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Unconventional Origin of Metal Ion Rescue in the Hammerhead Ribozyme Reaction: Mn^{2+}-Assisted Redox Conversion of 2′-Mercaptocytidine to Cytidine

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Abstract: A specific oxygen atom in RNA is identified as a ligand for a metal ion when sulfur substitution of that atom shifts the metal ion specificity of the RNA-mediated process to a more thiophilic metal. Extensive discussion and debate have centered around whether a metal ion activates the 2′-oxygen nucleophile during the phosphotransesterification reaction catalyzed by the hammerhead ribozyme (HH). To test this possibility, we probed the metal ion specificity of HH reactions using a substrate that contained 2′-mercaptocytidine at the cleavage site. This substrate is generated in situ from a disulfide-protected precursor by treatment with tris(carboxyethyl)phosphine (TCEP). In HH reactions with this substrate, phosphotransesterification does not occur when Mg^{2+} is present as the only divalent cation but does occur in the presence of Mn^{2+}. These results are consistent with a direct interaction between the metal ion and the nucleophile. However, further analysis reveals that this switch in metal ion specificity does not arise because Mn^{2+} coordinates sulfur more readily than Mg^{2+} does, but because under the assay conditions, the 2′-mercaptocytidine residue is converted to a mixture of cytidyl-1-β-D-arabinofuranoside and cytidyl-1-β-D-ribofuranoside, the natural substrate for the ribozyme. This conversion occurs in the absence of HH ribozyme, requires Mn^{2+} (or Co^{2+}), O_2, and TCEP, and is inhibited by a free radical scavenger. The mechanism presumably involves a multistep free radical process, in which the key step is homolytic fission of the C2′-sulfur bond induced by TCEP. The putative 2′-carbon radical then reacts with an oxygen species to produce the cytidyl-1-β-D-arabinofuranoside and ribocytidine products. To our knowledge, this chemical transformation is unprecedented in the literature and represents a new reaction for nucleic acids. If O_2 is excluded from the HH reactions, the 2′-sulfur is not modified in the presence of Mn^{2+} but is still blocked in the phosphotransesterification reaction, both in the forward direction as the mercapto (−SH) group or in the reverse direction as part of a cyclic phosphorothiolate. Although we are unable to provide evidence for metal ion activation of the nucleophile in the HH ribozyme reaction, this work establishes the groundwork for further use of 2′-mercaptonucleotides in biochemical analyses.

Introduction

Sulfur substitution of the oxygen atoms in DNA and RNA has revealed some of the most elusive, yet fundamental features of nucleic acid structure and function.[1] For example, a specific oxygen atom in RNA can be identified as a ligand for a metal ion when sulfur substitution of that atom shifts the metal ion specificity of the RNA-mediated process to a more thiophilic metal ion.[2,3] 2′-Mercaptonucleosides may possess similar potential to reveal the functional roles of the 2′-hydroxyl groups in RNA-mediated processes, including their roles as ligands for

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metal ions. Although 2′-mercaptonucleosides have been known for almost two decades,[4] they rarely have been exploited as biochemical probes,[5] possibly because there has been no convenient method for incorporating them into oligonucleotides. Additionally, early reports that the glycosidic linkage of 2′-mercaptonucleosides is highly labile under physiological conditions[6] may have discouraged biological chemists from using them.

We recently developed a method to incorporate 2′-mercaptonucleosides into oligonucleotides using phosphoramidite chemistry.[7] We found that oligonucleotides containing 2′-mercaptonucleosides are relatively stable under physiological conditions, although they do undergo slow fragmentation of the ribose phosphate backbone ($t_{1/2}$ = 3000 h)[7] by a two-step process—deglycosylation (step 1) through internal nucleophilic attack of sulfur at C-1′ to displace the heterocyclic base followed by backbone cleavage (step 2) of the abasic oligonucleotide.[8,9] To characterize further the behavior of 2′-mercapto oligonucleotides in biochemical reactions and to investigate their potential as probes for metal ion catalysis by enzymes and ribozymes, we examine herein the metal ion specificity of the hammerhead (HH) ribozyme reaction upon sulfur substitution of the 2′-nucleophile. In HH reactions with this substrate, phosphotransesterification does not occur when Mg^{2+} is present as the only divalent cation, but does occur in the presence of Mn^{2+}. However, this "rescue" of activity does not arise because of the ability of Mn^{2+} to directly coordinate sulfur, but because under the assay conditions, the 2′-mercaptocytidine residue is converted to a mixture of cytidyl-1-β-D-arabinofuranoside (aC) and cytidyl-1-β-D-ribofuranoside (rC), the natural substrate in the HH reaction. This conversion occurs in the absence of ribozyme and involves a multistep free radical process that requires Mn^{2+} (or Co^{2+}), O_2 and tris(carboxyethyl)phosphine. Although this process rendered the metal specificity switch inconclusive with respect to the mechanism of HH catalysis, our results underscore the importance of careful product analysis in biological assays, establish new experimental protocols for further use of 2′-mercaptonucleosides as biochemical probes, and reveal new, fundamental chemistry for this class of nucleotide analogues.

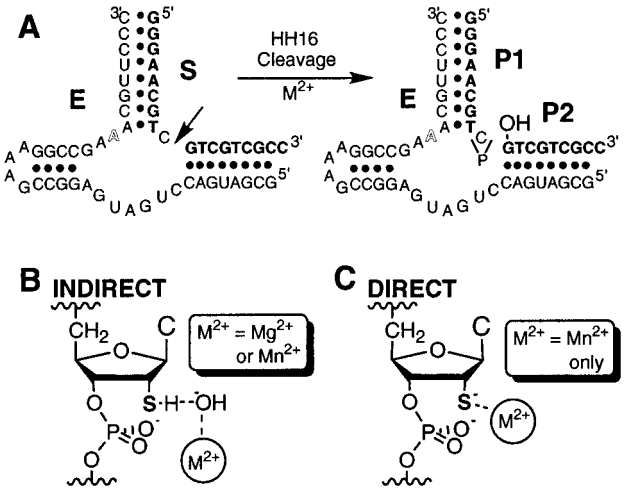

Figure 1. (A) The HH cleavage reaction. Primary and secondary structure of the hammerhead 16 ribozyme (**E**) and substrate (**S**). In the presence of divalent metal ions (M^{2+}) substrate cleavage occurs by a phosphotransesterification reaction, generating a 5′-product (**P1**) that contains a 2′,3′-cyclic phosphate, and a 3′-product (**P2**) that contains a 5′-hydroxyl group. The substrates used in this work contained all deoxyribonucleotides (bold) except at the cleavage site (indicated by the arrow). The Watson−Crick base pairs are denoted with dots. Replacement of A14 (bold outline) with G inactivates the ribozyme. Hammerhead-catalyzed cleavage of a modified substrate containing a 2′-mercapto group at the cleavage site is expected to be nonspecific for Mg^{2+} and Mn^{2+} if reaction occurs by the indirect mechanism (B), but specific for Mn^{2+} if reaction occurs by the direct mechanism (C).

Results and Discussion

The HH ribozyme is a small self-cleaving RNA derived from the genomes of several plant pathogens that replicate by a rolling circle mechanism.[10] To achieve strand cleavage, the ribozyme catalyzes a phosphotransesterification reaction in which a distinct 2′-hydroxyl attacks the adjacent phosphate to produce cleavage products containing 2′,3′-cyclic phosphate and 5′-hydroxyl termini (Figure 1A). Divalent metal ions are required for cleavage and are thought to play a direct role in catalysis through activation of the nucleophile and interactions with the scissile phosphodiester.[11] Two models for nucleophile activation have generally been considered:[12] (1) a metal-bound hydroxide abstracts the proton from the attacking 2′-hydroxyl, increasing the nucleophilicity of the oxygen, or (2) a metal ion coordinates directly to the 2′-hydroxyl, lowering the pK_a to provide a metal alkoxide, which is a stronger nucleophile than the corresponding alcohol. No study to date has provided compelling evidence to confirm or refute either model.

The two models might be distinguished by analyzing the metal ion specificity of a modified hammerhead substrate in which the nucleophilic 2′-hydroxyl group is replaced by a 2′-mercapto group. If an active-site metal hydroxide abstracts the

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(1) For examples see: (a) Sontheimer, E. J.; Steitz, J. *Science* **1993**, *262*, 1989. (b) Rao, T. S.; Durland, R. H.; Seth, D. M.; Myrick, M. A.; Bodepudi, V.; Revankar, G. R. *Biochemistry* **1995**, *34*, 765. (c) Woisard, A.; Favre, A.; Clivio, P.; Fourrey, J.-L. *J. Am. Chem. Soc.* **1992**, *114*, 10072. (d) Zhou, D.-M.; Usman, N.; Wincott, F. E.; Matulic-Adamic, J.; Orita, M.; Zhang, L.-H.; Komiyama, M.; Kumar, P. K. R.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 5862. (e) Kuimelis, R. G.; McLaughlin, L. W. *Biochemistry* **1996**, *35*, 5308. (f) Hancox, E. L.; Connolly, B. A.; Walker, R. T. *Nuc. Acids Res.* **1993**, *21*, 3485. (g) Herschlag, D.; Piccirilli, J. A.; Cech, T. R. *Biochemistry* **1991**, *30*, 4844. (h) Moore, M. J.; Sharp, P. A. *Nature* **1993**, *365*, 364. (i) McSwiggen, J. A.; Cech, T. R. *Science* **1989**, *244*, 679. (j) Rajagopal, J.; Doudna, J. A.; Szostak, J. W. *Science* **1989**, *244*, 692.

(2) (a) Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature* **1993**, *361*, 85. (b) Sontheimer, E. J.; Sun, S.; Piccirilli, J. A. *Nature* **1997**, *388*, 801. (c) Weinstein, L. B.; Jones, B. C. N. M.; Cosstick, R.; Cech, T. R. *Nature* **1997**, *388*, 805. (d) Sontheimer, E. J.; Gordon, P. M.; Piccirilli, J. A. *Genes Dev.* **1999**, *13*, 1729. (e) Yoshida, A.; Sun, S.; Piccirilli, J. A. *Nature Struct. Biol.* **1999**, *6*, 318. (f) Gordon, P. M.; Sontheimer, E. J.; Piccirilli, J. A. *RNA* **2000**, *6*, *199*.

(3) (a) Shan, S.; Yoshida, A.; Sun, S.; Piccirilli, J. A.; Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12299. (b) Yoshida, A.; Shan, S.; Herschlag, D.; Piccirilli, J. A. *Chem. Biol.* **2000**, *7*, 85.

(4) (a) Imazawa, M.; Ueda, T.; Ukita, T. *Chem. Pharm. Bull.* **1975**, *23*, 604. (b) Divikar, K. J.; Reese, C. B. *J. Chem. Soc., Perkin Trans. 1* **1982**, 1625.

(5) Coves, J.; Le Hir de Fallois, L.; Le Pape, L.; Decout, J.-L.; Fontecave, M. *Biochemistry* **1996**, *35*, 8595.

(6) (a) Patel, A. D.; Schrier, W. H.; Nagavary, J. *J. Org. Chem.* **1980**, *45*, 5, 4830. (b) Johnson, R.; Reese, C. B.; Pei-Zhuo, Z. *Tetrahedron* **1995**, *51*, 5093.

(7) Hamm, M. L.; Piccirilli, J. A. *J. Org. Chem.* **1997**, *62*, 3415.

(8) Reese, C. B.; Simons, C.; Pei-Zhou, Z. *J. Chem. Soc., Chem. Commun.* **1994**, 1809.

(9) Dantzman, C. L.; Kiessling, L. L. *J. Am. Chem. Soc.* **1996**, *118*, 11715.

(10) Symons, R. H. *Annu. Rev. Biochem.* **1992**, *61*, 641.

(11) Pyle, A. M. *Science* **1993**, *261*, 709.

(12) Pontius, B. W.; Lott, W. B.; von Hippel, P. H. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 2290.

proton from the 2′-nucleophile (Figure 1B), then HH-catalyzed cleavage of the 2′-mercapto substrate should proceed with similar efficiency in the presence of either Mg^{2+} or Mn^{2+}, relative to the unmodified substrate. Alternatively, if catalysis requires direct metal ion coordination to the 2′-nucleophile (Figure 1C), cleavage of the 2′-mercapto substrate is expected to be more efficient in Mn^{2+} than in Mg^{2+} because Mn^{2+} coordinates to sulfur much more effectively than does Mg^{2+}.[13,14]

The HH Catalyzes Phosphotransesterification of dHH16SH in the Presence of Mn^{2+}. We synthesized two substrates (5′dGGGAACGTC$_{2'X}$GTCGTCGCC3′) for the HH16 ribozyme, each containing 2′-deoxynucleotides (bold in Figure 1) at all positions except for a ribocytidine (X = OH; dHH16OH) or a 2′-mercaptocytidine (X = SH; dHH16SH) at the cleavage site. To maintain the integrity of the mercapto group, dHH16SH was radiolabeled and stored as the 2′-(2-pyridyl)dithio derivative (dHH16SSpyr).[7] A well-known disulfide cleaving agent, tris-(carboxytheyl)phosphine (TCEP),[15] was added to each HH reaction to generate in situ the free 2′-mercapto group. We chose the HH16 ribozyme because it is well characterized kinetically and thermodynamically,[16,17] and we used substrates in the 2′-deoxyribose background to simplify product analysis. In general, HH substrates that contain 2′-deoxynucleotides at all positions except at the cleavage site react about an order of magnitude more slowly than their all-ribose counterparts.[18] In the case of HH16, the cleavage rate of dHH16OH in the presence of saturating ribozyme (50 mM Tris-HCl, pH 7.5; 10 mM Mg^{2+}) was roughly 30-fold slower (data not shown) than that reported for reaction of an all-ribose substrate under similar conditions.[16]

The oligonucleotide substrates were radiolabeled at their 3′-ends using [α-^{32}P] cordycepin (Co; 3′-deoxyadenosine) triphosphate and terminal deoxynucleotidyl transferase. We carried out HH reactions in 50 mM NaMOPS (pH 7.5) instead of the more commonly used Tris-HCl buffer because the latter is known to complex divalent metal ions.[13] In the presence of saturating ribozyme and either Mg^{2+} or Mn^{2+}, dHH16OH cleaved to give a single radiolabeled product **P2** (Figure 2, lanes 8 and 9), which comigrated with an independently synthesized standard 5′dGTCGTCGCC*pCo3′ (Standard A, lane 10; *p indicates ^{32}P-phosphate) in a denaturing 20% polyacrylamide gel. No product formed in control reactions lacking divalent metal ions (Figure 2, lane 7) or ribozyme (Figure 2, lanes 2−4), or in reactions with an inactive mutant ribozyme (A14G)[19] in which the essential A14 (see Figure 1A) was replaced with G (Figure 2, lanes 5 and 6).

In the absence of HH, 3′-radiolabeled dHH16SH underwent slow fragmentation to give a single radiolabeled product **F2** (Figure 2, lanes 17−19) that comigrated with the independently synthesized standard, 5′p-dGTCGTCGCC*pCo3′ (Standard B; lane 11). In the presence of Mg^{2+} (or Ca^{2+}; see Supporting Information), the HH accelerates the fragmentation of dHH16SH by ~10^2-fold, but no product **P2** was formed, even at high pH, temperature or Mg^{2+} concentration (Figure 2, lane 13).[7] In the presence of Mn^{2+} (or Co^{2+}; see Supporting Information)

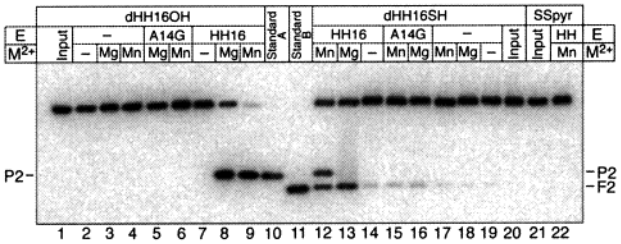


Figure 2. HH reactions of 3′-radiolabled substrates in the presence of Mg^{2+} and Mn^{2+}. Substrates (dHH16OH, lanes 1−9; dHH16SH, lanes 12−20; dHH16SSpyr, lanes 21−22) were incubated in 50 mM NaMOPS (pH 7.5) and 1 mM TCEP (except dHH16SSpyr) for 20 h at 25 °C with no exogenous divalent metal ions, 10 mM $MgCl_2$, or 10 mM $MnCl_2$ as indicated. Reactions contained either no ribozyme (lanes 2−4 and 17−19), inactive A14G mutant ribozyme (lanes 5, 6, 15, and 16), or wild-type ribozyme (lanes 7−9, 12−14, and 22). The products were compared to independently synthesized Standards A (5′dGTC-GTCGCC*pCo3′; lane 10) and B (5′p-dGTCGTCGCC*pCo3′; lane 11) as described in the text. Lanes labeled "Input" contain the substrates before incubation. dHH16Sspyr is a derivative of dHH16SH in which the 2′-mercapto group is protected as the 2-pyridyl disulfide.

however, the ribozyme not only accelerated the fragmentation reaction but also catalyzed the formation of an additional product (Figure 2, lane 12) that had the same gel mobility as **P2** from dHH16OH (lanes 8 or 9). Neither this product nor the acceleration of the fragmentation reaction was observed in control reactions lacking divalent metal ions (Figure 2, lane 14) or ribozyme (lanes 17−19), or in reactions with the inactive A14G ribozyme (lanes 15 and 16). The disulfide-protected derivative, dHH16SSpyr (Figure 2; lane 21), formed neither product upon incubation with active hammerhead and Mn^{2+} (lane 22), suggesting that the free 2′-mercapto group was required for both reactions.

The products of phosphotransesterification and fragmentation were isolated and examined by partial S1 nuclease digestion. The S1 ladder of **P2** obtained from dHH16SH (Figure 3A, lane 9) was identical to that of **P2** derived from dHH16OH (lane 6) and Standard A (lane 2). The S1 ladder of the fragmentation product **F2** from dHH16SH (Figure 3A, lane 12) was identical to Standard B (lane 4). Moreover, **F2** could be converted to a product that has the same mobility as **P2** and Standard A by phosphatase treatment (Figure 3A; lane 13). Conversely, **P2** could be converted to a product that has the same mobility as **F2** and Standard B by kinase treatment (Figure 3A, lanes 7 and 10). These results indicate that **F2** differs from **P2** by the presence of a 5′-phosphate and establishes their identities as p-dGTCGTCGCC*pCo and dGTCGTCGCC*pCo, respectively (Scheme 1).

Results of ribozyme and control reactions with 5′-radiolabeled dHH16OH and dHH16SH paralleled those with the 3′-radiolabeled substrates: only in the presence of active HH and Mn^{2+} did dHH16SH yield a radiolabeled product that comigrated with the product from dHH16OH (data not shown). These products (dHH16-P1), which are expected to contain a 2′,3′-cyclic phosphate at their 3′-termini, gave identical S1 nuclease ladders (Figure 3B, lanes 2 and 4), consistent with strand cleavage of dHH16SH and dHH16OH at the same site. However, we were unable to monitor by gel electrophoresis the opening of the cyclic phosphate following treatment with 0.1 N NaOH at 95 °C for 30 min, conditions known to hydrolyze 2′,3′-cyclic phosphates. Apparently, the nine-nucleotide cyclic phosphate has the same gel mobility as the corresponding 2′- or 3′-phosphomonoester products. To circumvent this, we used the shorter HH8 system[20] and isolated the 5′-cleavage products

(13) Sigel, R. K. O.; Song, B.; Sigel, H. *J. Am. Chem. Soc.* **1997**, *119*, 744.
(14) (a) Jaffe, E. K.; Cohn, M. *J. Biol. Chem.* **1979**, *254*, 10839. (b) Pecoraro, V. L.; Hermes, J. D.; Cleland, W. W. *Biochemistry* **1984**, *23*, 5262.
(15) Burns, J. A.; Butler, J. C.; Moran, J.; Whitesides, G. M. *J. Org. Chem.* **1991**, *56*, 2648.
(16) Hertel, K. J.; Herschlag, D.; Uhlenbeck, O. C. *Biochemistry* **1994**, *33*, 3374.
(17) Baidya, N.; Uhlenbeck, O. C. *Biochemistry* **1997**, *36*, 1108.
(18) Dahm, S.; Uhlenbeck, O. C. *Biochimie* **1990**, *72*, 819.
(19) Ruffner, D. E.; Stormo, G. D.; Uhlenbeck, O. C. *Biochemistry* **1990**, *27*, 10695.

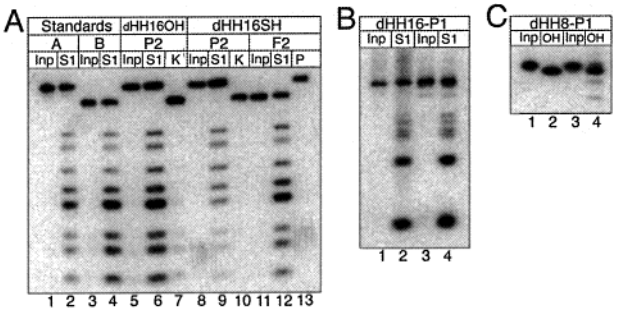
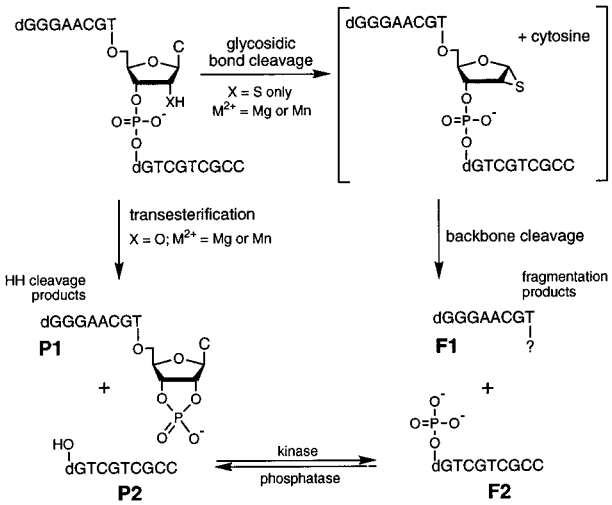


Figure 3. (A) Identification of the 3′-products of fragmentation and phosphotransesterification. Standards A and B and cleavage products from ribozyme reactions with dHH16OH (**P2**) and dHH16SH (**P2** and **F2**) were partially digested with S1 nuclease (lanes 2, 4, 6, 9, and 12). Incubation with ATP and polynucleotide kinase (K) converted **P2** from dHH16OH and dHH16SH to faster migrating species (lanes 7 and 10, respectively) that comigrated with **F2** and Standard B. Incubation with calf intestine alkaline phosphatase (P) converted **F2** to a slower migrating species (lane 13) that comigrated with **P2** and Standard A. (B) Nuclease digestion of the 5′-products from phosphotransesterification. The products (dHH16-P1) from ribozyme catalyzed cleavage of 5′-radiolabeled dHH16OH (lane 1−2) and dHH16SH (lane 3−4) in the presence of Mn^{2+} were partially digested with S1 nuclease (lanes 2 and 4, respectively). (C) Evidence for cyclic phosphate termini. Products from HH8 cleavage of 5′-radiolabeled dHH8OH (lanes 1−2) and dHH8SH (lanes 3−4) were treated with 0.1 N NaOH at 95 °C for 10 min (lanes 2 and 4, respectively). **P2** and **F2** are defined in Scheme 1. Lanes labeled "Inp" contain input oligonucleotide samples before incubation.

Scheme 1



(dHH8-P1), which are only six nucleotides in length. Treatment with base converted these products to slightly faster-migrating species (Figure 3C, lanes 2 and 4), consistent with the opening of a cyclic phosphate to a monophosphate.

These results show prima facie evidence for a metal specificity switch with dHH16SH. It is tempting to conclude from the data that the HH employs a metal ion to activate the nucleophile (Figure 1C). However, further characterization of the reaction of dHH16SH in Mn^{2+} reveals that the chemical basis for the switch in metal specificity is more complex than these initial experiments would suggest.

(20) We also performed the entire set of experiments for the HH8 system as described in the text for the HH16 system. The HH8 ribozyme[17] was $^{5'}$CGACCCUGAUGAGGCCGAAAGGCCGAAACAUU$^{3'}$, and the substrates were $^{5'}$AAUGUC$_x$GGUCG$^{3'}$, where C$_x$ is ribocytidine or 2′-mercaptocytidine. Results with both 5′- and 3′-^{32}P-labeled substrates closely paralleled those for HH16.

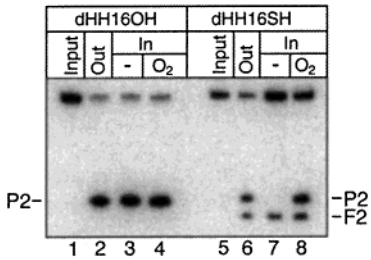


Figure 4. The phosphotransesterification reaction of dHH16SH requires O_2. Substrates (dHH16OH, lanes 1−4; dHH16SH, lanes 5−8) were incubated for 24 h at 25 °C with 1 μM HH, 10 mM Mg^{2+}, 50 mM NaMOPS (pH 7.5), and 1 mM TCEP. All solutions were degassed with nitrogen before use. Reactions were initiated in an anaerobic nitrogen chamber and incubated either in the air (Out; lanes 2 and 6) or in the nitrogen chamber with (In; lanes 4 and 8) or without (lanes 3 and 7) added O_2.

In Mn^{2+} the HH Catalyzes the Formation of P2 from dHH16SH only when Oxygen is Present. With the hope of obtaining direct evidence that a metal ion lowers the pK_a of the 2′-mercapto group, we intended to characterize the phosphotransesterification reaction of dHH16SH by determining the pH dependence of **P2** formation in the presence of different metal ions. However, because softer metal ions such as Mn^{2+} and Co^{2+} oxidize readily at high pH,[21] we carried out reactions in a nitrogen chamber. The exclusion of oxygen (O_2) had no effect on the reaction of dHH16OH in the presence of HH and Mn^{2+} (Figure 4, compare lanes 2 and 3), but remarkably, with dHH16SH there was a complete loss of **P2** formation (Figure 4, compare lanes 6 and 7). The difference in reactivity can be attributed directly to the absence of oxygen because addition of oxygen to the chamber environment restored **P2** formation (Figure 4, lane 8).[22] Only phosphotransesterification of dHH16SH was affected by the exclusion of oxygen; the rate of fragmentation of dHH16SH remained unchanged (data not shown). The different reaction requirements for phosphotransesterification of dHH16OH and dHH16SH in Mn^{2+} suggest that the two reactions occur through different mechanisms and raise the possibility that an O_2-dependent modified form of dHH16SH, and not dHH16SH itself, gives rise to **P2**. Additionally, the lack of **P2** formation in the absence of oxygen indicates that Mn^{2+} is not able to rescue HH-catalyzed phosphotransesterification of dHH16SH (Figure 4).

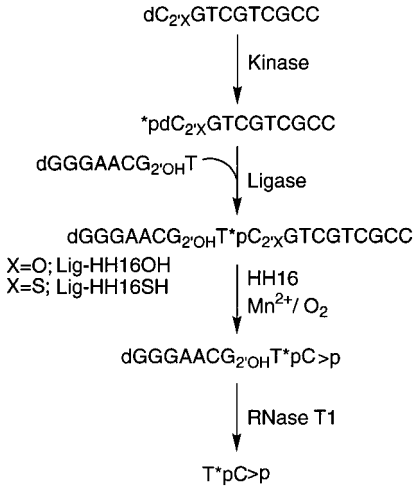
If dHH16SH itself does not give rise to **P2**, then what does? The answer to this question could reveal new and interesting chemistry of nucleic acids and could provide clues about the behavior of 2′-mercaptonucleotides under biochemical conditions. This information, in turn, may ultimately help to decipher what features of nucleic acid structure and function are testable with 2′-mercaptonucleotides. Accordingly, we developed strategies and tools to characterize 2′-mercapto oligonucleotides and the potential products they give rise to. We began by attempting further characterization of the 5′-phosphotransesterification products of dHH16SH.

The 5′-Phosphotransesterification Product from dHH16SH Does Not Contain a 2′-*S*,3′-*O*-Cyclic Phosphorothiolate.

(21) Dahm, S.; Derrick, W. B.; Uhlenbeck, O. C. *Biochemistry* **1993**, *32*, 13040.

(22) The reaction of dHH16SH was highly sensitive to the presence of oxygen. Consequently, we took extreme care to remove diatomic oxygen completely; all solutions were degassed with nitrogen and kept in an anaerobic environment at all times. Even with these precautions however, it was necessary on occasion to add an oxygen scavenger (0.1 mM) such as $Na_2S_2O_3$ (see: Lam, X. M.; Yang, J. Y.; Cleland, J. C. *J. Pharm. Sci.* **1997**, *86*, 1250) or $Na_2S_2O_4$ (see: Magnuson, J. K.; Paustian, T. D.; Shah, V. K.; Dean, D. R.; Roberts, G. P.; Rees, D. C.; Howard, J. B. *Tetrahedron* **1997**, *53*, 11971) to abolish **P2** formation completely.

Scheme 2



Although PAGE analysis of the six-nucleotide product dHH8-P1 after exposure to basic conditions suggested ring opening of a cyclic phosphate product (Figure 3C), the oligonucleotide was too long for single-atom differences to be detected by PAGE. Thus, we developed a strategy by which we could analyze the 3′-terminus of the 5′-product in a dinucleotide background, a size that might allow us to detect modifications in the cyclic phosphate product more easily. As outlined in Scheme 2, we synthesized two dHH16 substrates (dGGGAAC-$G_{2'OH}$T*p$C_{2'X}$GTCGTCGCC) that contained a radiolabeled phosphate (*p) immediately 5′ of the cleavage site, and a riboguanosine residue ($G_{2'OH}$) two nucleotides 5′ of the cleavage site. Analogous to dHH16OH and dHH16SH, the substrates contained either ribocytidine (X = OH; Lig-HH16OH) or a 2′-mercaptocytidine (X = SH; Lig-HH16SH) at the cleavage site. HH cleavage of these substrates in the presence of Mn^{2+} and O_2 followed by digestion with RNase T1 generated radiolabeled dinucleotide products ($^{5'}$T*pC>p$^{3'}$) that were isolated by PAGE (Scheme 2). The dinucleotide products of Lig-HH16OH and Lig-HH16SH should terminate with a 2′,3′-*O*-cyclic phosphate or a 2′-*S*,3′-*O*-cyclic phosphorothiolate if oxygen or sulfur, respectively, acts as the nucleophile during phosphotransesterification.

Similar to results for the 5′ products of dHH8-P1 (Figure 3C), the dinucleotide products derived from Lig-HH16OH and Lig-HH16SH comigrated during PAGE analysis (Figure 5, lanes 1 and 5). Additionally, base treatment (0.1 M NaOH, 90 °C, 10 min) converted them to faster comigrating bands (Figure 5, lanes 2 and 6), consistent with the opening of a cyclic phosphate to a monophosphate. Incubation of these monophosphates with calf intestine alkaline phosphatase (CIAP) generated slower comigrating bands, consistent with the loss of the phosphate (Figure 5, lanes 3 and 7). If the 2′-sulfur acts as the nucleophile during phosphotransesterification, the CIAP product of the dinucleotide isolated from Lig-HH16SH should contain a free 2′-mercapto group and therefore should react with iodoacetamide, a well-known thiol-modifying agent.[23] However, this product, like the dinucleotide isolated from Lig-HH16OH, was inert to iodoacetamide modification (Figure 5, lanes 4 and 8),[24] inconsistent with the presence of a free mercapto group. Additionally, both the cyclic phosphate and monophosphate derivatives of the dinucleotide derived from Lig-HH16SH should contain an S−P bond;[25] however, both derivatives were inert to treatment with silver and iodine, two reagents that are known to cleave S−P bonds (data not shown).[26]

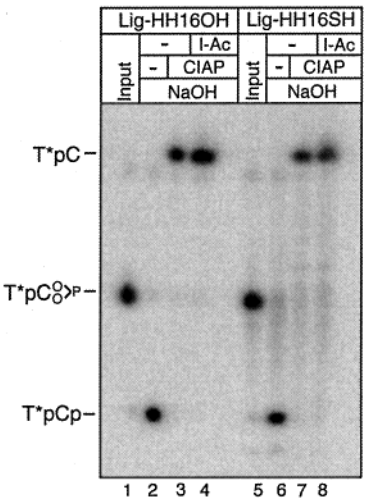


Figure 5. Characterization of the dinucleotide products derived from Lig-HH16OH (lanes 1−4) and Lig-HH16SH (lanes 5−8). The dinucleotides were obtained by incubating the substrates with HH/Mn^{2+} followed by RNase T1 digestion (Scheme 2). They were then incubated for 10 min at 90 °C with 0.1 M NaOH (lanes 2 and 5), neutralized with HCl, treated with calf intestine alkaline phosphatase (CIAP) for 1 h at 37 °C (lanes 3 and 7), and incubated with 20 mM iodoacetamide (I-Ac) for 20 min at 30 °C (lanes 4 and 8). Lanes labeled "Input" contain the dinucleotide products before incubation.

At the time of this study, the synthesis of oligonucleotides terminating with a 2′-*S*,3′-*O*-cyclic phosphorothiolate moiety had not been reported. To obtain authentic standards for comparison to the Lig-HH16SH-derived products, we developed a strategy for the synthesis of oligonucleotides containing 2′-*S*,3′-*O*-cyclic phosphorothiolate termini.[27] We synthesized a dinucleotide terminating in a 2′-*S*,3′-*O*-cyclic phosphorothiolate and examined its response to acid, base, and sulfur specific reagents such as iodoacetamide and silver.[27] This authentic standard behaved differently than the product dinucleotide derived from Lig-HH16SH, but similarly to previously characterized phosphorothiolates; it migrated more slowly than its phosphate counterpart and was labile to silver treatment.[27] These results strongly question the presence of an S−P bond in the isolated dinucleotide product from Lig-HH16SH and further diminish the possibility that sulfur acts as the nucleophile during phosphotransesterification, even though the data suggest that the dinucleotide terminates with some type of cyclic phosphate. Curiously, the dinucleotide cyclic phosphate products from Lig-HH16OH and Lig-HH16SH undergo base-catalyzed ring opening at the same rate. This finding, along with the strikingly similar behavior of the dinucleotide products in both reactivity and PAGE mobility, hints at the possibility that these products may have precisely the same identity, both terminating in a 2′,3′-*O*-cyclic phosphate. This, in turn, would imply that the 2′-sulfur of dHH16SH is somehow modified to a 2′-oxygen atom during incubation with Mn^{2+} and HH.

dHH16SH Is Modified by Mn^{2+} and O_2 in the Absence of HH. We preincubated dHH16SH with Mn^{2+} in the absence

(23) Coleman, R. S.; Kesicki, E. A. *J. Am. Chem. Soc.* **1994**, *116*, 11636.
(24) Modification of the sulfur by iodoacetamide should result in a slower migrating band.[23]
(25) Basic conditions cleave preferentially the O−P bond over the S−P bond in cyclic phosphorothiolates.[27]
(26) (a) Vyle, J. S.; Connolly, B. A.; Kemp, D.; Cosstick, R. *Biochemistry* **1992**, *31*, 3012. (b) Cosstick, R.; Vyle, J. S. *Tetrahedron Lett.* **1990**, *18*, 829.
(27) Hamm, M. L.; Piccirilli, J. A. *J. Org. Chem.* **1999**, *64*, 5700.

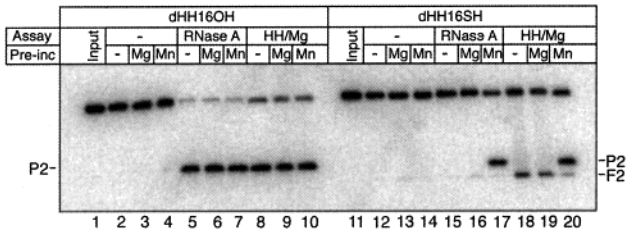


Figure 6. Mn^{2+} renders dHH16SH susceptible to cleavage by RNase A and the HH. dHH16OH and dHH16SH were incubated for 24 h in buffer containing 50 mM NaMOPS (pH 7.5), 1 mM TCEP, and either no exogenous metal ions, 10 mM Mg^{2+} or 10 mM Mn^{2+} as indicated. After 24 h 15 mM EDTA was added, and each reaction was divided into thirds: one-third was loaded directly onto the gel (lanes 2−4 and 12−14), one-third was incubated with RNase A for 6 h at room temperature (lanes 5−7 and 15−17), and one-third was incubated with HH and Mg^{2+} for 14 h at 25 °C (lanes 8−10 and 18−20). Lanes labeled "Input" contain the substrates before incubation.

of HH to determine whether modification of dHH16SH requires ribozyme. We then treated the products of the preincubation reactions with RNase A or Mg^{2+}-activated HH to test if the cleavage site 2′-mercaptocytidine is modified to a ribocytidine during Mn^{2+} treatment. Preincubation in 1 mM TCEP, 10 mM Mn^{2+}, and 50 mM NaMOPS (pH 7.5) at 25 °C converted dHH16SH (at a rate of 8×10^{-3} min^{-1}) to a species that was a substrate for phosphotransesterification with both RNase A and Mg^{2+}-activated HH (Figure 6, lanes 17 and 20). In contrast, control reactions in which the preincubation was carried out in the absence of divalent metal ions or in the presence of 10 mM Mg^{2+} produced no such species upon addition of RNase A or HH (Figure 6, lanes 14−15 and 18−19). Additionally, we found that both RNase A and the HH cleaved the product of the dHH16SH preincubation at the same rate as dHH16OH, although the extent of reaction was ∼20% less (data not shown). These results suggest that, in the presence of oxygen, Mn^{2+}, and buffer, dHH16SH is converted to a species that, analogous to dHH16OH, contains a ribocytidine at the cleavage site.

Incubation with Mn^{2+} Converts 2′-Mercaptocytidine to Cytidyl-1-β-D-ribofuranoside (rC) and Cytidyl-1-β-D-arabinofuranoside (aC). We obtained definitive evidence for the Mn^{2+}-catalyzed conversion of 2′-mercaptocytidine to rC through reverse phase high-pressure liquid chromatography (RP-HPLC) and mass spectral analysis. We synthesized 2′-mercaptocytidine-(3′→5′)-2′-deoxyguanosine ($^{5'}C_{2'SH}pdG^{3'}$) as described previously,[7] and analyzed its reactivity in Mn^{2+} by RP-HPLC. Incubation with 10 mM Mn^{2+}, 1 mM TCEP and 50 mM NaMOPS (pH 7.5) for 12 h at 25 °C converted $^{5'}C_{2'SH}pdG^{3'}$ (Figure 7A) to two new peaks in an 80:20 ratio (Figure 7B). The larger peak had a faster mobility and coeluted with cytidine-(3′→5′)-2′-deoxyguanosine ($^{5'}$rCpdG$^{3'}$; Figure 7C), and the smaller peak, which migrated more slowly, coeluted with cytidyl-β-D-arabinofuranoside-(3′→5′)-2′-deoxyguanosine ($^{5'}$aCpdG$^{3'}$; Figure 7D). Furthermore, mass spectral analysis revealed that the two peaks had the same mass (m/z (MH^-) = 571; Figure 8B) as the two standards (data not shown and Figure 8C). The results show that Mn^{2+} converts $^{5'}C_{2'SH}pdG^{3'}$ to $^{5'}$rCpdG$^{3'}$ and $^{5'}$aCpdG$^{3'}$ in an 80:20 ratio. The biochemical data suggest a similar conversion ratio for dHH16SH. The extent of HH-catalyzed phosphotransesterification was only 80% of that with dHH16OH, presumably because Mn^{2+} converts 20% of the substrate to the unreactive cytidyl-β-D-arabinofuranoside derivative (dHH16aOH; $^{5'}$dGGGAACGTaCGTCGTCGCC$^{3'}$, where aC is cytidyl-β-D-arabinofuranoside).

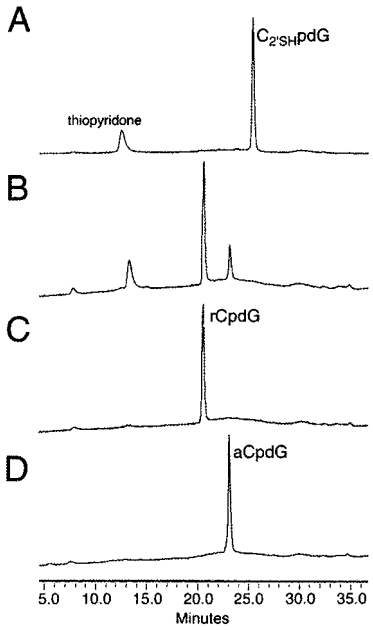


Figure 7. RP-HPLC analysis of $^{5'}C_{2'SH}pdG^{3'}$ following incubation with Mn^{2+}. $^{5'}C_{2'SH}pdG^{3}$ was incubated for 12 h at 25 °C in 50 mM NaMOPS (pH 7.5) and 1 mM TCEP with either no divalent metal ions (A) or 10 mM Mn^{2+} (B). Products were compared to authentic standards of $^{5'}$rCpdG$^{3'}$ (C) and $^{5'}$aCpdG$^{3'}$ (D). The peak at 13 min in (A) and (B) is thiopyridone and results from deprotection of the 2′-mercaptocytidine with TCEP.[7]

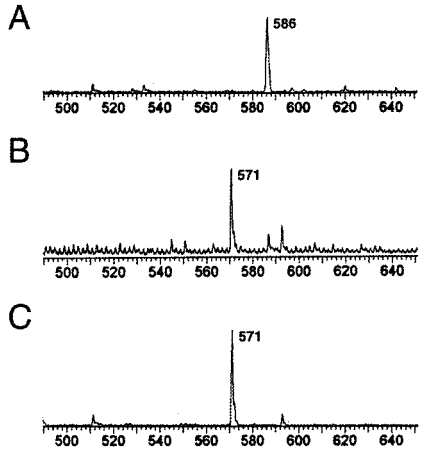


Figure 8. Negative ion electrospray mass spectra of $^{5'}C_{2'SH}pdG^{3'}$. (A) $^{5'}C_{2'SH}pdG^{3'}$, (B) $^{5'}C_{2'SH}pdG^{3'}$ incubated in 50 mM NaMOPS (pH 7.5), 1 mM TCEP, and 10 mM Mn^{2+} for 12 h at 25 °C, and (C) $^{5'}$rCpdG$^{3'}$ following purification by RP-HPLC. For (A) MH^- m/z = 586 due to disulfide formation following purification.

Modification of the 2′-Mercapto Group Can Be Monitored by APM-PAGE. We could also monitor modification of the mercapto group directly through PAGE analysis if the acrylamide contained [(*N*-acryloylamino)phenyl]mercuric chloride (APM).[28] The mercury present in APM has a high affinity for sulfur and retards oligonucleotides containing 2′-mercaptonucleotides relative to natural RNA. While dHH16OH and dHH16SH migrate similarly during normal PAGE (Figure 2, lanes 1 and 20), dHH16SH migrates more slowly than dHH16OH during APM-PAGE (Figure 9, lanes 1 and 2). Consistent with the necessity of a free 2′-mercapto group for retarded migration during APM-PAGE, the mobility of dHH16SH was no longer retarded after modification (20 min at 30 °C) with iodoacetamide

(28) Igloi, G. L. *Biochemistry* **1988**, *27*, 3842.

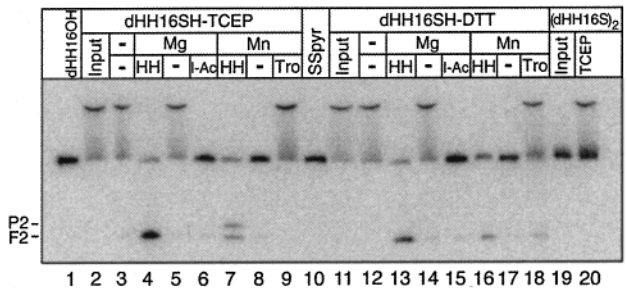


Figure 9. Monitoring the modification of dHH16SH by APM-PAGE. APM retards the gel mobility of oligonucleotides containing 2′-mercapto groups. All samples were incubated for 24 h at 25 °C and contained 3′-radiolabeled dHH16SH, 50 mM NaMOPS (pH 7.5), and either 1 mM TCEP (lanes 2−9) or DTT (lanes 11−18). Divalent metal ions, 10 mM Mg^{2+} or Mn^{2+}, were added as indicated. Reactions containing Mg^{2+} were incubated with no added reagents (lanes 5 and 14) before incubation with 20 mM iodoacetamide (I-Ac; lanes 6 and 15), or were incubated with 1 μM HH (lanes 4 and 13). Reactions containing Mn^{2+} were incubated with either 1 μM HH (lanes 7 and 16), no additional reagent (lanes 8 and 17), or 1 mM Trolox and 10% ethanol (Tro); lanes 9 and 18). Control reactions incubated with 10% ethanol gave the same results as those lacking ethanol (data not shown). dHH16SH oxidized to the disulfide $(dHH16S)_2$ following purification (lane 19), but could be regenerated upon addition of 1 mM TCEP (lane 20). dHH16OH (lane 1) and dHH16SSpyr (a derivative of dHH16SH in which the 2′-mercapto group is protected as the 2-pyridyl disulfide; lane 10) are shown for comparison. The gels contained 0.75 μg of APM for each mL of 20% acrylamide. Immediately before gel loading, 10 mM DTT was added to all samples except those in lanes 1, 10, and 19. This enabled more uniform electrophoresis of the products, but did not affect the results of this study. Lanes labeled "Input" contain oligonucleotide samples before incubation.

(I-Ac; Figure 9, lane 6). Incubation without divalent metal ions or with Mg^{2+} did not alter the mobility of dHH16SH (Figure 9, lanes 3 and 5), suggesting that under these conditions dHH16SH remains intact. However, incubation of dHH16SH in the presence of Mn^{2+} and O_2 converted it to a new species (Figure 9, lane 8) that migrated similar to dHH16OH (lane 1), indicating that the 2′-mercapto group was modified. These results demonstrate that APM-PAGE can be used to monitor the fate of the 2′-mercapto group of dHH16SH. We therefore used this assay to probe further the mechanism of the mercaptan modification.

A Free Radical Scavenger Inhibits Modification of the 2′-Mercapto Group. To test whether the modification of dHH16SH proceeds through a free radical intermediate, we preincubated dHH16SH in the presence of Trolox, a free radical scavenger.[29] Addition of 1 mM Trolox inhibited modification of dHH16SH in the presence of Mn^{2+} (Figure 9, lane 9), consistent with a mechanism that involves free radicals.

One-electron oxidants such as Mn^{3+} and Co^{3+} can catalyze the autoxidation of a sulfide to a sulfur radical.[30] These highly active species are formed from Mn^{2+} and Co^{2+} by reaction with oxygen as shown:

$$M^{2+} + O_2 \rightarrow M^{3+} + O_2^{-} \quad (1)$$

$$M^{3+} + RS^{-} \rightarrow M^{2+} + RS\bullet \quad (2)$$

To test whether modification of the 2′-mercapto group could proceed through a similar mechanism, we incubated dHH16SH and $^{5'}C_{2'SH}dpG^{3'}$ with Mn^{3+}. Products formed in the presence of Mn^{3+} paralleled those formed in the presence of Mn^{2+}: $^{5'}C_{2'SH}dpG^{3'}$ was converted to both $^{5'}rCpdG^{3'}$ and $^{5'}aCpdG^{3'}$, and dHH16SH was converted to an RNase A-labile product, presumably dHH16OH (data not shown). Additionally, the modification reaction occurred in a variety of buffers, but the rate decreased as the pH of the buffer decreased; the modification did not occur in the absence of buffer, presumably because of the low pH of an unbuffered solution (data not shown).

TCEP Facilitates the Loss of Sulfur. In all of the reactions described so far, we generated the 2′-mercapto group in situ by adding tris(carboxyethyl)phosphine (TCEP) just before initiation of the reaction. However, we can also generate the free mercapto group using dithiothreitol (DTT), which cleaves disulfide bonds via disulfide exchange chemistry.[31] In the absence of Mn^{2+}, dHH16SH generated by incubation with DTT behaved in a manner similar to dHH16SH generated by incubation with TCEP; it was still retarded in APM-PAGE relative to dHH16OH (Figure 9, compare lanes 3 and 14), and the HH catalyzed its fragmentation with an efficiency similar to that of the TCEP generated substrate (compare lanes 4 and 13). In contrast, when Mn^{2+} was present, dHH16SH generated with DTT behaved differently than dHH16SH generated with TCEP; HH-catalyzed fragmentation proceeded to a lesser extent (Figure 9, compare lanes 7 and 16), and although incubation in Mn^{2+} and DTT for 9 h caused dHH16SH to shift its APM-PAGE gel mobility to a band that comigrated with dHH16OH (Figure 9, lane 17), it did not give rise to **P2** in the presence of HH (lane 16). These observations suggest that incubation of dHH16SH with Mn^{2+} and DTT modifies dHH16SH to a species other than dHH16OH. To obtain further insight into the modification, we analyzed the behavior of the dinucleotide, $^{5'}C_{2'SH}pdG^{3'}$, in the presence of Mn^{2+} and DTT. RP-HPLC showed that rather than reacting to $^{5'}rCpdG^{3'}$ and $^{5'}aCpdG^{3'}$ as observed during TCEP/Mn^{2+} incubation, the dinucleotide is oxidized during DTT/Mn^{2+} incubation to the symmetrical disulfide, $(^{5'}C_{2'S}dpG^{3'})_2$ (Scheme 3).[32] These results suggest that during DTT/Mn^{2+} incubation, dHH16SH is oxidized to the disulfide $(dHH16S)_2$, which is inert both to interaction with the mercury in APM-PAGE and to the fragmentation and phosphotransesterification reactions catalyzed by the HH. Additionally, we found that at 25 °C in 1 mM DTT, 50 mM NaMOPS (pH 7.5) and 10 mM Mn^{2+}, dHH16SH was oxidized to $(dHH16S)_2$ with a half-life of 58 min. Consistent with disulfide formation, addition of TCEP following DTT/Mn^{2+} incubation restored the retarded mobility of dHH16SH during APM-PAGE (data not shown).

Why does incubation with DTT/Mn^{2+} convert dHH16SH to the disulfide, whereas incubation with TCEP/Mn^{2+} converts the 2′-mercaptocytidine residue to a mixture of rC and aC? An intriguing answer to this question was suggested by literature reports showing that phosphines catalyze the conversion of alkylthio radicals to alkyl radicals.[33] The phosphorus atom interacts with the alkylsulfur radical, leading to homolytic bond cleavage between the sulfur and the alkyl group:

(29) Holler, T. P.; Hopkins, P. B. *Biochemistry* **1990**, *29*, 1953.

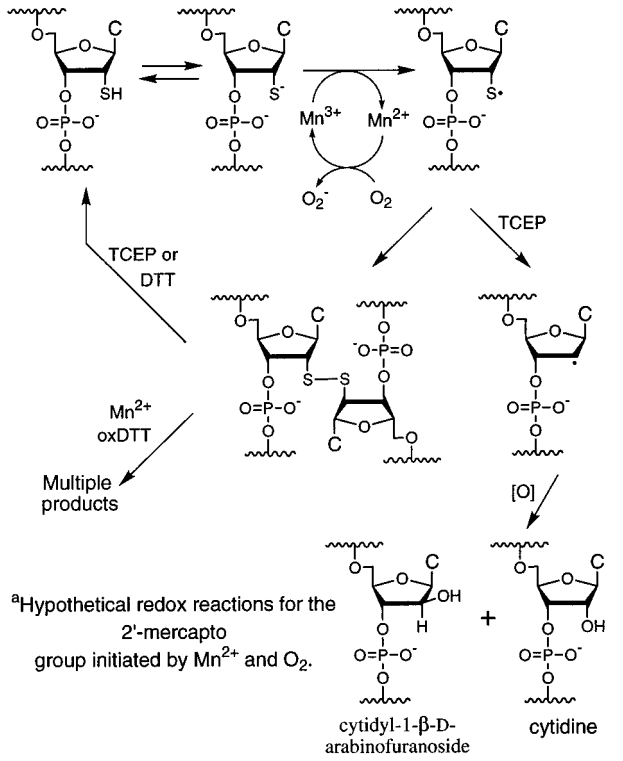
(30) (a) Misra, H. P. *J. Biol. Chem.* **1974**, *249*, 2151. (b) McEldoon, J. P.; Dordick, J. S. *J. Biol. Chem.* **1991**, *266*, 14288.

(31) Cleland, W. W. *Biochemistry* **1963**, *3*, 480.

(32) Ordinarily, the DTT present in the reaction would reduce the symmetrical disulfide back to the free mercaptan or prevent its formation in the first place. However, RP-HPLC showed that DTT itself also is oxidized to a disulfide (oxDTT) under these conditions ($t_{1/2}$ = ~4.5 h; 1 mM DTT; 10 mM Mn^{2+}; 50 mM NaMOPS, pH 7.5). Longer incubations (>24 h) of $(^{5'}C_{2'S}dpG^{3'})_2$ and oxDTT in the presence of Mn^{2+} resulted in decomposition of both disulfides to unknown products (data not shown).

(33) Walling, C.; Rabinowitz, R. *J. Am. Chem. Soc.* **1959**, *81*, 1243.

Scheme 3[a]



[a]Hypothetical redox reactions for the 2'-mercapto group initiated by Mn^{2+} and O_2.

$$RS\bullet + P(R)_3 \rightarrow RS{-}\bullet P(R)_3 \quad (3)$$

$$RS{-}\bullet P(R)_3 \rightarrow R\bullet + S{=}P(R)_3 \quad (4)$$

Analogously, TCEP might facilitate homolysis of the 2′-C−S bond in dHH16SH to produce a carbon radical (Scheme 3), which could then lead to the production of both dHH16OH and dHH16aOH by combining with an oxygen species.[34−36] Formation of more rC than aC may be due to steric hindrance from the heterocyclic base on the top face of the sugar.

2′-Sulfur Substitution Blocks the Reverse Hammerhead Reaction. Figure 4 showed that in the absence of oxygen, Mn^{2+} is not able to rescue HH-catalyzed phosphotransesterification of dHH16SH. Other soft metal ions such as Co^{2+}, Zn^{2+}, or Cd^{2+} also were unable to rescue dHH16SH (see Supporting Information).[37] These experiments therefore cannot provide evidence that a metal ion interacts directly with the 2′-nucleophile during the HH reaction. Alternatively, it may be possible to detect a metal ion interaction with a nucleophile in a phosphoryl transfer reaction by analyzing the reverse reaction, in which the nucleophile becomes the leaving group. Microscopic reversibility dictates that the transition state for the forward reaction is the same as that for the reverse reaction. Therefore, the observation of a metal specificity switch upon sulfur substitution of the leaving group in the reverse reaction implies that a metal directly coordinates to the nucleophile during the forward reaction.

To test for a metal ion leaving group in the reverse HH reaction, we constructed an oligonucleotide terminating in a 2′-*S*,3′-*O*-cyclic phosphorothiolate using our previously published method.[27] The reverse reaction of HH phosphotransesterification involves ligation of an oligonucleotide containing a 2′,3′-cyclic phosphate (P1) to another containing a free 5′-hydroxyl (P2).[16] We found that the 2′-*S* modified P1 bound to the HH·P2 complex with the same affinity as the unmodified P1 (see Supporting Information), but no reaction was observed for the dHH16S-P1 substrate under any of the conditions tested, including low temperatures, different pHs, and several metal concentrations (see Supporting Information). Other thiophilic metal ions such as Co^{2+}, Cd^{2+}, and Zn^{2+} also were unable to support ligation (data not shown).

Conclusions and Implications

2′-Sulfur Substitution Blocks the Hammerhead Ribozyme Reaction. Using a "metal specificity switch" approach, we addressed the long-standing and controversial question of whether the catalytic mechanism for the hammerhead ribozyme reaction involves direct coordination of the 2′-oxygen nucleophile with a metal ion. A substrate analogue, in which the 2′-OH nucleophile was replaced by a 2′-SH, was generated in situ from the corresponding disulfide by treatment with TCEP. The 2′-SH blocks the hammerhead-catalyzed phosphotransesterification if Mg^{2+} is present as the only divalent cation, but the reaction is restored if Mn^{2+} is present. This apparent switch in metal ion specificity occurs not because Mn^{2+} coordinates to sulfur more effectively than Mg^{2+} does, but because under the assay conditions, the 2′-mercaptonucleoside is converted partly to the 2′-ribonucleoside, the natural substrate in the ribozyme reaction. The conversion requires Mn^{2+}, O_2, and TCEP and involves a complex set of redox processes that generate a carbon radical at C2′, which then combines with an oxygen species to yield both aC and rC. If O_2 is excluded from the hammerhead reactions, the 2′-SH substrate is resistant to redox processes, but is still unable to undergo phosphotransesterification in the presence of Mn^{2+} or any other metal ion, possibly because a 2′-thiolate anion is an intrinsically poor nucleophile toward the adjacent 3′-phosphodiester.[9] Consequently, we tested for a metal specificity switch in the reverse HH reaction, whereby the 2′-sulfur would act as a leaving group. Even though sulfur is a better leaving group than oxygen at phosphate centers,[38] 2′-sulfur substitution also blocked the reverse reaction, regardless of the divalent cation present.

2′-Mercaptonucleotides as Biochemical Probes. Although our results provide no evidence to confirm or refute hypotheses concerning metal ion activation of the nucleophile in the hammerhead ribozyme reaction, we have established a potential

(34) To test whether the 2′-hydoxyl produced in this reaction is derived from water or diatomic oxygen, we carried out reactions on the dinucleotide, $^{5'}C_{2'SH}pdG^{3'}$, in ^{18}O water and isolated the products by RP-HPLC. Mass spectral analysis revealed that no ^{18}O was present in either $^{5'}rCpdG^{3'}$ or $^{5'}aCpdG^{3'}$, suggesting that the 2′-hydroxyl is not derived from water (see Supporting Information for further discussion).

(35) O_2 can react with an alkyl radical to form an alkylperoxy radical (ROO•), which could undergo phosphine-induced scission to form an alkoxy radical (RO•) in a manner analogous to the reaction between TCEP and the alkylthiyl radical (See: Furminsky E.; Howard J. A. *J. Am. Chem. Soc.* **1973**, *95*, 369). Alternatively, a mercapto group can help quench the alkoxy radical to produce the hydroxyl group, or Mn^{2+} can reduce the alkoxy radical to the alkoxide.

(36) The 2′-carbon radical may react directly with a hydroxyl radical to form the products. Hydroxyl radicals can form from O_2^- through a complex set of reactions involving Mn^{2+} and hydrazine, which acts as a source of H• (See: Ito, K.; Yamamoto, K.; Kawanishi, S. *Biochemistry* **1992**, *31*, 11606). Our reactions do not contain hydrazine, but mercapto groups also can act as a source of H• (see: Kice, J. L. In *Free Radicals*; Kochi, J. K., Ed.; John Wiley and Sons: New York, 1973; Vol. 2.).

(37) Co^{2+} is also redox active and behaves in a manner similar to that of Mn^{2+} in HH reactions with dHH16SH. In the absence of O_2, Co^{2+} facilitates formation of the fragmentation products, whereas in the presence of O_2 both fragmentation and phosphotransesterification products are formed. Zn^{2+} and Cd^{2+}, which are not redox active, do not give rise to phosphotransesterification products. In contrast to the other divalent metal ions tested, Zn^{2+} and Cd^{2+} inhibit the fragmentation of dHH16SH. These metal ions may coordinate so strongly to the sulfur that they weaken its nucleophilicity toward C-1′ (see Supporting Information).

(38) Barth, C.; Anjaneyulu, P. S. R.; Piccirilli, J. A. Manuscript in preparation.

experimental protocol for addressing this question in other enzyme/ribozyme systems. Additionally, we have characterized the behavior of nucleic acids containing 2′-mercaptonucleosides under conditions commonly used in biochemical experiments, thereby permitting the use of these analogues to probe RNA structure and function. In assays that use metal ions such as Mg^{2+}, Ca^{2+}, Cd^{2+}, and Zn^{2+}, which do not promote oxidation of sulfur, either DTT or TCEP may be used to deprotect in situ the 2′-mercapto group, and no additional precautions are required. However, in buffered solutions containing O_2 and Mn^{2+}, Co^{2+}, or other redox active metal ions, additional precautions must be taken as these metals initiate a complex series of redox reactions involving the 2′-SH group. In the absence of reducing agents, a symmetrical disulfide forms. Although DTT can slow this process, it also is oxidized under these conditions, and incubation of the symmetrical disulfide with oxidized DTT in the presence of Mn^{2+} leads to decomposition of both species (Scheme 3).[32] In contrast, TCEP, which can reduce the symmetrical disulfide, facilitates conversion of the 2′-mercaptocytidine to a mixture of rC and aC. To preserve the integrity of a 2′-mercapto group in the presence of redox-active metal ions, it is best to carry out reactions in an anaerobic environment using degassed solutions that contain an oxygen scavenger. If these precautions are taken, either DTT or TCEP may be used to deprotect in situ the 2′-mercapto group in the presence of Mn^{2+} or Co^{2+}.

A New Chemical Reaction. Although there is precedence for each of the subreactions that presumably occur in the conversion of the 2′-mercaptonucleotide to the corresponding ribonucleotide and arabinonucleotide, the overall process is fundamentally a new reaction. It will be interesting to explore the generality and the structural requirements for this process and to determine whether it will be useful synthetically. On the basis of the mechanistic evidence we have gathered, it may even be possible to use mercaptonucleotides to generate carbon radicals site specifically within structured RNA molecules, thereby providing possible probes for cross-linking and other biophysical studies.

Experimental Section

General. All chemicals were from Sigma unless otherwise indicated. Solutions of DTT were stored at −20 °C and were discarded after 2−3 months of use. $MgCl_2$, $MnCl_2$, $CoCl_2$, $CdCl_2$, $ZnCl_2$, $CaCl_2$, and $Mn(OAc)_3$ were purchased from Aldrich at their highest purity. Reverse phase high-pressure liquid chromatography (RP-HPLC) was performed using a Beckman Ultrasphere ODS C_{18} column (5 μM × 10 mm × 250 mm). The HPLC solvent gradient was performed with 0.01 M NH_4OAc (pH 6.5; solvent A) and CH_3CN (solvent B) as follows: 0−10% B from 0 to 35 min and 10−30% B from 35 to 40 min. Mass spectral analysis was performed in the lab of Richard B. van Breemen at the University of Illinois at Chicago on a Micromass (Manchester, UK) Quattro II triple quadruple electrospray mass spectrometer in the negative ion mode. Nondenaturing polyacrylamide gel electrophoresis (PAGE) was performed using 20% polyacrylamide (Fisher; acrylamide: bis-acrylamide 29:1) with 7 M urea (VWR) as a 1 × TBE solution. APM-PAGE was performed similarly but included APM (0.75 μg/mL)[28] in the gel solution. We found that more uniform electrophoresis occurred if DTT (10 mM) was added to those reactions containing dHH16SH just before loading on the APM gel. Gel loading buffer contained 8 M urea, 50 mM EDTA (pH 8.0, Fisher), 0.02% bromophenol blue (EM Science), and 0.02% xylene cyanol FF (Kodak).

Oligonucleotide Synthesis. Ribozymes were purchased from Dharmacon Inc. All substrates and standards were synthesized as described on a 1 μmol scale with standard phosphoramidites (Perseptive Biosystems) using a Millipore Expidite Nucleic Acid Synthesis System and standard DNA and RNA protocols. Synthesized oligonucleotides were deprotected at 55 °C for 14−18 h with concentrated aqueous NH_3 and evaporated to dryness. Oligonucleotides that contained ribonucleotides were desilylated by treatment with 1.0 M TBAF in THF solution (0.5 mL), gently shaken in the dark for 24−36 h, neutralized with 1.0 M TEAAC (pH 6.9), and purified by elution through a NAP-10 gel-filtration column (Pharmacia Biotech).

2′-Mercaptocytidine was incorporated into oligonucleotides by coupling 4-*N*-benzoyl-5′-*O*-(dimethoxytrityl)-2′-deoxy-2′-(tritylthio)-cytidin-3′-yl β-cyanoethyl *N*,*N*-diisopropylphosphoroamidite[7] to the growing oligonucleotide chain. The resulting oligonucleotide contained a 2′-tritylthio-2′-deoxycytidine which was converted to the 2′-dipyridyl disulfide by incubation with $AgNO_3$ and Aldrithiol-2 at pH 8.0 as described previously.[7] $^{5'}$aCpdG$^{3'}$ was synthesized using DNA protocols and 4-*N*-benzoyl-5′-*O*-(dimethoxytrityl)-2′-*O*-acetyl-1-β-D-arabinofuranosidecytidyl-3′-yl β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (Glenn).

Lig-HH16OH and Lig-HH16SH were synthesized by ligation of two oligonucleotides. A 10-mer oligonucleotide that contained all 2′-deoxynucleotides except for the 5′-end ($^{5'}C_{2'X}$GTCGTCGCC$^{3'}$) which contained either a cytidine (X = OH) or 2′- (2-pyridyldithio) cytidine (X = SSpyr), acted as the 3′-oligonucleotide. An 8-mer oligonucleotide that contained all 2′-deoxynucleotides except for a riboguanosine at one position, $^{5'}$dGGGAAC$G_{2'OH}$T$^{3'}$, acted as the 5′-oligonucleotide. A 26-mer 2′-deoxyoligonucleotide, $^{5'}$dTTTTCCGGACGACGACGTTCCCTTTT$^{3'}$, acted as the bridge. The 3′-oligonucleotide (2 μM) was 5′-radiolabeled in a 5 μL volume by incubation with [γ-^{32}P]ATP (4 μM), $MgCl_2$ (10 mM), Tris-HCl (100 mM; pH 8.0), and PNK (6 units) at 37 °C for 30 min. Cold ATP (1 μL of a 100 mM solution), template (0.5 μL of a 250 μM solution), and the 5′-oligonucleotide (1 μL of a 500 μM solution) were mixed. and the reaction was heated at 90 °C for 2 min before cooling on ice for 10 min. Finally, $MgCl_2$ (1 μL of a 100 mM solution) and T4 DNA ligase (15 units) were added to make a 10 μL volume, and the reaction was incubated at 37 °C for at least 3 h. Full-length oligonucleotides were separated from failure sequences by denaturing PAGE and visualized by autoradiography. The ligation products were then excised from the gel and eluted at 4 °C with 1 × TE. The eluent was applied to a Sep-Pak C_{18} cartridge (Waters) which was then washed with water (2 mL) followed by 50% aqueous CH_3CN (2 mL). Fractions containing oligonucleotide were evaporated to dryness and dissolved in water to a concentration of 20 nM as determined by specific radioactivity.

Radiolabeling of Oligonucleotides. Oligonucleotides (20 pmol) were 3′-end labeled with [α-^{32}P−CoTP] and terminal deoxynucleotidyl transferase (TDT). Reactions included [α-^{32}P−CoTP] (30 μCi), TDT (17 units), oligonucleotide (20 pmol) in a 10 μL volume. After a 1 h incubation at 37 °C, the reactions were quenched with gel loading buffer (10 μL). Oligonucleotides (20 pmol) were 5′-radiolabeled with [α-^{32}P]-ATP (NEN) and T4 Polynucleotide Kinase (USB) according to the manufacturer's (USB) protocol (10 μL scale). After 30 min at 30 °C, the reactions were quenched with gel loading buffer (10 μL). The radiolabeled oligonucleotides were isolated by denaturing PAGE and purified by Sep-Pak as described above before being concentrated and dissolved in water to a concentration of 20 nM as determined by specific radioactivity.

Preparation of Standard B. To obtain a standard for the 3′ fragmentation product ($^{5'}$p-dGTCGTCGCC*pCo$^{3'}$) $^{5'}$dGTCGTCGCC$^{3'}$ was 3′-radiolabeled as described above and then 5′-phosphorylated with T4 Polynucleotide Kinase (PNK) and adenosine triphosphate (ATP) by addition of the following ingredients directly to the TDT reaction: 0.5 M Tris-HCl (pH 7.5; 1.5 μL), 0.1 M $MgCl_2$ (1.5 μL), 50 mM ATP (1.7 μL), and 30 units/μL PNK (0.3 μL). The reaction mixture was incubated at 37 °C for 30 min before quenching with gel loading buffer. The product was purified by PAGE/Sep-Pak C_{18} as described above.

Ribozyme Reactions. Ribozyme reactions were performed at 25 °C essentially as described.[16] Saturating ribozyme (1 μM) and radiolabeled substrate (2 nM; dHH16OH or dHH16SSpyr) in NaMOPS buffer (50 mM; pH 7.5) were heated at 90 °C for 1.5 min and then cooled at 25 °C for 10 min. Solutions of DTT or freshly prepared TCEP (1 mM) were then added to deprotect the 2′-sulfur and obtain dHH16SH (TCEP was not added if dHH16SSpyr itself was required). After ∼5 min, reactions were initiated by the addition of $MgCl_2$ or $MnCl_2$ (10 mM). After a 24 h incubation, reactions were quenched with an equal volume of gel loading buffer and analyzed by denaturing 20% PAGE.

Gels were dried and visualized using a Molecular Dynamics Phosphorimaging System or by autoradiography.

Conversion of dHH16SH to dHH16OH and dHH16aOH. Modification reactions were run similar to the ribozyme reactions. Radiolabeled substrates (2 nM; dHH16OH or dHH16SSpyr) were added to a solution of NaMOPS buffer (50 mM; pH 7.5) before addition of either DTT or freshly prepared TCEP (1 mM) to deprotect the 2′-sulfur and obtain dHH16SH. After ~5 min, reactions were initiated by the addition of $MgCl_2$ or $MnCl_2$ (10 mM). Where appropriate, Trolox (1 mM) was added as a 10 mM solution in ethanol just before addition of the metal. After 12 h, EDTA (15 mM) was added, and the reactions were treated at 25 °C with RNase A (4 ng/μL), or HH (1 μM) and $MgCl_2$ (10 mM), and incubated for 10 h. Substrates treated with iodoacetamide (50 mM; Sigma) were incubated at 30 °C for 20 min without the addition of EDTA. $(dHH16S)_2$ or $(^{5'}C_{2'S}pdG^{3'})_2$ reduced to dHH16SH or $^{5'}C_{2'SH}$-$pdG^{3'}$, respectively, by incubating with 1 mM TCEP or DTT and 50 mM NaMOPS (pH 7.5) for 5 min as indicated. $^{5'}C_{2'SH}pdG^{3'}$ (10 pM) was incubated at 25 °C for 12 h with NaMOPS (50 mM; pH 7.5), TCEP (1 mM) and $MnCl_2$ (10 mM), before analysis by RP-HPLC or mass spectrometry.

HH Reactions under Anaerobic Conditions. The environment in the anaerobic chamber (Coy) consisted of industrial grade N_2 and 2−3% H_2. The oxygen and hydrogen concentrations were monitored by an oxygen/hydrogen analyzer (Coy). All reagents were dissolved or diluted in water that had been degassed for 30 min with N_2. The resulting solutions were kept under anaerobic conditions at all times, even when stored at −20 °C. Ribozyme (1 μM) and substrate (2 nM) were heated for 1.5 min at 90 °C and left at room temperature for 30 min to cool. Freshly prepared TCEP (1 mM) was added before initiation of the reaction by addition of $MnCl_2$ (10 mM). Reactions were incubated either under atmospheric conditions or under anaerobic conditions with or without added diatomic oxygen.

Product Isolation and Characterization. The 3′- and 5′-products (including dHH16O-P1) were isolated from large-scale HH reactions (150 μL) which contained $MgCl_2$ and about 10 pmol of a radiolabeled substrate. PAGE analysis and autoradiography were used to locate the individual product bands which were cut out and soaked in 1 × TE at 4 °C before Sep-Pak C_{18} purification. To obtain S1 nuclease digestion ladders, oligonucleotides (20 fmol) were incubated with S1 nuclease (25 units) in aqueous buffer (10 μL) containing 50 mM sodium acetate (pH 5.2), 1 mM $ZnCl_2$, and 0.25 NaCl. After 5 min, the reaction was stopped by the addition of gel loading buffer (10 mL) followed by cooling on dry ice. 5′-Phosphorylation of **P2** was performed as described for 5′-radiolabeling except that cold ATP (Pharmacia Biotech) was used. Dephosphorylation of **F2** with calf intestine alkaline phosphatase (CIAP; Amersham) was performed as described by the manufacturer. To assay for the presence of a cyclic phosphate or phosphorothiolate in **P1**, we incubated the isolated 5′-products from dHH8 substrates with 0.1 N NaOH at 95 °C for 10 min.

To obtain the dimer products of Lig-dHH16OH and Lig-dHH16SH, substrates were incubated for 36 h at 25 °C in a large-scale reaction (150 μM) that contained 10 mM $MnCl_2$, 25 μM HH, 1 mM TCEP, and 50 mM NaMOPS (pH 7.5). The reactions were then concentrated to ~10 μL, nuclease T1 (5 units) was added, and the reaction was incubated at 25 °C for another hour. Gel loading buffer (10 μL) was then added, and the dimer products were isolated by PAGE/Sep-Pak C_{18} as described above. The dimer products were characterized by treatment with 0.1 N NaOH at 90 °C for 10 min before neutralization with 0.1 N HCl and incubation with CIAP as described above. Finally, the reactions were incubated with iodoacetamide (50 mM) for 20 min at 30 °C before analysis by 20% denaturing PAGE.

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Supporting Information Available: Further data and discussion for the reverse HH reaction and for dHH16SH, including the mechanism of oxidation, the HH reaction with other divalent cations, and the stability in the absence of reducing agents (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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