Co}(\text{NH}_3)_6^{3+}$ as the crosslinking mediator eliminates the need for short-wavelength ultraviolet excitation of the RNA molecule and greatly reduces the amount of damage done to the system. Additionally, the crosslink formed most probably does not involve significant rearrangement of the base structures, as some of these isolated crosslinks retain catalytic activity and are reversible to the uncrosslinked form.

As with any method, this procedure does have its disadvantages. While the crosslinks in the hairpin ribozyme have yielded important structural information about the folded, active conformation of the molecule under specific experimental conditions, not all crosslinks will elucidate or probe the active fold of the molecule. Additionally, the number of crosslinks produced by this method is generally small, elucidating only a small portion of the structure of the molecule. However, this method is relatively quick and straightforward, and requires only a small amount of material. Based on our results, application of $\text{Co}(\text{NH}_3)_6^{3+}$ -mediated photocrosslinking in conjunction with other analyses such as activity assays or high-resolution structural data of the molecule under study can provide precise, sensitive structural constraints for either the active fold or important misfolds along or off the folding pathway. Additionally, this procedure can identify further experiments for probing the structure-function relationship in the molecule.

This crosslinking procedure has also proven useful for the identification of specific $\text{Co}(\text{NH}_3)_6^{3+}$ -binding sites on the hairpin ribozyme as well. A $\text{Co}(\text{NH}_3)_6^{3+}$ molecule must be specifically bound to the RNA in order for a photocrosslink of this type to be formed, although the number of crosslinks produced by this method is generally small and the yield of crosslinked species is in the femtomolar range. Therefore, the usefulness of this technique for structural analysis is somewhat limited. The results presented herein indicate that there are $\text{Co}(\text{NH}_3)_6^{3+}$ -binding sites around the G8/a-1 and u+2/G36 regions of the folded hairpin ribozyme molecule. Other studies utilizing ruthenium-labeled oligonucleotides for crosslinking indicate that guanine is the only natural nucleobase capable of donating an electron to a tethered ruthenium complex [58–60]. These results are consistent with our theory that G8 must be coordinating a $\text{Co}(\text{NH}_3)_6^{3+}$ complex. G36 may also be coordinating the same $\text{Co}(\text{NH}_3)_6^{3+}$ complex, or may be interacting with a different molecule entirely.

Whether or not this information can be extrapolated to identify sites where other metals such as magnesium(II) binds is questionable. Both $\text{Co}(\text{NH}_3)_6^{3+}$ and magnesium(II) tend to interact with the N7 and O6 of G residues, the nonbridging oxygens of the phosphate groups and (to a lesser extent) the O4 of U residues [although no hydrogen bonds are formed with the latter, the carbonyl groups do add to the negative surface of the ion binding pocket [48]]. However, $\text{Co}(\text{NH}_3)_6^{3+}$ tends to bind more strongly to Z-DNA than B-DNA because of the increase in the ability to form hydrogen bonds in the Z-form [47]. Small alterations in the binding pocket of the RNA such as site geometry and charge density may also provide

discrimination between the binding of the two cations [61]. Several seminal studies have elucidated the difference between $\text{Co}(\text{NH}_3)_6^{3+}$ and hexahydrated magnesium(II) binding to RNA. Cech and coworkers [39] found that magnesium(II) and $\text{Co}(\text{NH}_3)_6^{3+}$ do not bind RNA identically; they identified several $\text{Co}(\text{NH}_3)_6^{3+}$ -binding sites that are not usually occupied by magnesium(II). Dumas and coworkers [62] also found that $\text{Co}(\text{NH}_3)_6^{3+}$ did not replace hexahydrated magnesium(II) ions bound to the five strongest binding sites inside the duplex formed by the HIV-1 RNA dimerization initiation site. However, Doudna and coworkers [61] found that $\text{Co}(\text{NH}_3)_6^{3+}$ did tend to displace weakly bound magnesium(II) ions involved strictly in outer-sphere coordination at two of the three binding sites in the *T. thermophila* group I intron (P4–P6 domain). This loss in binding upon removal of the N7 position of G8 may explain partially why the activity of Z8 in Mg^{2+} is lower than the activity of the wild type under the same conditions, and bolsters the theory that a metal-binding site is indeed located by G8, as mentioned above. It is harder to explain why Z8 has a higher activity in the uncrosslinked form in the presence of cobalt(III)hexaammine than in Mg^{2+} . In light of this information, we must use caution when extrapolating the location of $\text{Co}(\text{NH}_3)_6^{3+}$ -binding sites as indicated by this photocrosslinking assay alone to the location of sites involved in the binding of magnesium(II) ions.

The crosslinking process in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$ described herein appears to be similar to long-range electron transfer (ET) in DNA, which is capable of transferring an electron up to 14 Å through intervening bases [63], as well as the crosslinking process with ruthenium-labeled oligonucleotides mentioned above. Recent studies have shown that the ET properties of DNA can range from insulator- to “wire”-like, detailing the importance of orientation and distance between bases for efficient ET; specifically, ET in DNA has proved exquisitely sensitive to π – π stacking [63–69]. This mechanism is also dependent upon the oxidation potential of the donor base (as shown in this study to be potentially important for electron transfer in RNA) and the reduction potential of the acceptor [70–75]. It may also have parallels in other types of base oxidation such as the two-electron oxidation of dGMP by a Pt^{IV} complex [51], despite the differences in the results and the necessity of an N7 position on the guanine base for metal binding.

$\text{Co}(\text{NH}_3)_6^{3+}$ -dependent photocrosslinking has the potential to be a useful tool in structure/function analysis of small catalytic RNAs – specifically, to establish distant stacking relationships primarily for RNAs whose activity is supported by cobalt(III)hexaammine and whose structures have not been established by crystallography, or whose crystal structures may not represent the active conformation. To date, it has proved useful for the qualitative structural analysis of variants of the hairpin ribozyme. It is system-independent, as well; our lab has utilized this technique to obtain an active crosslink (and thus a better understanding of the active structure) of the hammerhead (HH) ribozyme, another small RNA enzyme [76]. The crosslink, between G8–C17, was inconsistent with the minimal HH crystal structure but closer to the structure of the extended HH ribozyme. RNA has a high potential for misfolding; the crosslinks obtained, if not active, can be potentially indicative of these misfolds, provided that the crosslinks do not alter the structure of the base or bases responsible for the chemistry of the reaction. The usefulness of activity data of the crosslinked species does, therefore, depend on whether the crosslinks involve specific portions of the bases that are important for the chemistry, and therefore does not necessarily yield straightforward conclusions. Analysis of these misfolded structures can lead to targeted mutagenesis of molecules in the hopes of avoiding or lessening the population of these off-pathway folds, a process similar to negative design in the study of proteins. In conjunction with cleavage and ligation assays, we are confident that it will continue to assist in the determination of the active folded structure of various RNA molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cobalt(III)hexaammine mediates pH-dependent formation of photocrosslinks in the hairpin ribozyme.
- The two main photocrosslinks formed in this fashion are catalytically active.
- The oxidation potentials of the bases involved affect the yield of the crosslinks.
- The crosslinks require stacking and close interaction of the two donor bases.
- This tool has proven useful for analysis of the hairpin ribozyme folded structure.

Figure 1. The secondary structure of the hairpin ribozyme from the negative strand of the tobacco ringspot virus satellite RNA bound to substrate SV5
 (Left, 1a) The hairpin ribozyme; the two domains are labeled A and B. The cleavage site is marked by an arrow. (Right, 1b) A closer view of the active site of the hairpin ribozyme [5].

Figure 2. Mutating selected bases changes the crosslinks formed in the hairpin ribozyme
 The hairpin ribozyme and selected variants were irradiated with either 312 (first six lanes) or 365 nm (last six lanes) light at pH 9.0 as described in Materials and Methods, section 2.3. From left – wild type, Z8, Z36, C8, purine 8 and inosine 8 (I8). The middle band, labeled as 8, indicates a crosslink formed between the base at position 8 and its partner (either A-1 or G+1); the lower band indicates a crosslink formed between u+2 and the base at position 36.

Figure 3. (Top) $\text{Co}(\text{NH}_3)_6^{3+}$ has specific physicochemical properties

The absorbance spectrum (absorbance units *versus* wavelength) of 10 mM $\text{Co}(\text{NH}_3)_6^{3+}$ at pH 5.75 and 7.5. The absorbance maxima of the two smaller peaks occur at 338 and 472 nm. (Bottom) Molecular orbital diagram of cobalt(III)hexaammine. Red or green lines indicate change in orbital energies upon six ammine ligands coordinating to central cobalt(III) ion.

Figure 4. Metal ions and the presence of varying concentrations of reducing agents affect the yield of crosslinks

The crosslink identities are labeled. All samples containing ascorbic acid or DTT were crosslinked in the presence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$.

Figure 5. Photocrosslinks in the hairpin ribozyme in the presence of $\text{Co}(\text{NH}_3)_5\text{Cl}^{2+}$ exhibit variable pH dependencies compared to those formed in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$ Mean normalized crosslinking intensity (arbitrary) for the +2/36 crosslink formed in the hairpin ribozyme in the presence of 1 mM chloropentaammine cobalt(III). The intensities are averaged between two separate crosslinking trials using 365 nm light excitation and are normalized to the pH 9 average intensity to mirror what is shown in Figure 10 in the text. Error bars indicate the standard deviations of the mean normalized crosslinking intensities of the crosslinked species.

Figure 6. Cobalt(III)hexaammine-mediated photocrosslinks in the hairpin ribozyme are pH-dependent

(Top, 6a) Crosslinks formed in the presence of $\text{Co}(\text{NH}_3)_6^{3+}$. The pH of each lane is marked, along with the identity of the buffer utilized. (Bottom, 6b) Mean normalized crosslinking intensity (arbitrary) for crosslinks formed in the hairpin ribozyme in the presence of 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$. The intensities are averaged between two separate crosslinking trials and are normalized to the pH 8 average intensity (for the 8/-1 crosslink) or the pH 9 average intensity (for the +2/36 crosslink). Error bars indicate the standard deviations of the mean normalized crosslinking intensities of the two crosslinked species.

Figure 7. Changing the oxidation potential of one of the nucleobases involved in the photocrosslink alters the yield of the photocrosslink itself

(Top, 7a) Comparison of crosslinks formed when 7-deazaguanosine 8 or 36 is substituted for G8 or 36. Crosslinking was performed with 312 or 365 nm light excitation in 50 mM Tris-acetate (pH 8 data shown here), 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ and 200 nM ribozyme. Substrate is 5'- ^{32}P -labeled. The 365 nm results are normalized to the pH 5 value for each construct; the 312 nm results are normalized to an internal standard for both constructs. (Bottom, 7b) Quantitative analysis of crosslinks formed with position 8 variants. For all results, $n=1$. The oxidation potential of guanine is from [72]. Oxidation potential of 7-deazaguanosine is from [74].

Figure 8. Mutation of the hairpin ribozyme to include 7-deazaguanosine at position 8 alters the reversal of the 8/-1 crosslink

The crosslinks were isolated and harvested after irradiation at 312 nm in 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ as described in Materials and Methods section 2.6. (Top) G8/A-1 crosslink reversal; the pH, metal composition and time of incubation at 25 °C of each sample are marked above the figure. The uncrosslinked noncleavable substrate is shown as a control in the last lane. Each sample was incubated in the presence of 0.1 mM ATA to reduce ribonuclease activity. None of the samples show reversal to uncrosslinked substrate under any condition. (Middle) Z8/A-1 crosslink reversal; the pH, metal composition and time of incubation at 25 °C of each sample are marked above the figure. The uncrosslinked noncleavable substrate is shown as a control in the last lane. Each sample was incubated in the presence of 0.1 mM ATA to reduce ribonuclease activity. (Bottom) Quantification of Z8/A-1 crosslink reversal under different pH and metal conditions (n=1). The fractional reversal of each sample was calculated as described in section 2.6.

Figure 9. The 7-deazaguanosine mutation exhibits marked metal ion concentration and identity dependencies for cleavage

Cleavage rate *versus* pH profile for the Z8 variant (uncrosslinked) of the hairpin ribozyme under four different conditions (12 and 30 mM Mg^{2+} and 0.1 and 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$). At pH 7, the Z8 variant was active for cleavage in magnesium(II) and $\text{Co}(\text{NH}_3)_6^{3+}$ in noncrosslinked form (0.14 times the rate of wild type in magnesium(II) and 1.9 times the rate of wild type in $\text{Co}(\text{NH}_3)_6^{3+}$). For all but the 12 mM Mg^{2+} values, the cleavage rate is an average of four separate experiments; for the 12 mM Mg^{2+} values, the rate is an average of three separate experiments.

Figure 10. Mutation of the hairpin ribozyme to include 7-deazaguanosine at position +2 also affects the yield of the crosslinks

The crosslinks were formed after irradiation at 312 nm as described in the text. The pH of each sample is between 5.0 and 9.0 and is identified at the top of each sample lane. The substrate (the cleavable SV5 z+2 variant or the noncleavable version of SV5) was labeled and is indicated above the pH indicator in each lane. The species are labeled end, 8/-1 and +2/36 to indicate the identities of the crosslinked bases.

Figure 11. The crosslinks exhibit marked pH dependencies

The 8/–1 crosslink versus pH for the wild-type and Z8 mutant. Both sets of data were normalized to the pH 8 value of each data set and were obtained with 312 nm excitation and 1 mM $\text{Co}(\text{NH}_3)_6^{3+}$ under the same conditions as described in Materials and Methods. The wild-type intensities are averaged between two separate crosslinking trials. Error bars indicate the standard deviations of the mean normalized crosslinking intensities of the crosslinked species. The Z8 mutant was the result of one trial only.

Figure 12. The photocrosslinking procedure described herein is mediated by $\text{Co}(\text{NH}_3)_6^{3+}$

Top left: The molecular orbital diagram of $\text{Co}(\text{NH}_3)_6^{3+}$ after irradiation at 312 or 365 nm.

The pink line indicates the excitation of the t_{1u} electron to the e_g^* level. The G8 base energy level is estimated and is located at the position of the base itself. Top middle: The postulated redox procedure, wherein an electron is donated from the donor base (here, G8) to the

excited $\text{Co}(\text{NH}_3)_6^{3+}$ molecule (the electron donation is marked with a thin green line). Top left: The oxidation of G8 results in a guanosine cation radical; the possible resulting radicals are from [77]. The guanosine cation radical forms a covalent bond with A-1 as shown by a thick green line, but the actual identity of the bond that forms between the two bases (transferable radical or true covalent bond) is unknown at this time. At the bottom, the

procedure is drawn in simplified terms. $\text{Co}(\text{NH}_3)_6^{3+}$ is depicted as a yellow sphere with a 3+ in the center; * denotes the excited metal ion complex. $\text{Co}(\text{NH}_3)_6^{2+}$ is depicted as a yellow sphere with a 2+ in the center. A green line indicates the new covalent bond formed between the bases.

Table 1
Crosslink activity varies in the tested mutants

Qualitative results of various studies with wild-type hairpin ribozyme or variants (n=1 unless mentioned otherwise in the text).

Variant	Results of Crosslink Sequencing	Ribozyme Activity of the Crosslinked Species
Wild-type (G8)	G8/a-1, u+2/G36	Yes (both)
Z8	Z8/a-1, u+2/G36	8/-1 no; +2/36 not tested
C8	C8/g+1; lower crosslink not sequenced	8/+1 no; lower crosslink not tested
A36	21/42, u+2/A36, g+1/RzA	-
c+2	c+2/G36	-
a+2	a+2/G36	-
Z36	u+2/Z36, u+2/G8	+2/36 yes; +2/8 no
I8	I8/g+1 (much decreased yield); lower crosslink not sequenced	8/+1 no; lower crosslink not tested
Purine 8	Purine 8 or A9/g+1 ^{§§} , u+2/G36 (both much decreased yield)	8/+1 unknown; +2/36 yes
U8	u+2/G36, g+1/A9	-
A8	g+1/A7, u+2/G36	-
z+2	G8/a-1, z+2/G36 (decreased yield)	Not tested
g+2	Much decreased yield, difficult to analyze results; appears to be +2/36	-

A '-' indicates that this study was not performed due to low yield of crosslinked species.

^{§§}The purine base utilized in the synthesis was in deoxyribose form and could not be sequenced. Mutation of position 8 to any base besides 7-deazaguanosine does not maintain the 8/-1 crosslink. None of these altered crosslinks were active. The +2/36 crosslink is maintained in the two tested mutants, but only one (Z36) retained activity.