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Application of a 5′-Bridging Phosphorothioate to Probe Divalent Metal and Hammerhead Ribozyme Mediated RNA Cleavage

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Abstract—This paper describes the preparation and application of a chimeric DNA/RNA oligonucleotide that contains a single 5′-bridging phosphorothioate linkage adjacent to a ribonucleotide and embedded in an otherwise all-DNA sequence. The influence of pH, divalent metal cation, hybridization, and secondary structure on the susceptibility of the thio linkage towards transesterification is investigated in an effort to better understand the metal–phosphorothioate interactions and the basis for catalysis. In addition to the chemical cleavage, we have examined the hammerhead ribozyme mediated cleavage of the 5′-bridging phosphorothioate linkage specifically to test the hypothesis that the ribozyme employs a second metal cofactor, which functions as a Lewis acid, to catalyze transesterification. The results of our kinetics experiments do not support this double-metal model. © 1997 Elsevier Science Ltd.

Introduction

The hammerhead ribozyme is derived from a structural motif present in the RNA genome of several plant pathogens where it is believed that the cleavage reaction is an essential step in the replication pathway.[1,2] In the in vivo reaction, cleavage occurs in *cis* from a monomolecular complex. However, the reaction can also function in *trans* when two[3] (or even three)[4] RNA fragments are used to form the catalytically competent complex. In the simplest view of cleavage in *trans*, a single ribozyme (non-cleaved) sequence binds to the substrate (cleaved) sequence, and in the presence of a metal cofactor, transesterification of the target phosphodiester linkage in the substrate sequence results. Recognition of the substrate by the catalytic (ribozyme) sequence occurs nominally through Watson–Crick interstrand hydrogen bonding interactions, and in this respect, the 'recognition' step of the process is not a mystery. However, the mechanism by which the cleavage event occurs has eluded formal explanation, even though two X-ray diffraction analyses of the ground state structure are now available.[5,6] In spite of this structural information, solution studies are still necessary in order to deconvolute the mechanistic pathway that describes RNA cleavage catalyzed by the hammerhead ribozyme. The use of nucleotide analogues offer another dimension to such solution studies in that they permit the alteration of single sites, in fact individual atoms, within the macromolecular complex. By making subtle atomic substitutions, particularly near or at the reaction center, it is possible to incrementally alter the nature of the biochemical process, and then observe, under a variety of conditions, the incremental response of the biochemical process to these changes.

Phosphorothioates have generally proven to be valuable analogues for studying a wide variety of biochemical processes.[7] The diastereomeric nature (Rp and Sp) of phosphorothioates, and the difference in the properties of the sulfur vs. oxygen ligands, has permitted the identification of metal coordination sites in a number of nucleotide processing enzymes.[8] Non-bridging phosphorothioate diesters have also been incorporated site-specifically into DNA sequences by chemical or enzymatic means, replacing one[9,10] or both[11,12] of the non-bridging oxygen atoms. The nucleophilicity of the sulfur atom can be exploited to introduce stereochemically oriented reporter groups at unique sites on the DNA backbone.[13,14] Alkylation of phosphorothioates permits site-specific cleavage and sequencing of DNA.[15] DNA sequences that have been uniformly modified with phosphorothioate linkages have received much attention as potential therapeutic agents.[16] Phosphorothioate analogues that contain sulfur in the 3′- or 5′-bridging positions of DNA phosphodiesters (i.e., P–S–C bonds) have more recently been reported.[17–20] Both non-bridging and bridging phosphorothioate linkages in DNA have helped elucidate the cleavage mechanisms of nucleases[21,22] and the *Tetrahymena* ribozyme.[23] RNA oligomers containing non-bridging phosphorothioates have also been reported.[24–28] Interference assays employing such RNA phosphorothioates have identified possible metal coordination sites in the hammerhead ribozyme,[29] as well as proof for an in-line SN2 type cleavage mechanism.[24,26] However, the related oligomeric RNA analogues containing bridging phosphorothioates have only very recently been described by us.[30] Such RNA phosphodiester analogues should prove to be quite useful for studying a variety of RNA cleaving agents or enzymes, metal–RNA interactions, and the mechanism of RNA cleavage itself.[30–33] We describe here some of our efforts to examine the divalent metal-assisted cleavage properties of a 5′-bridged phosphorothioate, and we also describe how we have applied this new linkage to investigate the cleavage mechanism and metal-cofactor requirements of the hammerhead

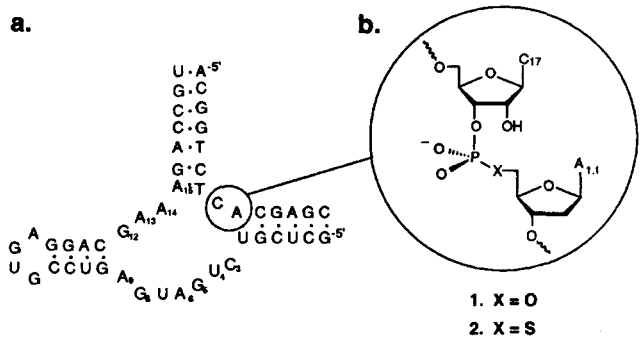

Figure 1. The hammerhead ribozyme. (a) Sequence and secondary structure of the hammerhead complex employed in this study. Bold letters represent conserved nucleotides. Circled portion indicates the cleavage site. (b) Expansion of the cleavage site between C_{17} and $A_{1.1}$, showing the native oxo substrate **1** and the 5′-bridging phosphorothioate substrate analogue **2**.

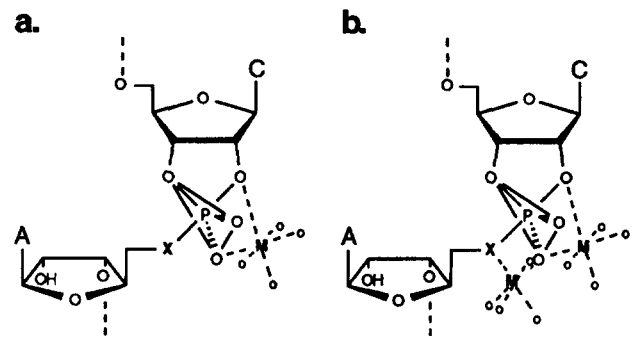

Figure 2. Single-metal versus double-metal mechanism. Transition state of the hammerhead ribozyme illustrating (a) the single-metal model and (b) the double-metal model. X represents sulfur or oxygen.

ribozyme. In these studies we have employed a novel oligonucleotide chimera that contains a single ribonucleotide linkage embedded in an otherwise all-deoxyribonucleotide strand, (see Fig. 1) an approach which simplifies both the synthetic methodology and the analysis of the cleavage event.

The hammerhead ribozyme structural motif consists of three helical stems and includes nine conserved nucleotides that are nominally single-stranded and would appear to be responsible for the formation of a catalytically active domain via a complex network of non-Watson–Crick hydrogen bonding interactions (see Fig. 1).[1-3,34-37] Base analogue substitutions have identified, by what amounts to atom-directed mutagenesis, many of the critical functional groups that are involved in these interactions.[33] The cleavage event itself, mediated by Mg^{2+}, produces 5′-hydroxy and 2′,3′-cyclic phosphate fragments consistent with an in-line transesterification pathway involving the adjacent, internal 2′-hydroxyl.[3] Although the hammerhead ribozyme is considered to be a metalloenzyme,[23,38,39] the precise role of the critical metal cofactor remains obscure. The exact number of metals that are involved in the actual chemical cleavage event, or in the organization of the tertiary structure, still remains a contentious issue.[40] Solvated metal hydroxide has been implicated as the base that deprotonates the 2′-hydroxyl,[25] and the proR non-bridging oxygen at the site of cleavage has been shown to be bound to the metal cofactor in the transition state;[4,24,26] a single metal could simultaneously satisfy both of these roles. However, by analogy with the crystal structures of proteinacious phosphoryl transfer enzymes, where the roles of the metal cofactors have been more clearly established, it has been proposed that a general two-metal mechanism may be operative for many such enzymes, including ribozymes.[41-43] Ab initio molecular orbital calculations of small model compounds in the gas phase have suggested that a second metal cofactor could function as a Lewis acid in the hammerhead ribozyme to stabilize the departing 5′-oxyanion leaving group and facilitate cleavage,[44-46] and the apparent lack of a solvent deuterium isotope effect has been offered as evidence for the participation of a second metal.[47] Unfortunately, two recent X-ray structures of hammerhead–inhibitor complexes[5,6] did not unambiguously identify the key catalytic metals, but Scott et al. used indirect methods to identify a single hydrated Mg^{2+} atom near the cleavage site and proposed a single-metal mechanism that involves only this metal.[5] We have employed the 5′-bridging phosphorothioate linkage to probe the cleavage mechanism of the hammerhead ribozyme and to directly test the possibility of a second metal cofactor interaction with the 5′-leaving group (the double-metal model, compare Fig. 2a, b) by examining the cleavage kinetics with a series of metals under a variety of conditions.[31-33]

Results and discussion

Design and synthesis

Our substrate sequence consists of an oligonucleotide chimera containing a single ribonucleotide linkage embedded in an otherwise all-deoxyribonucleotide strand. This design simplifies both the preparation of the 5′-bridging phosphorothioate containing sequence and the analysis of the cleavage event itself. Such substrates are efficiently cleaved by the hammerhead ribozyme and behave very much like the all-ribo sequences.[32,48-52] It is well known that DNA/RNA heteroduplexes, such as would occur when the hammerhead ribozyme forms a complex with sequence **1** or **2**, adopt an A-type helix similar to the type found in RNA/RNA homoduplexes.[53,54] Moreover, the two recently solved X-ray crystal structures of hammerhead–inhibitor complexes, one employing an all-DNA substrate[6] and another employing an all-RNA substrate[5] give virtually identical three-dimensional structures. Therefore, the embedded ribonucleotide chimera substrate should be a good model to study RNA cleavage mediated either by ribozymes or other catalysts. In fact, it has recently been suggested that a single ribonucleotide embedded in an oligodeoxynucleotide and flanked by multiple T residues is far superior to the diribonucleotides that have traditionally been used to examine RNA cleavage and screen potential RNA cleaving agents.[55]

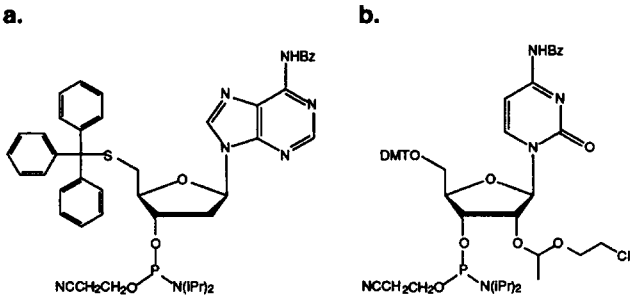

Figure 3. Special phosphoramidite monomers. (a) 5′-Thio dA nucleoside with triphenylmethyl protecting group on the sulfur atom. (b) Ribo C nucleoside with Cee protecting group on the 2′-hydroxyl.

Figure 3(a) shows the monomer that we have used to incorporate the thio linkage. Details on the synthesis of this monomer have been reported elsewhere;[30] its preparation is fairly straightforward and follows literature precedents.[56,57] The key transformation is the displacement of a 5′-arylsulfonyl group by sodium triphenylmethyl mercaptide. Several additional steps afford the fully protected phosphoramidite building block, which can be used directly with an automated DNA synthesizer employing conventional solid-supported, β-cyanoethyl synthesis methodology with a few important modifications, as outlined below.

A critical issue in the synthesis of the 5′-bridging phosphorothioate adjacent to a ribonucleotide was the choice of protecting group for the 2′-hydroxyl adjacent to the thio linkage. A suitable protecting group for this functionality must be stable under the conditions of oligomer assembly, but must subsequently be removable under conditions that will not degrade the labile 5′-bridging phosphorothioate diester. The conventional *t*-butyldimethylsilyl ether (TBDMS) group[58] was incompatible due to substantial chain cleavage upon treatment with the known deprotection reagents; either triethylamine trihydrofluoride[59,60] or tetrabutyl ammonium fluoride. Other 2′-hydroxy protecting groups, such as Fpmp,[61,62] Thp, and Mthp[63] require a prolonged acid treatment for deprotection, conditions which can cause substantial depurination of deoxynucleotides (these protecting group were originally developed for RNA, which is much more resistant to depurination)[64] and 2′-, 3′-isomerization of the 5′-bridging phosphorothioate.[65] Fortunately, the recently introduced chloroethoxyethyl (Cee) group[66–68] possessed nearly ideal properties for our application; the acetal remained intact during oligomer assembly, but was subsequently easily removed without compromising the sensitive thio linkage. The structure of the Cee monomer is shown in Figure 3(b). To our surprise, the single Cee group flanked by DNA bases was completely lost during the post-synthesis oligomer isolation steps and thus required no separate deprotection per se.[30] Most likely, the adjacent phosphate moieties catalyze the hydrolysis of the single Cee group, but the precise mechanism and participating functionalities remain to be determined. In contrast, monomers, dimers, and trimers that contain a single Cee-protected ribonucleotide require ca. 4 h at pH 2.0 for complete deprotection.

The thio residue is incorporated into the oligomer in the normal fashion, but the acid detritylation step is skipped since the triphenylmethyl group is not acid labile. Instead, the cartridge containing the solid support is removed from the instrument and treated for 45–60 min with and aqueous solution of 50 mM silver nitrate for 'detritylation'.[69] We originally used a 30 min treatment, but this amount of time was not always sufficient for complete deprotection. The support is next treated with 50 mM DTT for 5 min to reduce any disulfide that may have formed during deprotection; longer DTT treatments cause discoloration of the support and degradation of the oligomer. The synthesis cartridge, now containing a 5′-thiol, is reattached to the synthesizer and the Cee-protected RNA monomer is coupled in the normal fashion to form the P–S bond. From this point on, if not from the beginning of the synthesis, it is prudent to use dichloroacetic acid as opposed to tricholoracetic acid for detritylation to preserve the labile 2′-Cee linkage and prevent branching. After complete oligomer assembly the solid support is treated with conc. ammonium hydroxide for 12 h at 50 °C to release the oligomer from the support and remove the base protecting groups. The oligomer is desalted by size exclusion chromatography (Sephadex G-10) and lyophilized to dryness. Analysis at this stage reveals that the single Cee protecting group is completely gone.

The crude oligonucleotide is initially purified by strong anion exchange chromatography using an FPLC system (Pharmacia Mono-Q column, 10 mM KH_2PO_4, pH 6.35, 30% acetonitrile with a KCl gradient). The low pH is necessary to minimize degradation of the bridging phosphorothioate linkage. A representative FPLC chromatogram of a crude oligomer is shown in Figure 4. The incorporation of the 5′-bridging phosphorothioate does cause substantial degradation of the oligo, and it is not yet clear if this occurs during sequence assembly or deprotection, but the desired full-length product can be easily isolated. The appropriate fraction is collected, concentrated to a low volume and desalted. Denaturing PAGE can be used to analyze or partially purify the thio-modified oligomers, but degradation will occur if the conventional TBE buffer system (pH 8.4) is used. Therefore, gels must be specially prepared and run at pH 6.8 with a Tris-NaOAc buffer, which reduces the resolution somewhat. Standard techniques can be used to characterize the thio-containing sequence, as previously described.[30] Perhaps the most conclusive analytical data to confirm the identity of the thio linkage comes from the quantitative HPLC analysis of an enzymatic digestion of the oligomer performed with snake venom phosphodiesterase and bacterial alkaline phosphatase, which yields free nucleosides that can be chromatographically compared to authentic standards. ^{31}P NMR has also been used to characterize the 5′-bridging (or 3′-bridging) phosphorothioate since the sulfur bonded to the phosphorus imparts a unique and characteristic chemical shift of ca. 20 ppm.[20,56] The purified oligomer is successfully end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase only if the pH is reduced from 8.3 (Tris) to 6.8 (PIPES), the Mg^{2+}

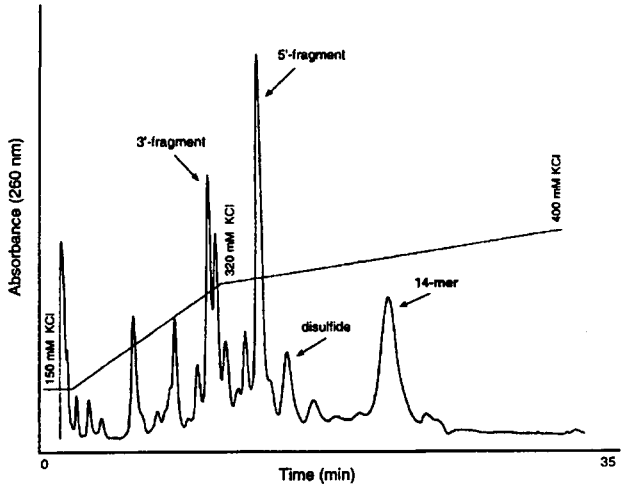

Figure 4. Preparative anion-exchange chromatography. Chromatogram of the crude 5′-bridging phosphorothioate substrate (10 OD) after ammonia deprotection and reduction with TCEP. Mono-Q HR 5/5 column with a KCl gradient, pH 6.35.

concentration is lowered from 10 to 1 mM, and the reaction is carried out at 27 °C instead of 37 °C. If these alterations to the conventional reaction conditions are not made, substantial degradation of the thio linkage will occur during the labeling.

Chemical cleavage properties

We originally anticipated that a bridging 5′-phosphorothioate adjacent to an RNA linkage would be exceptionally labile, given the much greater leaving group ability of sulfur vs. oxygen, and this expectation was realized in our initial unsuccessful efforts to isolate oligomers containing the modified linkage free from degradation or cleavage products. To investigate the influence of pH on the stability of the thio linkage, the radiolabeled oligomer was incubated in 50 mM Tris-HCl solution adjusted to pH 7.5, 8.0, or 9.0. To avoid the possible contribution from contaminating metal ions, 5 mM EDTA was also added as a sequestering agent. Aliquots were removed at intervals, re-buffered to pH 6.8 at 0 °C, and the cleavage products were separated by denaturing PAGE (pH 6.8). The cleavage was quantified by phosphorimaging. Figure 5(a) shows a representative gel. The cleavage rate at pH 7.5 (1.5×10^{-4})[30] is roughly 10^6-fold greater than estimates for the spontaneous cleavage of natural RNA linkages in diribonucleoside monophosphates.[70,71] This degree of rate acceleration is consistent with the known pK_a differences between the oxo and thio moieties (the pK_a of methanol is 15.5, vs. 10.0 for methanethiol).[72]

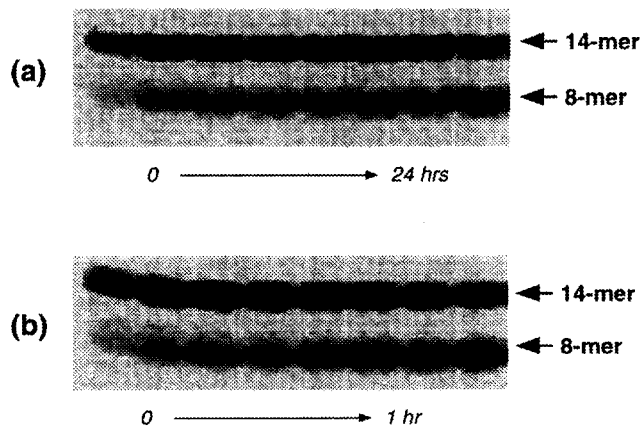

Figure 5. Analysis of the 5′-bridging phosphorothioate cleavage reaction. (a) Autoradiogram of a gel run at pH 6.8 showing product (8-mer) formation as a function of time in the presence of EDTA and Tris-HCl, pH 8.5. (b) Time-course of product formation in the presence of Mn^{2+} at pH 7.5.

Divalent metals (5 mM metal dichloride) were observed to dramatically accelerate the cleavage rate of the bridged 5′-phosphorothioate. Relative cleavage rates at pH 7.5 for the metals Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+} and Cd^{2+} were 10, 24, 71, 98, 370, and 3400, respectively, compared to an EDTA containing reaction at the same pH.[30] Figure 6 shows a graph of product formation vs. time for several of the metals. Comparison of these cleavage rates with some physical properties of the divalent metals, for example the pK_a of the hydrated metal cations or the ionic radii, do not reveal an obvious correlation. The pK_a values (ranging from 9.0 to 12.8) would be important if the metal is acting as a general base catalyst, and the ionic radii (ranging from 0.65 to 0.97) could be important if the nucleic acid is obligated to provide specific coordinating ligands to precisely position the metal and catalyze the cleavage event, but neither of these scenarios seem to be important. However, if Pearson's HSAB principle is invoked,[73–75] the relationship that does emerge is the correlation between the 'softness' character of the metal and the increased cleavage rate. It would appear that increasingly soft metals have a correspondingly higher affinity for the soft mercapto ligand.[30] Such an interaction between the metal and the sulfur atom could facilitate cleavage by improving the leaving group ability of the

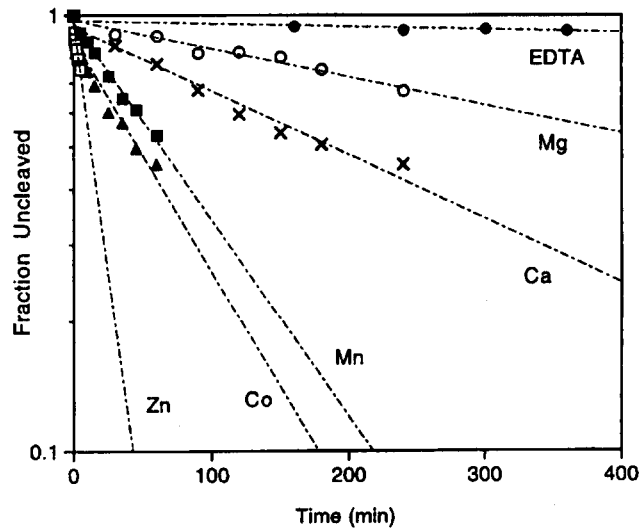

Figure 6. Effect of divalent metals on the chemical cleavage rate of the 5′-bridging phosphorothioate. Graph showing the fraction of uncleaved substrate versus time in the presence of various divalent metals (5 mM metal dichloride) at pH 7.5 (50 mM Tris-HCl).

mercapto substituent, stabilizing the transition state, or rendering the phosphorus center more electrophilic.[76]

All of the above results pertain only to the cleavage of a bridging 5′-phosphorothioate adjacent to an RNA linkage in a single-stranded, unhybridized state. We have found that hybridization and secondary structure can dramatically influence the Mn^{2+} mediated cleavage rates, and presumably cleavage mediated by other metals as well. Figure 7 illustrates some of the structures that we have examined. The duplex structure formed between **2** and a 14bp DNA complement substantially lowers the cleavage rate of **2**, relative to its single stranded form. An interior loop opposite the 5′-phosphorothioate linkage provides only a moderate stabilizing effect. These secondary structures most likely limit the accessibility of the metal to the sulfur atom and/or restrict the motions and flexibility of the thio linkage itself, relative to the single-stranded species, and thereby hinder the metal-promoted cleavage. In contrast to the simple duplex and opposite strand interior loop, an interior loop at the site of the thio linkage itself substantially accelerates the metal-promoted cleavage. In this case, the accessibility of the metal to the sulfur atom may be increased, or the structural constraints of the internal loop structure may induce P–S bond strain, giving rise to an increased cleavage rate. An interior bulge at the site of the thio linkage has the same effect as the interior loop opposite to the thio linkage—an increased cleavage rate. It will be interesting to examine the effects of other structures and helix types on the cleavage rate, and also to determine if the same metal trends hold for the single-stranded vs. hybridized structures.

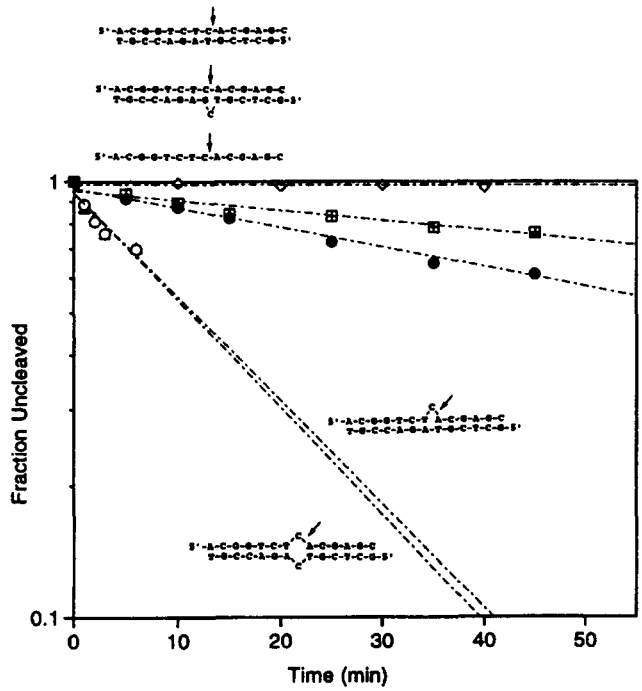

Figure 7. Effect of hybridization on the Mn^{2+} mediated cleavage of the 5′-bridging phosphorothioate. Graph showing the fraction of uncleaved substrate versus time in the presence of various complementary oligonucleotides. Arrow indicates the cleavage site.

The 5′-bridging phosphorothioate linkage in an all-DNA strand, which lacks the adjacent 2′-hydroxyl group, did not exhibit the extraordinary lability described above for sequence **2**. This observation implies that the likely mode of cleavage is one that involves participation of the 2′-hydroxyl group; namely, internal transesterification in which the adjacent, nucleophilic 2′-hydroxyl displaces the mercapto substituent to give fragments that contain a 2′,3′-cyclic phosphate and a 5′-thiol. Although this is the most likely cleavage pathway, other cleavage mechanisms are also possible. For example, two hydrolytic pathways, one involving P–S(5′) bond cleavage and one involving P–O(3′) bond cleavage, would result in fragments containing a 3′-monophosphate or a 2′,3′-diol, respectively; the products of cleavage are dependent upon the cleavage mechanism (see Fig. 8). To experimentally establish the mode of cleavage for the 5′-bridging phosphorothioate, we have isolated and characterized the termini of the cleavage products. Under all the conditions examined, the cleavage reaction affords the expected 2′,3′-cyclic phosphate and the 5′-thiol. High resolution PAGE provided a convenient means to evaluate the 3′-end of the 5′-cleavage fragments.[30,31]

Ribozyme cleavage properties

All of our hammerhead ribozyme mediated cleavage reactions have been conducted under single-turnover conditions, where the ribozyme is in large excess (ca. 1000-fold) over the substrate. This assures that the observed rate constants represent the actual chemical cleavage step and that all of the substrate exists as part of the ribozyme–substrate complex. In addition, we have used the conventional reaction conditions consisting of 50 mM Tris-HCl (pH 7.5) and 10 mM divalent metal. Chemical cleavage not caused by the ribozyme is eliminated by quenching the reaction mixtures with EDTA and a pH 6.8 buffer/gel loading mixture. The reaction products are separated by electrophoresis at pH 6.8 to further minimize cleavage of the labile 5′-bridging phosphorothioate.

In the presence of the ribozyme and the absence of spermine or a metal cofactor (5 mM EDTA), there is no increase in the cleavage rate of the 5′-bridging phosphorothioate substrate analogue over the control

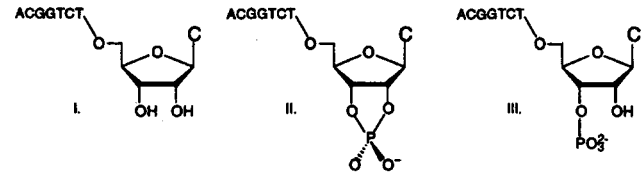

Figure 8. Possible 5′-cleavage fragments (8-mers) that could be formed from the cleavage of the 5′-bridging phosphorothioate: (I) 2′,3′-dihydroxy terminus, (II) 2′,3′-cyclic phosphate terminus, and (III) 3′-phosphate terminus.

Table 1. Half-lives for the chemical and ribozyme-mediated cleavage of the 5′-bridging phosphorothioate linkage in the presence of various divalent metals

	$t_{1/2}$ (min)						
	EDTA	Mg^{2+}	Ca^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}	Cd^{2+}
Chemical cleavage[a]	4600	460	187	63	46	12	1
Ribozyme cleavage[b]	5800	11 [20]	15	9 [15]	29	53	5
Ribozyme + spermine[c]	30	8 [15]	11	8 [5]	18	28	3

[a]5 mM EDTA or metal dichloride and 50 mM Tris–HCl, pH 7.5.
[b]0.5 μM ribozyme, 10 mM metal dichloride, 50 mM Tris–HCl, pH 7.5, trace amount of substrate. Ribozyme concentration is far in excess of the substrate concentration.
[c]0.5 mM spermine added to the ribozyme reaction. Bracketed values are for the oxo substrate.

rate, which is defined here as the rate of spontaneous chemical cleavage of the thio linkage in the absence of ribozyme. In fact, the ribozyme seems to provide a certain degree of protection from cleavage in the absence of a metal cofactor (see Table 1; compare $t_{1/2}$ = 4600 min vs. 5800 min). Not unexpectedly, the addition of a divalent metal cofactor dramatically increases the ribozyme-mediated cleavage of the thio linkage. A 10 mM concentration of Mg^{2+} decreases the $t_{1/2}$ over 500-fold, whereas the half-life of the control reaction is only decreased by a factor of 10. As summarized in Table 1, other divalent cations also increase the ribozyme-mediated cleavage of the 5′-bridging phosphorothioate. For example, Mn^{2+} decreases the ribozyme-mediated $t_{1/2}$ by over 600-fold, but the same metal only decreases the control $t_{1/2}$ by a factor of 70. Several of the metals, most notably Zn^{2+} and Cd^{2+}, appear to effect cleavage more slowly in the presence of the ribozyme, which implies that the labile thio linkage is shielded from the metal ions in solution by the folded tertiary structure of the complex, or that the ribozyme does not use these metals effectively as catalytic cofactors. Some representative gels are shown in Figure 9.

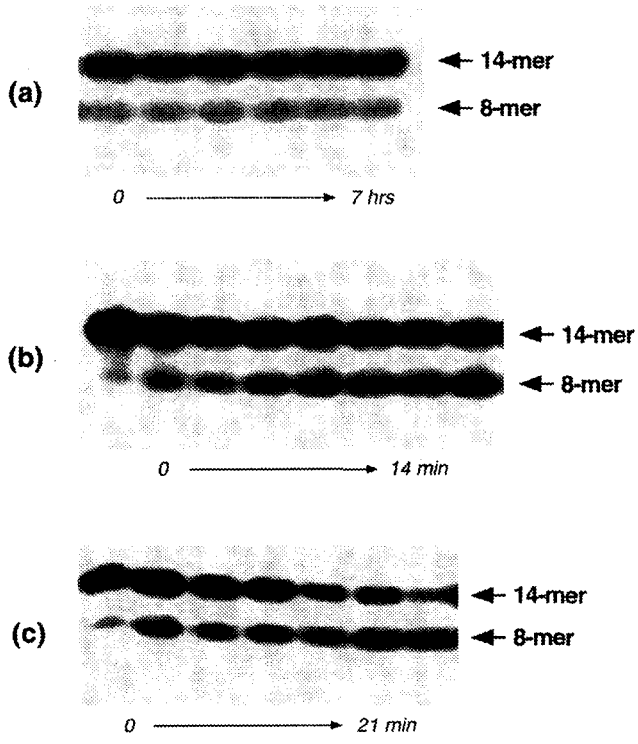

Figure 9. Ribozyme mediated cleavage of the 5′-bridging phosphorothioate. Autoradiograms of gels run at pH 6.8 showing time-course of product (8-mer) formation: (a) ribozyme reaction containing 5 mM EDTA, (b) ribozyme reaction containing 10 mM Mg^{2+}, and (c) ribozyme reaction containing EDTA and 0.5 mM spermine.

In the presence of a metal cofactor and the polycation spermine (0.5 mM), the half-lives are somewhat reduced (see Table 1). Surprisingly, however, spermine can also promote extremely effective ribozyme-mediated cleavage of the 5′-bridging phosphorothioate in the complete absence of a metal cofactor (5 mM EDTA).[31] Neither the related polyamine spermidine (0.5 or 1.0 mM) nor NaCl (500 mM) were found to have this ability, despite the fact that both spermidine and NaCl are known to stabilize higher-order nucleic acid structures.[54] These observations suggest a special role for spermine beyond the simple organization of the proper complex conformation. The polycation may even participate in the cleavage event itself, and we have initiated experiments to investigate this possibility by using spermine analogues. Although spermine also increased the cleavage rate of the native oxo linkage in the presence of a metal cofactor, no cleavage could be detected with spermine alone.

As revealed in Table 1, the half-life for the ribozyme-mediated cleavage of the 5′-bridging phosphorothioate in the presence of 10 mM Mg^{2+} at pH 7.5 is only marginally lower than the half-life for the native oxo linkage (compare $t_{1/2}$ = 11 min vs 20 min). Thus, there is no appreciable thio effect at pH 7.5 despite the fact that the thio analogue is cleaved about a million-fold faster than an oxo linkage in the absence of the ribozyme.[30] Moreover, the thio analogue does not exhibit a noticeable preference for the 'softer', more thiophilic Mn^{2+} cofactor (compare $t_{1/2}$ = 11 min vs. 9 min). Ribozyme reactions containing both Mg^{2+} and Mn^{2+} also do not show appreciable changes in the cleavage kinetics for either the oxo or the thio linkage.[33] Taken together, these observations provide compelling evidence that the metal cofactor does not interact with the 5′-leaving group in the transition state. Similar phosphorothioate experiments performed with the *Tetrahymena* ribozyme clearly established that a metal cofactor coordinates to the 3′-leaving group; in that case, the Mg^{2+} vs. Mn^{2+} rate differential was 1000-fold.[23] We observe only a 1.2-fold rate differential at pH 7.5. Therefore the hammer-

head and *Tetrahymena* catalytic RNA, although both metalloenzymes, appear to use very different strategies to facilitate cleavage. Moreover, since we see no evidence of metal–ion interaction with the 5′-leaving group, our data do not support the double-metal model for hammerhead ribozyme-mediated RNA cleavage (see Fig. 2).[31,32]

Experiments similar to ours have recently been repeated with an all-RNA substrate, but in contrast to our findings, these investigators observed a large thio effect and concluded that the departure of the 5′-leaving group is the rate-limiting step of the cleavage reaction.[77] However, it is not clear how meaningful these results are since the substrate contained a substantial and unexplained impurity and the substrates were >30% cleaved at the start of the reaction. Moreover, the reactions were conducted at pH 6.0 and contained only 0.3 mM metal, compared to the conventional conditions of pH 7.5 and 10 mM metal. Such sub-optimal reaction conditions could dramatically amplify the differences in the cleavage rate between the oxo and thio linkages, especially in light of our pH-rate data, which indicate a possible change in mechanism for the 5′-bridging phosphorothioate at such a low pH (vide infra).

The oxo substrate exhibits a linear pH vs. rate profile between pH 6.0 and 8.0 in the presence of 10 mM Mg^{2+} or Mn^{2+} (see Fig. 10a). Above pH 8.0, the rate begins to drop off. The slopes of the curves are 1.1 and 0.78 for Mg^{2+} and Mn^{2+}, respectively, which is in agreement with values obtained for all-RNA substrates.[25] The linear pH rate profiles suggest that the chemical cleavage step, as opposed to a conformational change, is the rate determining step. Ribozyme-mediated cleavage of the 5′-bridging phosphorothioate also exhibits a linear pH rate profile (between pH 6.5 and 8.5) with slopes of 0.36 and 0.24 for Mg^{2+} and Mn^{2+}, respectively (see Fig. 10b). Although the slope for the thio analogue is lower than for the oxo linkage, this may be due to the leveling off of the curve at the lower pHs. For both the oxo and the thio linkages, Mn^{2+} gives the smaller slope and the Mg^{2+} and Mn^{2+} curves intersect at about pH 8.5. The leveling off of the pH rate profile at pH 6.0 and 6.5 for the 5′-bridging phosphorothioate suggests that there may be a change in mechanism at these lower pHs. Therefore, it would be prudent to perform rate determinations and other analyses at the conventional pH 7.5, which is in the linear region of the pH rate profile. Because of the differences in the slopes of the pH-rate profiles for the oxo and thio linkages, and because the slope levels-off for the thio linkage, the rate differential between the two substrates is exaggerated at the lower pH values. For example, although we observe a thio effect of only 1.9 at the conventional reaction condition of pH 7.5, this is inflated to 100 at pH 6.0. The magnitude of the thio effect at pH 6.0 is similar to that recently reported for similar all-RNA substrates at pH 6.0, which independently confirms that there is very little difference between the all-RNA substrate and our simplified chimera model.[77] However, since there appears to be a change in mechanism at this low, non-standard pH, the validity of drawing conclusions from kinetic data obtained at low pH must be questioned.

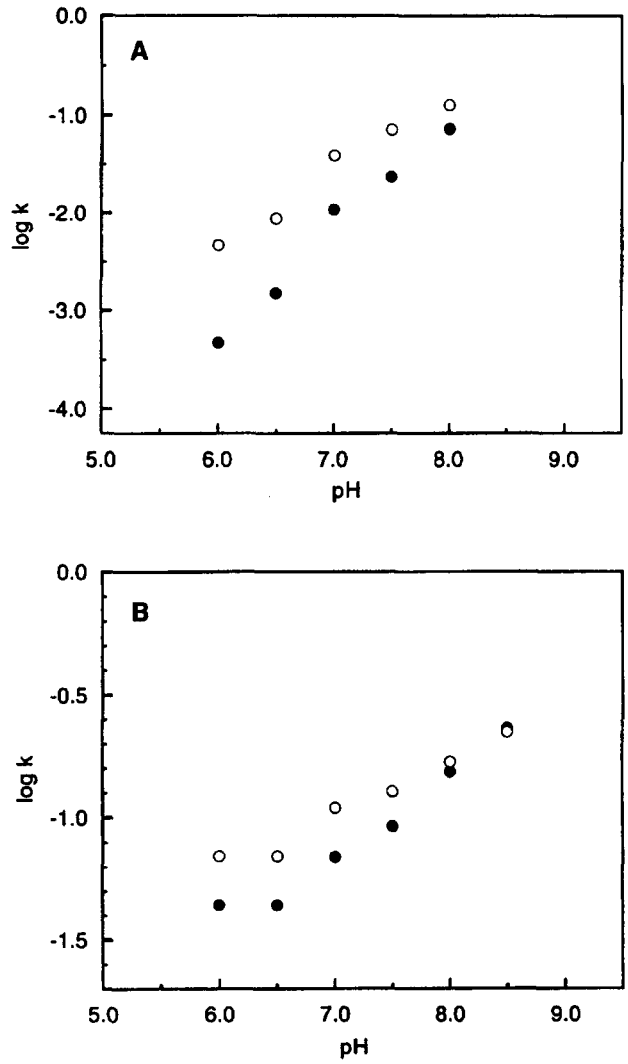

Figure 10. The influence of pH on the ribozyme-mediated cleavage. (a) Log k versus pH for the native oxo linkage in the presence of 10 mM Mg^{2+} (filled circles) or Mn^{2+} (open circles). (b) Log k versus pH for the 5′-bridging phosphorothioate linkage in the presence of 10 mM Mg^{2+} (filled circles) or Mn^{2+} (open circles).

Figure 11 illustrates the effect of increasing divalent metal-ion concentration (Mg^{2+} and Mn^{2+}) on the ribozyme-mediated cleavage of the substrate at pH 7.5. The Mg^{2+} curves indicate cooperative metal binding with saturation near 100 mM. The use of Mn^{2+} at concentrations greater than 10 mM caused reductions in the cleavage rate, possibly due to precipitation of metal hydroxides at these higher metal concentrations.[25] Both the oxo and thio linkages exhibit very similar metal titration curves for both the 'hard' Mg^{2+} cation and the 'soft' Mn^{2+} cation. This suggests that both ribozyme–substrate complexes interact with the divalent metal similarly despite the different metal binding characteristics of the two leaving groups (oxygen vs. sulfur).

We have also investigated the influence of the reaction temperature on the ribozyme-mediated cleavage of the

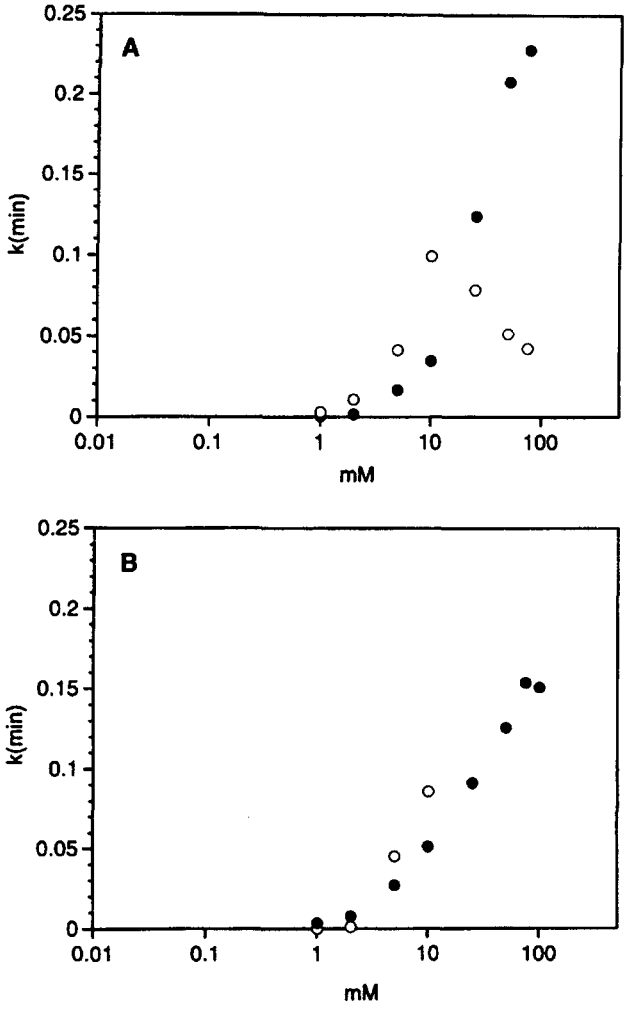

Figure 11. The influence of divalent metal ion concentration on the ribozyme-mediated cleavage. (a) Cleavage rate versus Mg^{2+} concentration (filled circles) or Mn^{2+} concentration (open circles) for the native oxo linkage. (b) Cleavage rate versus Mg^{2+} concentration (filled circles) or Mn^{2+} concentration (open circles) for the 5′-bridging phosphorothioate linkage.

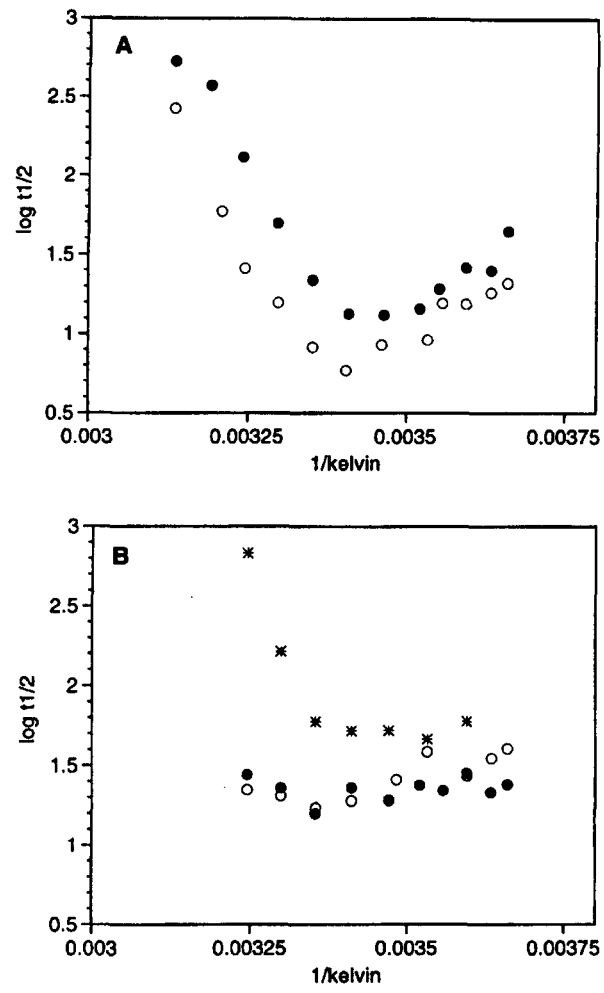

Figure 12. The influence of temperature on the ribozyme-mediated cleavage. (a) Log $t_{1/2}$ versus temperature for the cleavage of the native oxo linkage at pH 7.5 in the presence of 10 mM Mg^{2+} (filled circles) or Mn^{2+} (open circles). (b) Log $t_{1/2}$ versus temperature for the cleavage of the 5′-bridging phosphorothioate linkage at pH 7.5 in the presence of 10 mM Mg^{2+} (filled circles), Mn^{2+} (open circles), or 5 mM EDTA and 0.5 mM spermine (asterisks)

oxo and thio linkages at pH 7.5 in the presence of either Mg^{2+} or Mn^{2+} (see Fig. 12). Cleavage rates were measured at temperatures between 0 °C and 50 °C. The effect of temperature on the rate of cleavage of the thio linkage in the presence of spermine and the absence of metal ions (5 mM EDTA) was also examined, since spermine can replace the metal cofactor when the leaving group is a sulfur atom. The cleavage rates increase linearly as the reaction temperature is raised from 0 to 30 °C. In the case of the oxo linkage, the cleavage rate drops off sharply at temperatures above 30 °C due to the melting of the tertiary structure that is required for catalysis. For the thio linkage, the rate reduction at the higher temperatures is not nearly as pronounced because the substrate strand that is displaced from the ribozyme is susceptible to metal-assisted chemical cleavage, which cannot be distinguished from the ribozyme-mediated cleavage and is accelerated by the elevated temperatures. The lack of a substantial rate reduction also reflects the protection that is afforded to the phosphorothioate linkage in the folded ribozyme–substrate complex. In the presence of spermine and the absence of metal ions the temperature–rate profile for the ribozyme-mediated cleavage of the thio linkage mirrors that of the oxo linkage and shows the same rate drop-off near 30 °C. In the absence of a divalent metal, the displaced substrate is not especially prone to chemical cleavage.

Conclusion

We have synthesized a chimeric oligonucleotide that contains a bridging 5′-phosphorothioate adjacent to a ribonucleotide. The cleavage of this labile linkage proceeds by a transesterification pathway and is dramatically accelerated by divalent metal ions. The relative ability of the divalent metals to promote cleavage is correlated with the softness character of the metal, which implies that the metal cation interacts with the 5′-mercapto leaving group to facilitate cleavage. Hybridization and secondary structure influence

the Mn^{2+} cleavage of the thio linkage. In contrast to the chemical cleavage, the hammerhead ribozyme-mediated cleavage of the 5′-bridging phosphorothioate does not exhibit an appreciable metal preference or thio effect at pH 7.5. Metal concentration experiments show that ribozyme complexes containing either the native oxo linkage or the thio linkage interact with Mg^{2+} or Mn^{2+} similarly, and pH-rate profiles reveal a possible change in reaction mechanism at low pHs. The kinetic results for the simplified chimeric substrates are shown to be identical to all-RNA substrates. The departure of the 5′-leaving group in the cleavage reaction catalyzed by the hammerhead ribozyme is not rate-limiting and a metal cofactor does not interact with the 5′-leaving group under standard reaction conditions. These results do not support the double-metal model for hammerhead ribozyme-mediated RNA cleavage.

Experimental

General

Reagents and buffers were from Sigma or Aldrich Chemical Co. (Milwaukee, WI) and were used as supplied. Deoxynucleoside phosphoramidite monomers and solid supports were from CPG, Inc. (Lincoln Park, NJ). Ribonucleoside phosphoramidite monomers were from Biogenex (San Ramon, CA) The Cee-protected cytidine phosphoramidite was a gift of Glen Research (Sterling, VA). Enzymes were from Boehringer (Mannheim, Germany) or New England Biolabs (Beverly, MA). [γ-^{32}P]ATP was from NEN/DuPont (Wilmington, DE). Oligonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer. Anion exchange chromatography was performed on a Pharmacia FPLC system. Electrophoresis gels were analyzed with a Molecular Dynamics Phosphorimager 425 and Image-Quant v3.3 software.

Oligo synthesis and isolation

Oligonucleotide substrate sequences were synthesized on CPG supports at 1 μmol scale with conventional β-cyanoethyl methodology. Detritylation was accomplished with 2% dichloroacetic acid in anhydrous CH_2Cl_2. All oxidations were performed with the standard aqueous iodine/pyridine/THF mixture. Oligonucleotides were synthesized 'trityl off.' The coupling time for the thio A and Cee-protected rC phosphoramidite was 9 min. After coupling, oxidizing, and capping the 5′-thio A residue, the synthesis cartridge was removed from the instrument, washed with H_2O, and treated with aqueous $AgNO_3$ (50 mM, 3 mL) for 45–60 min in the dark. The cartridge was then rinsed with H_2O and treated with DTT (50 mM, 3 mL) for 5 min, rinsed again with H_2O followed by acetonitrile, and finally dried in a vacuum desiccator for 1–3 h. The dried synthesis cartridge was reattached to the instrument and the next phosphoramidite monomer was coupled to the 5′-thiol for 9 min. Subsequent monomer additions were made in the conventional, automated manner. After complete oligomer assembly, the solid support was treated with concentrated NH_4OH (5 mL) at 50 °C for 12 h. Ammonia was removed under reduced pressure and the oligomer was desalted with a Sephadex G-10 column (2 × 40 cm), and lyophilized.

The crude sulfur-containing oligonucleotide was isolated and purified by anion-exchange chromatography with a Mono-Q HR 5/5 column (Pharmacia). The optimum buffer was 10 mM KH_2PO_4, pH 6.35, containing 30% acetonitrile. Prior to purification the oligomer was adjusted to 10 mM KH_2PO_4 (pH 5.5) and a small amount of tris carboxyethylphosphine (TCEP) was added. After 5 min the solution was loaded onto the column. Elution was accomplished with a two-step KCl gradient (15–320 mM in 10 min, followed by 330–400 mM in 25 min) at a flow rate of 1.5 mL/min. The full-length 14-mer eluted at 25 min, the 12-mer disulfide (formed by auto-oxidation of the 5′-thiol containing cleavage fragment) at 17 min, and the 8-mer cleavage fragment at 12 min. The appropriate fractions were collected, concentrated to a low volume, and desalted on a Sephadex G-10 column.

Sequences containing the 5′-bridging phosphorothioate were end-labeled with [γ-^{32}P]ATP (25 pmol) and T4 polynucleotide kinase (30 units) in a special buffer mixture consisting of 50 mM PIPES, pH 6.6, 1 mM $MgCl_2$, 1 mM 2-mercaptoethanol. After 15 min at 27 °C, the reaction was quenched with 10 volumes of a solution containing 10 mM EDTA and 50 mM PIPES, pH 6.6. This solution was applied to a Sep-Pak C-18 column, washed with 4% MeOH in H_2O (20 mL), eluted with CH_3CN/MeOH/H_2O (35:35:30, 4 mL) and lyophilized to dryness.

Chemical and ribozyme cleavage

The chemical pH experiments were conducted by incubating a small amount of the end-labeled oligomer at ambient temperature with EDTA (5 mM) and Tris (50 mM) at pH 7.5, 8.5, or 9.0. Aliquots of 5 μL were removed at time intervals and quenched at 0 °C with 10 μL gel loading buffer containing the following: 20% glycerol, 0.01% bromophenol blue and xylene cyanol, 7 M urea, 80 mM Tris, 40 mM NaOAc, 100 mM EDTA, pH 6.8. Reaction products were separated as described below. The chemical, metal-assisted cleavage reactions were similarly carried out by incubating a small amount of the oligomer with metal dichloride (5 mM) and Tris-HCl (50 mM, pH 7.5). Aliquots were removed and quenched as described above. For the hybridization studies, a two-fold molar excess of the complement was mixed with the substrate in Tris-HCl (50 mM, pH 7.5) prior to adding the metal dichloride. Thermal UV melting experiments in the same buffer showed that the helix-coil transition for each of the duplexes was well above the ambient reaction temperature.

For the ribozyme-mediated cleavage reactions, two 20 μL solutions containing either 1 μM ribozyme or 1 nM radiolabeled substrate in 50 mM Tris-HCl (pH 7.5) were heated to 65 °C and slowly cooled to 27 °C. After cooling, the appropriate additive (e.g., EDTA, metal dichloride, spermine) was added to the ribozyme solution. Reactions were initiated by mixing the ribozyme and substrate solutions. Aliquots of 5 μL were removed at time intervals and were terminated by pipetting the solution into 1.5 volumes of the gel loading mixture described above. For the pH studies, the following buffers were used in place of Tris: MES (pH 6.0), PIPES (pH 6.5), MOPS (pH 7.0), TAPS (pH 8.0, 8.5).

Denaturing PAGE (20% acrylamide, 2% crosslinking, 7 M urea) was performed at pH 6.8 with a running buffer of 40 mM Tris, 20 mM NaOAc, 1 mM EDTA. The buffer was recirculated between the upper and lower chambers of a modified, vertical slab-gel apparatus (Hoeffer SE-400) with a peristaltic pump to maintain a constant pH during the run. The gels were pre-electrophoresed for 2 h prior to the run, which was carried out at 10 mA constant current for 8 h. Control experiments showed that electrophoresis at pH 6.8 did not cause degradation of the thio linkage, in contrast to the conventional TBE (pH 8.4) buffer system. The gels were exposed to a Kodak SO230 phosphor storage screen (Molecular Dynamics) for 12–24 h, and were analyzed and quantitated with ImageQuant (Molecular Dynamics) software. Pseudo-first-order rate constants were calculated from the half-lives of the substrates ($k = 0.693/t_{1/2}$).

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References

1. Forster, A. C.; Symons, R. H. *Cell* **1987**, *49*, 211.

2. Symons, R. H. *Trends in Biochemical Sciences* **1989**, *14*, 445.

3. Uhlenbeck, O. C. *Nature (London)* **1987**, *321*, 596.

4. Koizumi, M.; Ewai, S.; Ohtsuka, E. *FEBS Lett.* **1988**, *239*, 285.

5. Scott, W. G.; Finch, J. T.; Klug, A. *Cell* **1995**, *81*, 991.

6. Pley, H. W.; Flaherty, K. M.; McKay, D. B. *Nature (London)* **1994**, *372*, 68.

7. Eckstein, F.; Gish, G. *TIBS* **1989**, *14*, 97.

8. Eckstein, F. *Angew. Chem. Int. Ed. Engl.* **1983**, *22*, 423.

9. Connolly, B. A.; Potter, B. V. L.; Eckstein, F.; Pingoud, A.; Grotjahn, L. *Biochemistry* **1984**, *23*, 3443.

10. Vosberg, H. P.; Eckstein, F. *Biochemistry* **1977**, *16*, 3633.

11. Marshall, W. S.; Caruthers, M. H. *Science* **1993**, *259*, 1564.

12. Brill, W. K. D.; Nielsen, J.; Caruthers, M. H. *J. Am. Chem. Soc.* **1991**, *113*, 3972.

13. Fidanza, J. A.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1989**, *111*, 9117.

14. Fidanza, J. A.; Ozaki, H.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1992**, *114*, 5509.

15. Gish, G.; Eckstein, F. *Science* **1988**, *240*, 1520.

16. *Methods in Molecular Medicine: Antisense therapeutics*; Agrawal, S., Ed.; Humana: Totowa, 1996.

17. Rybakov, V. N.; Rivkin, M. I.; Kumarev, V. P. *Nucleic Acids Research* **1981**, *9*, 189.

18. Cosstick, R.; Vyle, J. S. *J. Chem. Soc., Chem. Commun.* **1988**, 992.

19. Cosstick, R.; Vyle, J. S. *Tetrahedron Lett.* **1989**, *30*, 4693.

20. Cosstick, R.; Vyle, J. S. *Nucleic Acids Res.* **1990**, *18*, 829.

21. Sayers, J. R.; Olsen, D. B.; Eckstein, F. *Nucleic Acids Res.* **1989**, *17*, 9495.

22. Grasby, J. A.; Connolly, B. A. *Biochemistry* **1992**, *31*, 7855.

23. Piccirilli, J. A.; Vyle, J. S.; Caruthers, M. H.; Cech, T. R. *Nature (London)* **1993**, *361*, 85.

24. Slim, G.; Gait, M. J. *Nucleic Acids Res.* **1991**, *19*, 1183.

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

26. van Tol, H.; Buzayan, J. M.; Feldstein, P. A.; Eckstein, F.; Bruening, G. *Nucleic Acids Res.* **1990**, *18*, 1971.

27. Koizumi, M.; Ohtsuka, E. *Biochemistry* **1991**, *30*, 5145.

28. Warnecke, J. M.; Furste, J. P.; Hardt, W. F.; Erdmann, V. A.; Hartmann, R. K. *Proc. Natl. Acad. Sci., U.S.A.* **1996**, *93*, 8924.

29. Ruffner, D. E.; Uhlenbeck, O. C. *Nucleic Acids Res.* **1990**, *18*, 6025.

30. Kuimelis, R. G.; McLaughlin, L. W. *Nucleic Acids Res.* **1995**, *23*, 4753.

31. Kuimelis, R. G.; McLaughlin, L. W. *J. Am. Chem. Soc.* **1995**, *117*, 11019.

32. Kuimelis, R. G.; McLaughlin, L. W. *Biochemistry* **1996**, *35*, 5308.

33. Kuimelis, R. G.; McLaughlin, L. W. In *Nucleic Acids and Molecular Biology*; Eckstein, F., Lilley, D. M. J., Eds.; Springer: Berlin, 1996; Vol. 10, pp 197–215.

34. Forster, A. C.; Symons, R. H. *Cell* **1987**, *50*, 9.

35. Symons, R. H. *Crit. Rev. Plant Sci.* **1991**, *10*, 189.

36. Haseloff, J.; Gerlach, W. L. *Nature (London)* **1988**, *334*, 585.

37. Ruffner, D. E.; Stormo, G. D.; Uhlenbeck, O. C. *Biochemistry* **1990**, *29*, 10695.

38. Yarus, M. *FAESB J.* **1993**, *7*, 31.

39. Pyle, A. M. *Science* **1993**, *261*, 709.

40. Scott, W. G.; Klug, A. *TIBS* **1996**, *21*, 220.

41. Freemont, P. S.; Friedman, J. M.; Beese, L. S.; Sanderson, M. R.; Steitz, T. A. *Proc. Natl. Acad. Sci., U.S.A.* **1988**, *85*, 8924.

42. Beese, L. S.; Steitz, T. A. *EMBO J* **1991**, *10*, 25.

43. Steitz, T. A.; Steitz, J. A. *Proc. Natl. Acad. Sci., U.S.A.* **1993**, *90*, 6498.

44. Taira, K.; Uebayasi, M.; Maeda, H.; Furukawa, K. *Protein Eng.* **1990**, *3*, 691.

45. Taira, K.; Uchimaru, T.; Tanabe, K.; Uebayasi, M.; Nishikawa, S. *Nucleic Acids Res.* **1991**, *19*, 2747.

46. Uebayasi, M.; Uchimaru, T.; Koguma, T.; Sawata, S.; Shimayama, T.; Taira, K. *J. Org. Chem.* **1994**, *59*, 7414.

47. Sawata, S.; Komiyama, M.; Taira, K. *J. Am. Chem. Soc.* **1995**, *117*, 2357.

48. Dahm, S. C.; Uhlenbeck, O. C. *Biochimie* **1990**, *72*, 819.

49. Perreault, J.-P.; Wu, T.-F.; Cousineau, B.; Ogilvie, K. K.; Cedergren, R. *Nature (London)* **1990**, *344*, 565.

50. Perreault, J. P.; Labuda, D.; Usman, N.; Yang, J. H.; Cedergren, R. *Biochemistry* **1991**, *30*, 4020.

51. Yang, J. H.; Perreault, J. P.; Labuda, D.; Usman, N.; Cedergren, R. *Biochemistry* **1990**, *29*, 11156.

52. Yang, J. H.; Usman, N.; Chartrand, P.; Cedergren, R. *Biochemistry* **1992**, *31*, 5005.

53. Hall, K. B.; McLaughlin, L. W. *Biochemistry* **1991**, *30*, 10606.

54. Saenger, W. *Principles of Nucleic Acid Structure*; Springer: New York, 1983.

55. Jenkins, L. A.; Bashkin, J. K.; Autry, M. E. *J. Am. Chem. Soc.* **1996**, *118*, 6822.

56. Mag, M.; Lking, S.; Engles, J. W. *Nucleic Acids Res.* **1991**, *19*, 1437.

57. Sproat, B. S.; Beijer, B.; Rider, P.; Neuner, P. *Nucleic Acids Res.* **1987**, *15*, 4837.

58. Usman, N.; Ogilvie, K. K.; Jiang, M.-Y.; Cedergren, R. J. *J. Am. Chem. Soc.* **1987**, *109*, 7845.

59. Gasparutto, D.; Livache, T.; Bazin, H.; Duplaa, A.-M.; Guy, A.; Khorlin, A.; Molko, D.; Roget, A.; Teoule, R. *Nucleic Acids Res.* **1992**, *20*, 5159.

60. Westman, E.; Stromberg, R. *Nucleic Acids Res.* **1994**, *22*, 2430.

61. Rao, V. M.; Reese, C. B.; Schehlmann, V.; Yu, P. S. *J. Chem. Soc. Perkin. Trans. 1* **1993**, 43.

62. Capaldi, D. C.; Reese, C. B. *Nucleic Acids Res.* **1994**, *22*, 2209.

63. Reese, C. B.; Saffhill, R.; Sulston, J. E. *J. Am. Chem. Soc.* **1967**, *89*, 3366.

64. Iocono, J. A.; Gildea, B.; McLaughlin, L. W. *Tetrahedron Lett.* **1990**, *31*, 175.

65. Liu, X.; Reese, C. B. *Tetrahedron Lett.* **1995**, *36*, 3413.

66. Sakatsume, O.; Ogawa, T.; Hosaka, H.; Kawashima, M.; Takaki, M.; Takaku, H. *Nucleosides Nucleotides* **1991**, *10*, 141.

67. Sakatsume, O.; Yamaguchi, T.; Ishikawa, M.; Hirao, I.; Miura, K.-i.; Takaku, H. *Tetrahedron* **1991**, *47*, 8717.

68. Yamakage, S.-i.; Sakatsume, O.; Furuyama, E.; Takaku, H. *Tetrahedron Lett.* **1989**, *30*, 6361.

69. Connolly, B. A.; Rider, P. *Nucleic Acids Res.* **1985**, *13*, 4485.

70. Komiyama, M.; Matsumura, K.; Matsumoto, Y. *J. Chem. Soc., Chem. Commun.* **1992**, 640.

71. Shapiro, R.; Vallee, B. L. *Biochemistry* **1989**, *28*, 7401.

72. Dean, J. A. *Lange's Handbook of Chemistry*; 13 ed.; McGraw-Hill: New York, 1985.

73. Pearson, R. G. *J. Am. Chem. Soc.* **1963**, *85*, 3533.

74. Pearson, R. G. *Coordination Chemistry Reviews* **1990**, *100*, 403.

75. Huheey, J. E. *Inorganic Chemistry: Principles of Structure and Reactivity*; Harper and Row: New York, 1983.

76. Bashkin, J. K.; Jenkins, L. A. *Comments Inorg. Chem.* **1994**, *16*, 77.

77. Zhou, D.; Usman, N.; Wincott, F. E.; Matulic-Adamic, J.; Orita, M.; Zhang, L.; Komiyama, M.; Kumar, P. K. R.; Taira, K. *J. Am. Chem. Soc.* **1996**, *118*, 5862.

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