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Sequence Specific Cleavage of Messenger RNA by a Modified Ribonuclease H

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Abstract—Ribonuclease H (RNase H) is an endonuclease that cleaves only the RNA strand of an RNA–DNA hybrid to produce 5'-phosphate and 3'-hydroxy termini and lacks useful sequence specific recognition properties. A mutant form of the *E. coli* enzyme has been prepared that is suited for selective chemical modification at a site proximal to the substrate binding region. The chemical derivatization involves the formation of a disulfide linkage to a modified octadeoxyribonucleotide. The conjugate retains only 0.3% of the normal sequence independent RNase H activity demonstrating that substrate recognition can be modulated by a covalent appendage. A β-globin RNA transcript containing a sequence complementary to that of the octadeoxyribonucleotide was cleaved in a catalytic fashion to two products upon treatment with the conjugate. The selectivity in the phosphodiester bond cleavage mediated by the conjugate was found to be different than that displayed by the nonderivatized enzyme. These results demonstrate the potential of semi-synthetic RNase H conjugates for mechanistic studies and their application as RNA targeted diagnostic or therapeutic agents.

Introduction

Agents that are capable of sequence selective cleavage of RNA have potential utility as diagnostic or therapeutic agents, and this goal has stimulated a variety of recent experimental approaches. In nature, many enzymes and cellular toxins elicit their biological effects through the modification or degradation of RNA.[1,2] This principle has been explored in the design of cell specific toxins that could have utility in chemotherapeutics.[3–7] Development of protein-based catalytic agents which cleave specific sequences of mRNA would complement these approaches and potentially allow for a broad range of applications. Semi-synthetic ribonucleases based upon engineered proteins offer the opportunity to target RNA sequences. Oligodeoxyribonucleotide-directed nucleases, including RNase S,[8] staphylococcal nuclease,[9] 2–5A dependent RNase,[10] and RNase H[11] have been reported. However, the general potential for this approach requires further investigation. Despite the initial advances, the design of modified ribonucleases with significant utility represents a challenge because most have no natural sequence specificity, and requires the absence of the normal background activity in the modified protein.[a] Also, the utility of such an agent would be enhanced if a high rate of catalytic turnover can be maintained after alteration. The results described here demonstrate the potential for the rational development of ribonucleases with tailored specificity for RNA sequences.

Ribonuclease H (RNase H), which is found in all cell types, cleaves only the RNA strand of an RNA–DNA hybrid to produce 5'-phosphate and 3'-hydroxy termini.[12,13] Although the enzyme behaves as an endoribonuclease, there is no inherent sequence selectivity displayed in the phosphodiester bond hydrolysis. Antisense oligodeoxyribonucleotides complementary to RNA sequences can direct *in vitro* cleavage events mediated by RNase H. This property has inspired the use of RNA cleavage by RNase H as a mechanism for inhibition of gene expression by antisense oligodeoxyribonucleotides. This is now a common design of modified oligodeoxyribonucleotides,[14–16] and similar rationales have been explored to cleave specific RNA structures by RNase P and ribozymes.[17] However, many modified antisense agents are not suitable substrates for these approaches, and function through a secondary mode of action by transcriptional arrest.[18] Stringent sequence specificity is required to avoid *in vivo* toxic side effects.[19,20] Finally, the cellular localization and specificity of RNase H has taken on an increased significance and may guide the design of agents directed at nucleic acids.[21]

An RNase H molecule that displays a high degree of sequence specificity might overcome some of the problems associated with antisense oligonucleotides. In addition, such an agent would be useful for a detailed understanding of the structure and mechanism of RNase H. Our interest in the mechanisms of endoribonucleases has led us to investigate *E. coli* RNase H oligodeoxyribonucleotide conjugates. During the course of these studies, a covalently modified RNase H molecule was reported that cleaved only a model nonaribonucleotide and had specificity similar to that of the non-modified enzyme.[11] In this communication, we report a novel RNase H conjugate that displays catalytic, sequence specific cleavage of an intact mRNA molecule but has negligible wild type activity.

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[a]The authors do not mean to imply that RNase H has no specificity in its substrate recognition. The use of the term sequence-independent refers to the context of the flanking sequences in the RNA strand of an RNA/DNA hybrid for which the wild type enzyme has no inherent specificity.

Results

Design and preparation of mutant RNase H

The choice of a site for covalent attachment of an octadeoxyribonucleotide was based upon a proposed model for binding of an RNA–DNA hybrid substrate to *E. coli* RNase H.[22,23] Cys13 was selected as the site for derivatization with the 5' modified oligodeoxyribonucleotide **1** because of its proximity to the putative substrate binding groove (Figure 1).[24] This design principle was incorporated in order to potentially minimize the normal sequence-independent catalytic properties of the protein.[a] A disulfide linkage to the oligonucleotide was chosen since reduction of this link provides a straightforward means of assessing the integrity of the folded enzyme.[25] The three cysteine residues in *E. coli* RNase H are not required for enzyme activity.[26] Therefore, Cys63 and Cys133 were mutated to Ser and Ala, respectively, and two unique restriction sites (Nde I and Bgl II) were introduced at the 5' and 3' ends of the coding sequence to allow for cloning into the *E. coli* expression vector pET 11a. The protein was overexpressed in the RNase H deficient *E. coli* strain MIC1066 at substantially higher levels than with previously reported systems.[24,27] From 1.5 L of cells, 62 mg of purified enzyme was obtained, which provided suitable quantities for derivatization. The mutant protein retained 90% of the wild type catalytic activity; the molecular weight was found by electrospray MS to be 17,549, which is consistent with the calculated value for C63S/C133A-RNase H. For each monomer of the protein, a single reactive thiol was identified by titration using Ellman's reagent. Modification of the cysteine with vinyl pyridine followed by amino acid sequence analysis allowed for verification of this residue at position 13.

Synthesis of the conjugate

The derivatization reaction between **1** and C63S/C133A-RNase H was monitored by SDS-PAGE as well as the standard enzyme assay.[28] As shown in Figure 2, the covalent modification of the protein resulted in a slower rate of migration under the electrophoresis conditions and provided a suitable method of analysis for optimizing the reaction. Regardless of the concentration of **1**, the disulfide exchange process with the protein always resulted in residual noncoupled protein (< 10% by scanning densitometry). Most importantly, a time dependent loss in sequence-independent catalytic activity with a poly(rA)•poly(dT) substrate occurred during the course of the covalent modification reaction (Figure 3).

A simple purification of the Cys13-conjugate away from excess **1** was effected by anion exchange chromatography on Mono Q (Pharmacia), since *E. coli* RNase H does not bind to this material, and the oligonucleotide components display a suitable affinity as shown in Figure 4.[27] The purified conjugate was found to retain only ≤ 0.3% of the normal RNase H activity indicating that the covalent appendage can prohibit the sequence-independent binding or cleavage of a RNA–DNA hybrid. To ensure that the protein had not incurred irreversible denaturation throughout the modification and purification process, the disulfide link in the RNase H Cys13-conjugate was reduced with excess DTT. Upon cleavage of the octadeoxyribonucleotide in this manner, the catalytic activity of the protein with the poly(rA)•poly(dT) substrate was restored to that of the unmodified mutant enzyme. This result confirms the stability of the RNase H molecule throughout the course of the modification process, and further substantiates that a covalent modification can modulate the normal catalytic function of the enzyme.

Figure 1. A ribbon diagram of the *E. coli* C63S/C133-RNase H protein modeled from the original X-ray coordinates.[22] Conserved Asp 10, Asp 70, Glu 49, and Asp 136 residues are colored in purple and Mg^{2+} is incorporated at the proposed active site. In red is the hydrocarbon linker covalently attached to Cys13. In yellow is position 135, which was the site of attachment for previous oligodeoxynucleotide conjugate.[11]

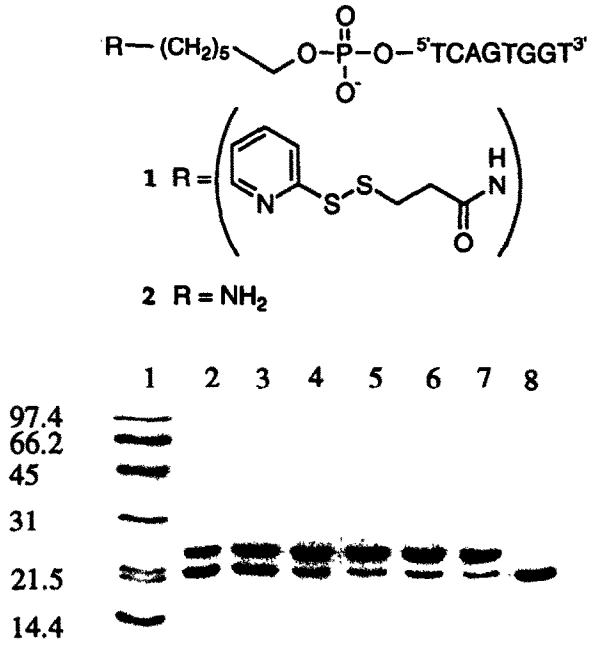

Figure 2. SDS-PAGE of C63S/C133-RNase H coupling reaction with octamer **1** in the absence of reducing agent. Samples were added to denaturing buffer and analyzed after 0.5 h, 1 h, 2 h, 4 h, 8 h, 20 h (lanes 2–7) and 20 h after treating sample with 2-mercaptoethanol lane 8. Lane 1: MW markers.

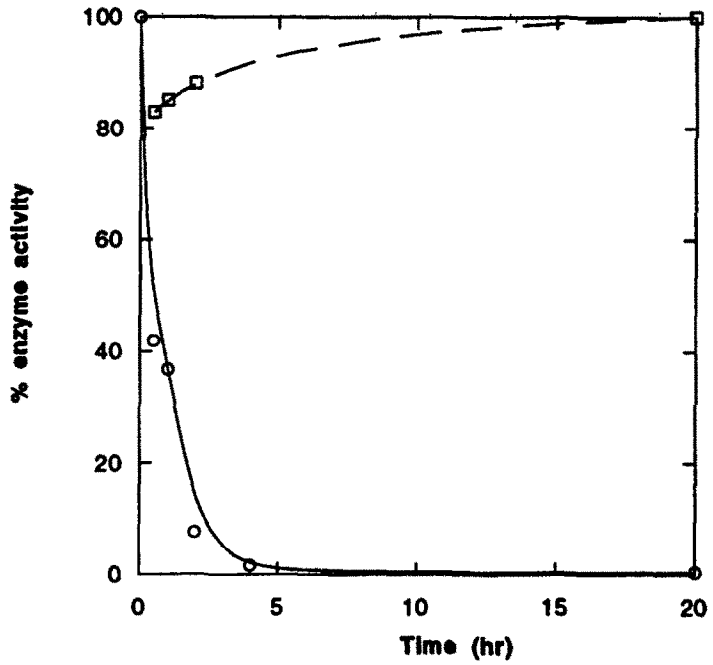

Figure 3. The sequence-independent enzyme activity of the C63S/C133-RNase H in the coupling reaction as a function of time: assayed in the presence (□) or absence (○). of 2-mercaptoethanol.

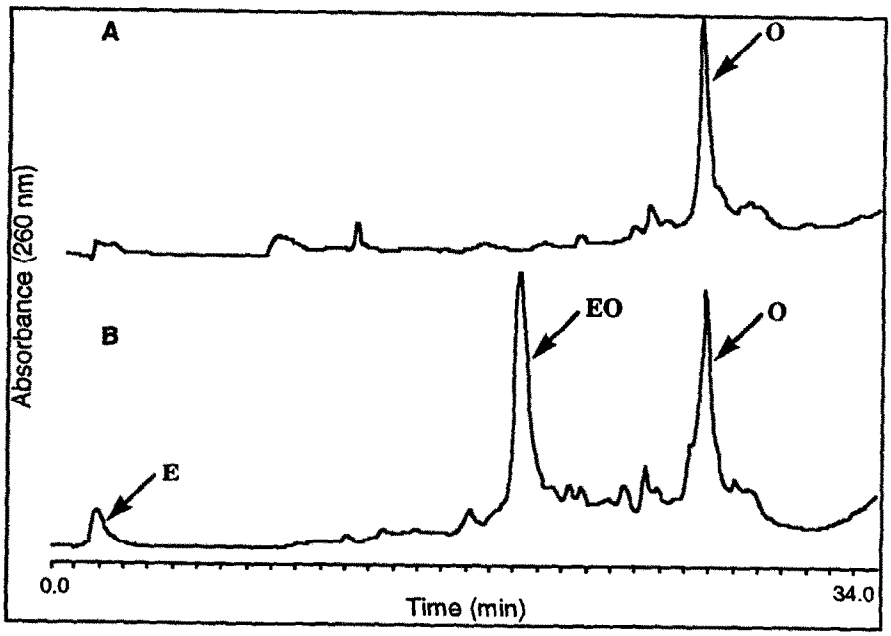

Figure 4. Purification of the RNase H Cys13-conjugate by FPLC Mono Q anion exchange chromatography. A. Octadeoxyriboonucleotide **1**. B. Coupling reaction mixture of **1** and C63S/C133-RNase H. Peaks of interest are identified as **E** (C63S/C133-RNase H), **O** (octadeoxyribonucleotide **1**), and **EO** (RNase H Cys13-conjugate).

Cleavage of mRNA

To test the sequence directed features of the RNase H Cys13-conjugate, a substrate was prepared by *in vitro* run-off transcription of DNA encoding the mouse β-globin gene using T3 RNA polymerase and [α-^{32}P]UTP as a source of radiolabel. The mRNA transcripts were 722 bases in length and included at position 560 a sequence complementary to that of octadeoxyribonucleotide **1**.[29] Phosphodiester bond hydrolysis at the targeted sequence predicted a fragment of approximately 162 and 560 bases. Antisense oligodeoxyribonucleotides to RNA sequences can direct the cleavage events mediated by RNase H and this property has been used in our studies as an RNA size standard control.[30] When the free C63S/C133A-RNase H was incubated with β-globin mRNA in the presence of octadeoxyribonucleotide **2**, two radiolabeled fragments were produced (Figure 5). The lengths of these fragments are consistent with the products expected from RNA cleavage in the region complementary to oligonucleotide **1**. In a similar manner, incubation of RNA with the Cys13-conjugate in the absence of exogenous oligodeoxyribonucleotide revealed two fragments of the same apparent length as those observed with the C63S/C133A-RNase H in the presence of **2**. These reactions were optimized for time, temperature and pH. A noteworthy point is that the RNA cleavage reactions with the Cys13-conjugate were optimal at pH 6.5, while the corresponding reaction with free enzyme and octadeoxyribonucleotide **2** was not affected over the range of pH 6.0 to 8.0.

In order to confirm the catalytic turnover of the proteins in these cleavage reactions, the time courses for the disappearance of the full-length β-globin mRNA and appearance of the 560 base fragment were analyzed. The results are shown in Figure 6. For both the Cys13-conjugate and the C63S/C133A-RNase H, the extent of these reactions under the same conditions indicate that turnover of the protein catalyst has occurred ≥ 8 times. The kinetics of each reaction indicates a burst over the first 5 min with a slower phase over the next 2 h. Nonspecific degradation of the mRNA was observed in control incubations without enzyme after 4 h, which prevented following these reactions to completion.

For direct comparison of the selectivity in phosphodiesterase activity of the Cys13-conjugate and C63S/C133A-RNase H, cleavage of an octaribonucleotide complementary to **1** (ACCACUGA) was investigated. The 5'-end of the octaribonucleotide was labeled by the use of [γ-^{32}P]ATP and polynucleotide kinase before digestion with a) C63S/C133A-RNase H plus **2**, and b) the Cys13-conjugate alone (Figure 7). These analyses revealed that under identical conditions, the phosphodiester bonds cleaved were different for the two enzymes. The cleavage by the C63S/C133A-RNase H and the complementary octadeoxyribonucleotide was not completely specific; the primary site was between A4–C5 and secondary sites were at C5–U6 and U6–G7. However, cleavage by the Cys13-conjugate showed equal extents of cleavage at C5–U6 and U6–G7 and only a trace at A4–C5.

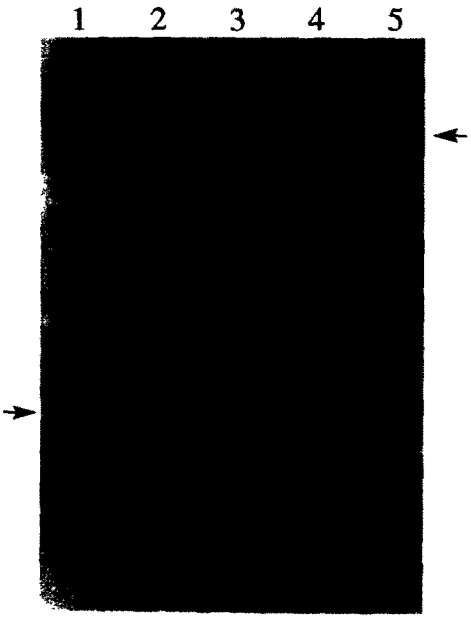

Figure 5. Autoradiogram of the [^{32}P]-RNA transcript cleavage reactions in 40 mM HEPES pH 6.5, 4 mM $MgCl_2$, 4% glycerol at 37 °C for 0.5 h. Lane: 1. Full RNA transcript of 722 bases alone; 2. transcript (0.6 μM). with RNase H Cys13-conjugate (0.15 μM); 3. same as lane 2 in the presence of 1 mM DTT; 4. transcript (0.6 μM) with C63S/C133-RNase H (0.06 μM). and octamer **2** (0.6 μM); 5. same as lane 4 in the presence of 1 mM DTT.

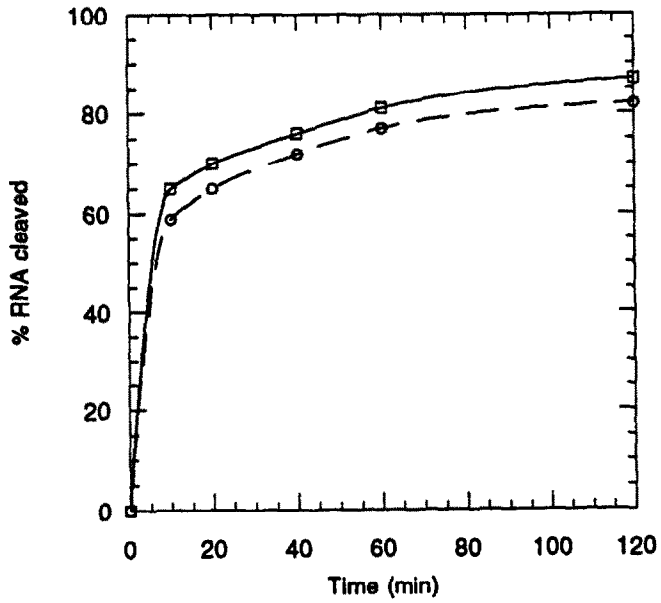

Figure 6. Time course for the cleavage reaction using a. (□) underivatized C63/C133 RNase H (0.17 μM) and octamer **2** (0.17 μM); b. (○) the RNase H Cys13-conjugate (0.17 μM.) Each incubation contained 1.9 μM [^{32}P]-RNA transcript under conditions described for Figure 1. Aliquots taken at the specified time points were heated to 100 °C to stop the reaction before the fragments were separated on a 5% polyacrylamide/7 M urea gel. Visualization was by autoradiography, and area integration was achieved by scanning densitometry.

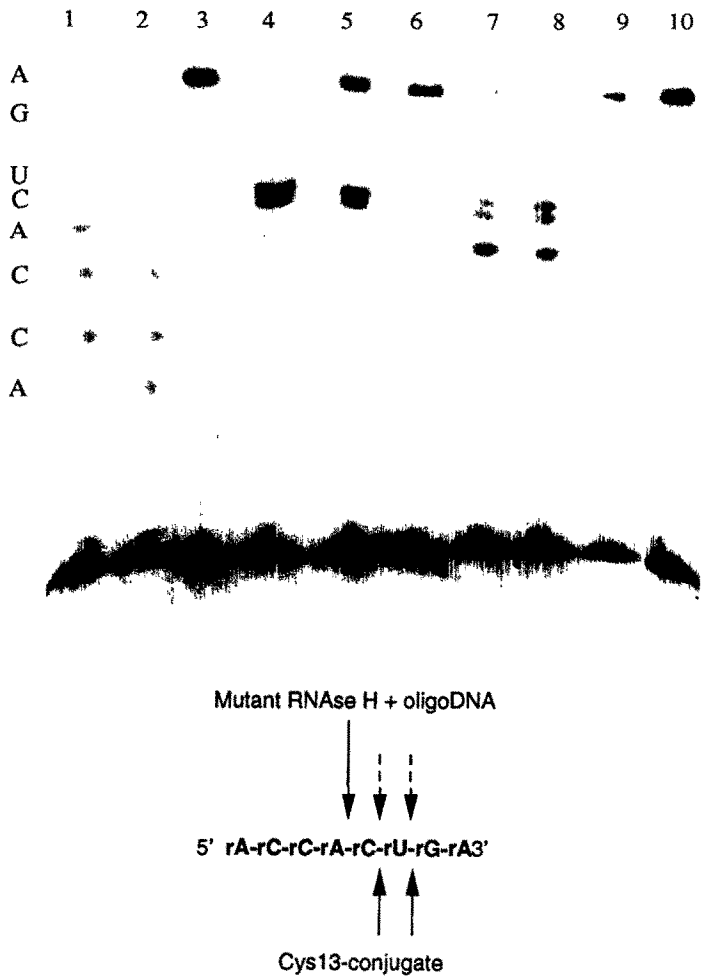

Figure 7. PAGE of octaribonucleotide cleavage products. The 5'-end-labeled octaribonucleotide (lanes 3 and 10) was digested with phosphodiesterase (lanes 1 and 2) and decreasing amounts of the RNAse H Cys13-conjugate (lane 4 = 5:1, lane 5 = 50:1, lane 6 = 100:1) or the octamer **2** plus underivatized C63/C133 RNase H lane 7 = 50:1, lane 8 = 100:1). Sample in lane 9 was incubated with C63/C133 RNase H alone. The solid arrows in the lower figure indicate where the cleavage occurred with highest frequency and the dashed arrows denote low frequency cleavage sites. Although there is no arrow indication, the PAGE does show a low frequency cleavage at A4–C5 for the RNase H Cys13-conjugate in the 5:1 molar ratio incubation.

Discussion

The results with the RNase H Cys13-conjugate and the β-globin mRNA demonstrate the potential of semi-synthetic ribonucleases to serve as catalytic site-specific RNA cleavage agents. The design of these enzymes requires consideration of the endonuclease, target RNA recognition element, the covalent linker, and the site of attachment to the endoribonuclease. For success in the use of these enzymes, a structural change that minimizes the sequence independent endoribonuclease activity must also be incorporated.

E. coli RNase H is an attractive candidate for synthetic modification since it is a relatively low molecular weight, stable protein which can be engineered and produced in suitable quantities. In the case of the RNase H Cys13-conjugate, covalent modification of the protein at a residue near the active site inhibits the catalytic properties required for nonspecific RNA hydrolysis. The reason for this inhibition is not established but may reflect competitive binding between the covalent ligand and an RNA–DNA hybrid substrate. When the attached octamer hybridizes with its complementary strand of RNA, the protein displays the catalytic properties for RNA strand hydrolysis.

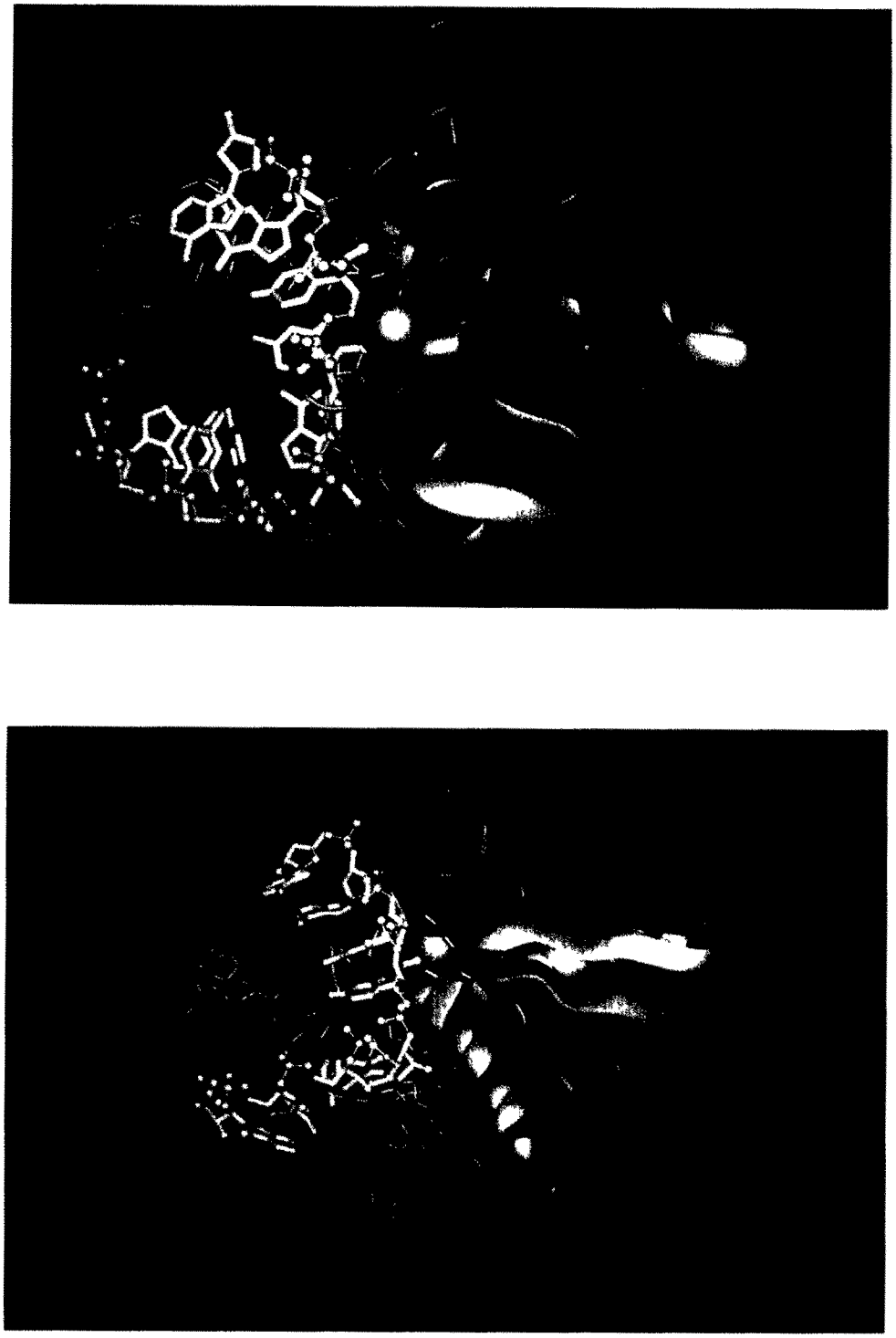

Figure 8. Ribbon diagrams of a modeled RNase H Cys13-conjugate hybridized with the octaribonucleotide ACCACUGA.. Coloring of the metal and amino acid residues are the same as those in Figure 1. The hydrocarbon linker (red) is linked to the 5'-phosphate group of the 2'-deoxy strand and the phosphodiester bonds that are cleaved in the octaribonucleotide strand have been positioned within 3–4 Å of the metal ion. The ribbon structures were constructed using QUANTA 3.3 and displayed on a Silicon Graphics 4D/120 GTX. The complex was minimized with CHARMm 22 (Molecular Simulations) using a conjugate gradient algorithm in which fixed constraints were applied to the protein and harmonic constraints with a mass weighted force constant of 50.0 were applied to the RNA/DNA duplex. No minimization constraints were applied to the Cys13 side chain, the linker arm, the 5'-phosphodiester group, and the magnesium ion. The views of the model are related by a 60° rotation about the horizontal axis in the plane of the graph.

The success of the Cys13-conjugate at distinguishing between RNA substrates indicates that judicious choice of the attachment site on the protein, and the length of the chemical linker can have major impacts on control of the molecular recognition by the protein. Future developments should provide for complete removal of the nonspecific catalytic activity.

The structure of the substrate RNA–DNA hybrids are known to influence the phosphodiester bond selectivity of RNase H.[14,20,31,32] For the RNase H Cys13-conjugate, the catalytic specificity is attributed to a restricted binding register of the RNA/DNA hybrid. The current model of the binary complex of RNA and the Cys13-conjugate is based upon the X-ray crystal structure of the protein and is consistent with the proposed role of the linker (Figure 8).[22,33] Despite the limited experimental information regarding substrate recognition by RNase H, the specificity of the Cys13-conjugate requires positioning of the correct RNA phosphodiester bonds within a suitable distance of the active site defined by the conserved basic residues and the Mg^{2+} atom. A favored enzyme–RNA/DNA conformation was selected by positioning the complex in a conformation similar to that of Yang[23] followed by minimization (see Figure 8). The model places the linker arm within a major groove in the RNA/DNA hybrid and imposes limitations on the position of the RNA strand within the active site. This conformation is presumed similar to a minor one that occurs in the unmodified enzyme–RNA/DNA complex since the same bonds are cleaved but at reduced frequency. Therefore, the position of covalent attachment and the length of the chemical linker impacts the orientation of the RNA/DNA hybrid on the enzyme and could account for the alternate cleavage sites on the RNA strand.

A second oligodeoxyribonucleotide–RNase H conjugate has been reported to cleave a model nonaribonucleotide.[11] The covalent sulfide linkage in this system was derived from an engineered cysteine residue at position 135 of the polypeptide chain, and the resultant catalytic chemistry is different from that displayed by the RNase H Cys13-conjugate. In the previous example, the major sites of cleavage and the steady-state kinetics displayed by the conjugate were similar to those of the free enzyme and oligodeoxyribonucleotide. A model for the Cys135 conjugate reveals that the covalent appendage is near the surface of the protein allowing the duplex form of the molecule to adopt conformations free from the proposed substrate binding sites. Using the model shown in Figure 8, it is apparent in the RNase H Cys13-conjugate that the conformational mobility of the appended oligodeoxyribonucleotide is restricted to the vicinity of the enzyme active site. This observation predicts that RNA/DNA substrate recognition would be reduced through steric exclusion and may explain the ability of the attached ligand to inhibit the nonspecific catalytic activity.

The enzyme–oligonucleotide conjugate approach to site specific cleavage of mRNA may enhance current antisense technology. An important principle implicit in this approach is the catalytic nature of the molecule which could lend the added potency required for the success of an antisense agent. Oligonucleotide length has also been associated with nonspecific events that are mediated by RNase H cleavage, and may lead to potential toxic traits. Woolf and coworkers have demonstrated that an antisense d-13mer was sufficient to produce cleavage on a fibronectin mRNA *in vivo*, and concluded that increased lengths of oligonucleotides produce additional nontargeted RNA destruction.[19] In our *in vitro* studies, the simple octamer in the Cys13-conjugate was sufficient to direct specific cleavage of the β-globin mRNA. Non-specific hybridization of a noncomplementary d-19mer to the β-globin mRNA was also detected and led to non-targeted mRNA cleavage by RNase H at an unidentified site (data not shown). Conversely, a non-complementary octamer (5'-dAdGdCdAdCdAdAdT-3') did not result in cleavage of the mRNA in these assays. Therefore, by choosing the appropriate target RNA sequence, the RNase H conjugate approach has the potential to overcome nonspecific antisense recognition.

Experimental

Materials

DNA restriction and modifying enzymes were purchased from Promega, New England BioLabs, Bethesda Research Laboratories, USB, and Stratagene. [2,5,8-^{3}H]ATP (44 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), [α-^{32}P]UTP (3000 Ci/mmol), and [α-^{35}S]ATP (> 1000 Ci/mmol) were from Amersham. Chloro-β-cyanoethoxy-*N*,*N*-diisopropyl-aminophosphine was purchased from American Bionetics. 6-(4-Monomethoxytritylamino)hexyl-(2-cyanoethyl)-(*N*,*N*-diisopropyl)-phosphoramidite (5' amino modifier C_6) was from Glen Research. The oligoribonucleotide octamer (r8mer), 5'-ACCACUGA-3', was purchased from Integrated DNA Technology. *N*-Succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) was from Pierce. 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB) was from Sigma Chemical Company. Isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were from Gold Biotechnology. *Crotalus durissus* phosphodiesterase was from Boehringer Mannheim. All other chemicals were of reagent grade. Bacterial strains used in this work were XL1-Blue (Stratagene), DH5α (B. Bachman), MIC1007/5, and MIC1066 (Robert Crouch). DNA plasmids used were Bluescript SK II or KS II (Stratagene), pRSV-β-globin (American Type Culture Collection), pET3a (William Studier), pETlla (Novagen), and pAR3040rnh (Robert Crouch). Synthetic oligodeoxyribonucleotides primers were synthesized by the Purdue Center for Macromolecular Structure and Function using phosphoramidite chemistry.

DNA Methods

Plasmid DNA isolations and restriction digest analyses were conducted using established methods (Maniatis, 1989). DNA fragments were separated by agarose gel electrophoresis (0.9% to 1.2%) and compared to known digested lambda or φX174 DNA standards. Preparative scale digests (5–10 μg plasmid DNA) for fragment isolation used 1.4% agarose gels in 1X TAE (0.1 M Tris-

acetate, 0.1 M boric acid, 2 mM EDTA (pH 8.4). Gels were stained with ethidium bromide and visualized by transillumination with long wavelength uv light. DNA fragments were excised in a minimal volume of gel and recovered using the glassmilk protocol (BIO 101). Vector DNA for ligations was digested to completion with the appropriate restriction enzymes and treated with calf intestinal alkaline phosphatase (Promega) followed by addition of 1 μL of 0.5 M EDTA, phenol/chloroform extraction and DNA precipitation. Vector and insert DNA (100 ng each) and the appropriate buffer were incubated in a total volume of 10 μL with 2.7 U T4 DNA ligase (Promega) for 4–12 h at ambient temperature. Templates for DNA sequencing were either recovered single-stranded (1–2 μg) or purified double stranded (3–5 μg) Bluescript DNA. All sequencing analyses were performed by the dideoxy chain termination method (Sanger, 1977) using Sequenase 2 (USB).

Construction of mutant RNase H

The *E. coli rnh* gene in pAR3040rnh was amplified by PCR to incorporate unique restriction sites using as the sense strand primer 5'CTAGGAATTCATATGCTT-AAACAGGTAGAA-3' and the antisense strand primer 5'CTTAAGCTTCCCGGGTTAAACTTCAACTTG-3'. The PCR product was cloned into Bluescript SK at the EcoR I and Hind III sites. Site-directed mutagenesis was carried out using a modified Kunkel oligodeoxyribonucleotide-directed *in vitro* mutagenesis protocol.[33] The mutagenic primers were 5'-CGGTACTCAAAATGACTTCGCTATGTTCTT-3' (C63S) and 5'GGATTCATCGCCGCGGCACGA-GCCAGTTCATCAGCGCGTTCG-3' (C133A). The single mutant plasmids, pBSKII-RNH(C63S) and pBSKII-RNH(C133A), were confirmed by DNA sequencing, and the appropriate fragments from a Dra III digestion of these two mutants were ligated to give the double mutant pBSK-RNH(C63S/C133A). The plasmid was then linearized by Hind III digestion, and an adaptor, 5'AGCTTC-GTAGATCTACGA-3', (underline shows Bgl II restriction site) was introduced into the plasmid by ligation, to yield pBSK-RNH-Bgl II. Plasmid pET11a was digested to completion with EcoR I and BamH I and treated with calf intestinal alkaline phosphatase. This vector was ligated with the EcoRI-BglII vector fragment from pBSK-RNH-Bgl II to give the final mutant plasmid pET11a-RNH(63S/133A). DNA sequencing of the entire coding region was performed to ensure the fidelity of the mutant DNA plasmid construct.

RNase H assays

[^{3}H]poly(rA)·poly(dT) was synthesized according to the standard protocol.[28] The crude substrate was extracted with an equal volume of phenol (saturated with 1 M Tris–HCl, pH 7.9) and with an equal volume of chloroform–isoamylalcohol (24:1). The nucleic acids were precipitated with an equal volume of cold 2-propanol and washed with 2 volumes of absolute ethanol. Final purification was achieved on a 1 x 5 cm column of Sephadex G-50 equilibrated with 50 mM NaCl, 10 mM Tris–HCl (pH 7.9), 0.1 mM EDTA.

Enzymatic activity was determined by measuring the radioactivity of the acid-soluble digestion product generated from the substrate [^{3}H]poly(rA)·poly(dT).[34] The assay mixture (50 μL) contained 40 mM Tris–HCl (pH 7.9), 4 mM $MgCl_2$, 1 mM dithiothreitol, 4% glycerol, 30 μg mL^{-1} BSA, 40 pmol [^{3}H]poly(rA)·poly(dT), and RNase H. After 20 min incubation at 37 °C, the reaction was terminated by addition of 25 μL of sonicated herring sperm DNA (1 mg mL^{-1}) and 25 μL of 30% trichloroacetic acid.[28] Acid-insoluble material was removed by centrifugation for 5 min, and a 75 μL aliquot of the supernatant was added to a scintillation vial containing 4 mL Budget Solve cocktail (ICN) before analysis by liquid scintillation. One unit of activity was defined as the amount of enzyme producing 1 nmol of acid-soluble material in 20 min at 37 °C.

Purification of RNase H-C63S/C133A

A strain of *E. coli* overproducing RNase H-C63S/C133A was constructed by transforming strain MIC1066 with pET11a-RNH(63S/133A). Growth and selection of this strain was accomplished on LB medium containing ampicillin (75 μg mL^{-1}) with shaking at 37 °C. One and one-half liters of medium was inoculated with a 25 mL saturated culture (12 h) and grown to an OD_{550nm} of 1.0, before addition of IPTG to a final concentration of 1 mM. Cells were harvested after an additional 5 h by centrifugation, washed, and frozen at –80 °C. The purification protocol was modified from a reported procedure.[35] Frozen cells were thawed and resuspended in 5 volumes of Buffer A (0.2 M KCl, 50 mM Tris–HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA) with 1 mM phenylmethylsulfonyl fluoride. Cells were ruptured by ultrasonication on ice using 5 x 15 s bursts with 10 s intervals to provide a soluble protein extract containing the mutant RNase H (175 mg, 32,000 U/mg), and debris was removed by centrification. The supernatant was diluted with Buffer B (50 mM Tris–HCl pH 7.9, 0.1 mM EDTA) to an ionic strength equivalent to that of Buffer C (50 mM NaCl, 50 mM Tris–HCl pH 7.9, 0.1 mM EDTA), before loading onto a DEAE–Sepharose column (5 cm x 6 cm) connected in series with a phosphocellulose P-11 column (2.5 cm x 20 cm). The columns were washed with 2 to 3 column volumes of Buffer C, and the DEAE column was removed. The P-11 column was washed with 1 column volume of 0.15 M NaCl in Buffer B before the RNase H was eluted with 0.7 M NaCl in Buffer B. Fractions containing the highest RNase H specific activity were pooled and concentrated using an Amicon PM 10 membrane. The purified enzyme (62 mg, 8.4 x 10^3 U mg^{-1}) was stored at –80 °C in 50% glycerol in a total of 12 mL.

Protein methods

SDS polyacrylamide gels (15%) were run using the method of Laemmli[36] and were stained with Coomassie brillant blue. Low range molecular weight markers (Bio-Rad) were used routinely as standards. Protein concentrations were determined by the modified Bradford method using BSA as a standard.[37] Titrations of thiol groups in the mutant

RNase H with Ellman's reagent were carried out according to the method previously reported.[38] Cysteine modification was modeled after the vinyl pyridine alkylation.[39] All purifications and assays of the RNase H Cys13-conjugate were conducted using diethylpyrocarbonate treated water.

N-Succinimidyl-3-(2-pyridyldithio)propionate octadeoxyribonucleotide

An oligodeoxyribonucleotide octamer with a hexylamino linker through the 5'-phosphate (5'-TCAGTGGT-3') was synthesized by addition of 6-(4-monomethoxytritylamino)hexyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite (20 mmol) in the final cycle of an automated synthesis starting from 1 mmol of *N*-benzoyldeoxycytidine attached to controlled pore glass.[40,41] In a 500 μL Eppendorf tube, the modified octamer (30 μmol) *N*-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) (3.0 mmol), DMF (120 μL), and 50 mM HEPES buffer (pH 8.0) (80 μL) were combined. The oligomer and active ester were allowed to react for 2 h at room temperature before the solvent was evaporated *in vacuo*. The residue was washed 10 times with 200 μL aliquots of ethanol by resuspension and centrifugation. After being dried for 10 min, the residue was dissolved in H_2O (200 μL) and analyzed by HPLC using a 4.1 x 250 mm PRP-1 column (Hamilton). Solvent A was 50 mM triethylammonium acetate (pH 6.5) and solvent B was acetonitrile and the sample was eluted with a linear gradient of solvent B from 5% to 70% in 24 min.

Coupling reaction

Modified octamer **1** (180 nmol) was combined with C63S/C133A-RNase H (1.5 mg, 85.5 nmol) in 1.5 mL of 50 mM triethylammonium acetate pH 7.5, and stirred at 4 °C for 20 h. Aliquots were taken at various time intervals and analyzed by 15% SDS-PAGE using nonreducing sample buffer and by the standard RNase H assay. The reaction mixture was purified using a Mono Q anion exchange column (Pharmacia). Solvent A was 10 mM HEPES pH 7.0, 0.1 mM EDTA and solvent B was 10 mM HEPES pH 7.0, 0.1 mM EDTA, 1 M NaCl. A linear gradient from 0% to 50% B over 30 min was used to elute the RNase H Cys13-conjugate (19 min) and the residual SPDP-octamer (27 min). Under these conditions, the unmodified C63S/C133A-RNase H was not retained on the column.

Preparation of ^{32}P labeled β-globin mRNA

The β-globin gene was recovered from plasmid pRSV-β-globin and subcloned into Bluescript KS II at the BamH I and Hind III sites. This DNA construct was digested with Pvu II, and the 920 bp fragment was purified by 1.2% agarose gel electrophoresis and used as a DNA template. A Megascript kit was purchased from Ambion for *in vitro* run-off transcription using [(α-^{32}P]UTP according to the manufacturer's instruction. Purified ^{32}P-labeled transcript was resuspended in 50 μL water.

Assay of RNase H Cys13-conjugate

The oligoribonucleotide octamer 5'-ACCACUGA-3' (r8mer) was first dephosphorylated by calf intestinal alkaline phosphatase, and the 5'-end was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. The labeled r8mer was redissolved in 1 mL H_2O and purified by passage through a NAP-10 column (Pharmacia). Hydrolysis of the ^{32}P-labeled β-globin mRNA (6 pmol) with the mutant RNase H Cys13-conjugate (1.5 pmol) was carried out at 37 °C for 4 h in 10 μL of 40 mM HEPES, pH 6.5, containing 4 mM $MgCl_2$, 0.03% BSA, and 4% glycerol. Reactions that were performed at pH values > 7.4 used 50 mM Tris–HCl as the buffer. After the reaction mixture had been heated at 100 °C for 1 min, the hydrolysates were fractionated on a 5% polyacrylamide/7 M urea gel (0.8 mm x 14 cm). When the complementary ^{32}P-labeled r8mer (36 pmol) was used in the assay, it was digested with varied amounts of the RNase H Cys13-conjugate (0.36–7.2 pmol) at 37 °C for 30 min in 10 μL of 40 mM Tris–HCl, pH 7.9, containing 4 mM $MgCl_2$, 0.03% BSA, and 4% glycerol. The ^{32}P-labeled r8mer (36 pmol) was also partially digested with 20 ng of *Crotalus durissus* phosphodiesterase, which served as a source of oligoribonucleotide length markers. After the reaction mixture had been heated at 100 °C for 1 min, the hydrolysates were fractionated on a 20% polyacrylamide/7 M urea sequencing gel (0.4 mm x 40 cm).

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