Stereochemical Course of *Escherichia coli* RNase H

Dedicated to Prof. Frank Westheimer on the occasion of his 90th birthday.

Agnieszka Krakowiak, Alina Owczarek, Maria Koziołkiewicz, and Wojciech J. Stec*[a]

A new enzymatic method has allowed the assignment of the stereochemistry of E. coli *RNase-H-assisted hydrolysis of RNA labelled within the scissile bond with (R_P)-phosphorothioate. This method is based on a stereospecific, two-step enzymatic conversion of cytidine 5′-[^{18}O]phosphorothioate into the corresponding 5′-α-[^{18}O]thiotriphosphate, which is then further used for stereospecific transfer of cytidine 5′-[^{18}O]phosphorothioate to the 3′-OH group of a short oligonucleotide with the aid of terminal deoxyribonucleotidyl transferase. Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry of the resulting elongated primer revealed that RNase-H-assisted hydrolysis proceeds with inversion of configuration at the phosphorus atom. This result is discussed in the context of current knowledge of the architecture of the active site of the enzyme.*

KEYWORDS:

chirality · DNA · enzymes · reaction mechanisms · ribonucleases

Introduction

Ribonucleases H (RNases H) are endonucleases that catalyse the hydrolysis of phosphodiester linkages in the RNA strand of an RNA/DNA duplex in the presence of divalent cations such as Mg^{2+} or Mn^{2+}. The products of this reaction are short oligoribonucleotides containing the 5′-phosphate residue and the 3′-hydroxy function.[1] These enzymes are found in a wide variety of organisms, which range from prokaryotes to eukaryotes, as well as retroviruses (e.g., human immunodeficiency virus; HIV-1).[2] It is generally accepted opinion that the action of antisense oligonucleotides depends on RNase H activity.[3] Furthermore, the RNase H domain of HIV-1 reverse transcriptase (HIV-1 RT) is regarded as an attractive target for anti-HIV therapies, as functional RNase H activity is essential for the replication of the retrovirus.[4] However, the catalytic mechanisms of these enzymes are not fully understood. Although some models of RNase H action have been suggested,[5] none of them takes account of the problem of the stereochemical course of the enzyme-catalysed reaction: inversion or retention of configuration at the phosphorus atom of the scissile bond. Elucidation of the stereochemical mode of action of nucleolytic enzymes provides information about the mechanism of phosphoryl transfer. Inversion of configuration supports the one-step, in-line mechanism of cleavage, while retention strongly speaks for a two-step mechanism, the first step of which involves a covalent enzyme – substrate complex, and the second step the hydrolysis of that complex by water. Moreover, the stereochemistry provides data that allows the elucidation of the architecture of the active site of the enzyme, or more precisely, the architecture of the enzyme – substrate complex. Stereochemical studies on the mode of action of sequence nonspecific *Serratia marcescens* endonuclease, for example, have recently confirmed one of two alternative mechanisms proposed for this enzyme.[6]

In this report we present the results of our studies on the stereochemistry of the RNase-H-assisted cleavage of internucleotide bonds in oligoribonucleotides. Earlier results by Uchiyama et al.[7] provided the important information that RNase H cleaves internucleotide phosphorothioate bonds of R_P configuration. The authors used conditions that forced this sequence nonspecific enzyme into site-specific cleavage. This was achieved by "gating" the cleavage site on the RNA strand with the aid of a chimeric complementary DNA strand modified with 2′-OMe ribonucleotides.[7, 8] These findings, together with a newly designed methodology for the assignment of absolute configurations at phosphorus atoms of isotopomeric oligonucleotide 5′-[^{18}O]phosphorothioates (Mizuuchi et al.[9]), kindled hopes for the elucidation of the stereochemistry of RNase H action.

In our earlier work we presented results on the higher efficiency of RNase-H-assisted cleavage of the **R-1** pentadecaribonucleotide complexed with complementary (all-R_P)-oligo(-deoxyribonucleoside phosphorothioate) (i.e., possessing all phosphorothioate linkages of R_P configuration), as compared with those measured for isosequential all-S_P congener.[10] RNase-H-catalysed hydrolysis of the **R-1**/**D-5** heteroduplex provided a

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mixture of numerous products that result from the cleavage of internucleotide bonds, as indicated by the arrows in Table 1.

For further analysis, the method designed by Mizuuchi requires products of defined length and sequence to ligate to especially designed templates. It was thus necessary to generate such products for the RNase-H-catalysed reaction. Numerous complementary DNA constructs with 2′-OMe-ribonucleosides incorporated at the preselected positions were therefore prepared, and their complexes with the **R-1** oligomer were treated with RNase H. Of these complexes, three (**R-1**/**D-6**, **R-1**/**D-7** and **R-1**/**D-8**) were cleaved at the single internucleotide bond of the **R-1** substrate. Subsequently, analogues containing internucleotide phosphorothioate bonds at the scissile positions (**R-2** – **R-4**) were prepared and separated into diastereomerically pure species. These oligomers were complexed with their appropriate DNA counterparts and treated with RNase H, which resulted in 5′-*O*-phosphorothioylated penta- or hexaribonucleotides: compounds psCAUCU and psCAUCUU, respectively. Unfortunately, these short oligoribonucleotides did not undergo ligation. That unexpected limitation of Mizuuchi's approach prompted us to adapt our recently designed method for the assignment of absolute configuration at phosphorus in isotopomeric adenosine 5′-[^{18}O]phosphorothioates.[11] The reaction was performed in a medium containing [^{18}O]water, so that the resulting oligoribonucleotide **9** (see the Experimental Section) with [^{18}O]phosphorothioate attached at the 5′-end (Scheme 1) provided cytidine 5′-[^{18}O]phosphorothioate (CMPS; **10**) after digestion with nuclease P1. The absolute configuration of **10** was assigned by a modified version of the methodology recently used for the elucidation of the stereochemical course of plasma 3′-exonuclease.[11] The mononucleotide **10** was converted by

Table 1. *Sequences of oligoribonucleotides used in this study*[a].

A	**RNA (5′→3′)** **DNA (3′→5′)**	**HETERODUPLEX**
	R-1 D-5	AGAGCUCAAACAUCU TC TCGAGTT TGTAGA
	R-1 D-6	AG A G C U CAAAC A U C U T $C_mU_mC_mG_mA_mGTT$ $TG_mU_mA_mG_mA_m$
	R-1 D-7	AG A G C U C AAACA U C U $TC_mU_mC_mG_mA_mG_mTTTGU_mA_mG_mA_m$
	R-1 D-8	AG A G C UCA A A C A U C U $TC_mU_mC_mG_mAGU_mU_mU_mG_mU_mA_mG_mA_m$
B	**PS-RNA (5′→3′)** **DNA (3′→5′)**	**HETERODUPLEX**
	R-2 [R_P] D-6	AG A G C U CAAA$_{PS}$C A U C U [R_P] T $C_mU_mC_mG_mA_mGTT$ T $G_mU_mA_mG_mA_m$
	R-3 [R_P] D-6	AG A G C U CAAA$_{PS}$C A U C UU [R_P] T $C_mU_mC_mG_mA_mGTT$ T $G_mU_mA_mG_mA_m$
	R-4 D-6	AG A G C U CAA$_{PS}$A$_{PS}$C A U C U [R_P, any] T $C_mU_mC_mG_mA_mGTT$ T $G_mU_mA_mG_mA_m$

[a] A) The oligoribonucleotide sequences (R) and complementary oligodeoxyribonucleotides with incorporated 2′-OMe-ribonucleotides (D) used for selection of the best model for stereochemical studies; B) sequences of oligoribonucleotides modified at preselected positions with internucleotide phosphorothioates (R), together with complementary oligodeoxyribonucleotide templates. N_m = 2′-OMe modification of nucleotide

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Wojciech J. Stec*, born in 1940 in Warsaw, majored in chemistry and then obtained a Ph.D. in organic chemistry at the Technical University of Lodz in the laboratory of Jan Michalski. After postdoctoral research with John R. Van Wazer from 1969 – 70, he continued his research in the Centre of Molecular and Macromolecular Studies of the Polish Academy of Sciences in Lodz, focusing on the stereospecific synthesis of P-chiral organophosphates, with an emphasis on biologically active compounds. His group initiated studies in chirotechnology, exemplified by the first synthesis of enantiomerically pure cyclophosphates of therapeutic relevance, such as cyclophosphamide, isophosphamide and trophosphamide. His contribution to the synthesis and stereochemistry of organophosphates includes the conformational analysis of dioxaphosphorinanes, stereospecific PN→PX conversion demonstrated by the first stereospecific syntheses of R_p- and S_p-cAMPS and the design of a novel approach to stereospecific synthesis of diesters and amidoesters of phosphorothioic and isotopically labeled [^{16}O, ^{17}O, ^{18}O] phosphoric acids. His oxathiaphospholane approach to the synthesis of P-chiral organophosphates flourished in the first stereocontrolled synthesis of phosphorothioate oligonucleotides, a class of compounds first chemically synthesized by Stec et al. and now broadly used in drug target validation and antisense therapy of viral and cancer diseases. He and his group designed numerous P-chiral compounds useful as chemical tools for studies of the mechanism of action of nucleolytic phosphoesterases and hydrolases. He spent 1983 – 84 working as Distinguished Visiting Scientist, in FDA, National Center for Drugs and Biologics, Bethesda, MD, USA. He was also invited as a Visiting Professor to the Max-Planck Institute of Experimental Medicine, Göttingen, (1976, 1986), Ruhr-University, Bochum, Germany (1981), Beijing Medical University, P. R. China (1998), and the National Institute for Advanced Interdisciplinary Research, Tsukuba, Japan (2000). His contribution to health sciences has been rewarded by the National Institutes of Health, Fogarty International Center, who elected Prof. Stec as the Fogarty Scholar-in-Residence (July 1992 – June 1993). In 1994 Prof. Stec was elected as the corresponding member of the Polish Academy of Sciences. He has also been appointed as a member of advisory boards of* Antisense and Nucleic Acid Drug Development, ChemBioChem *and* The European Journal of Organic Chemistry.

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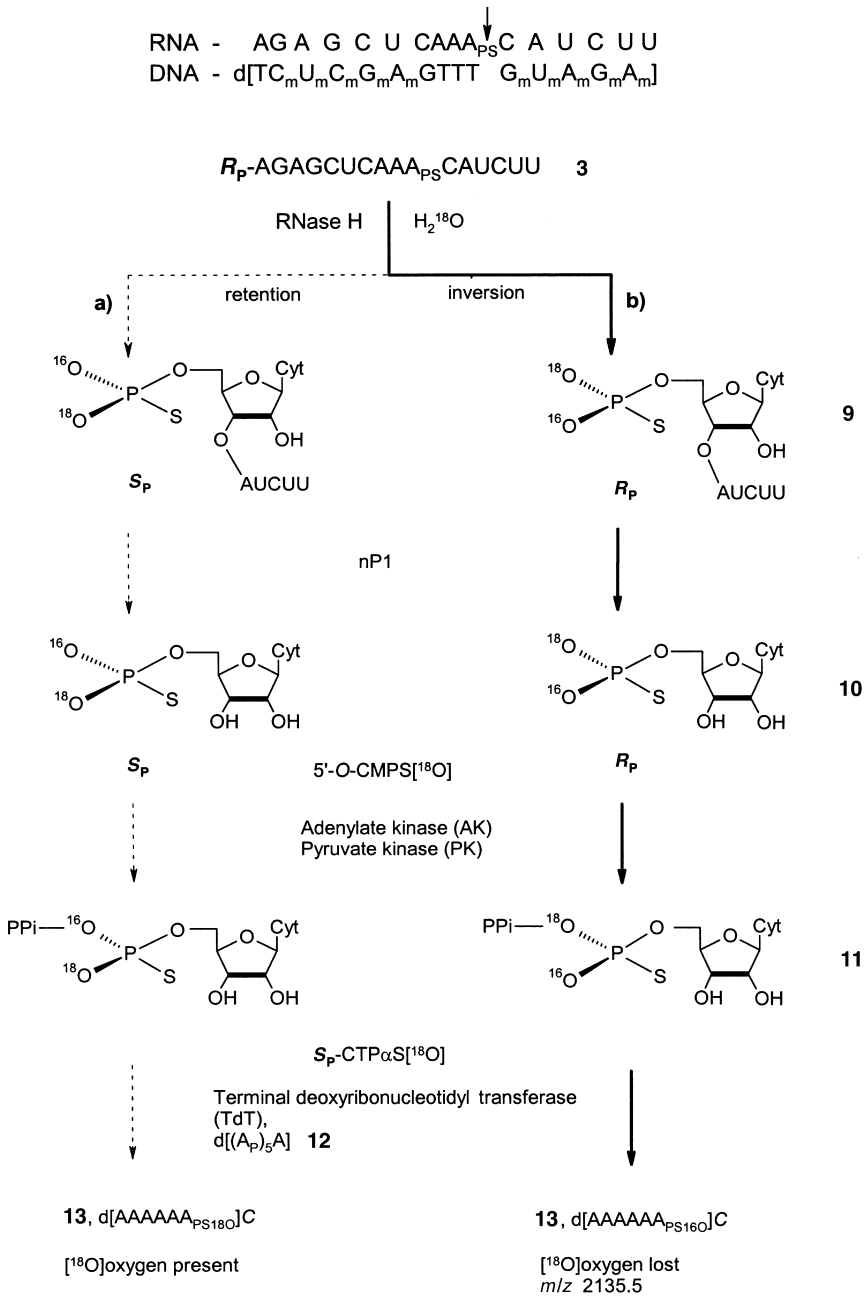


Scheme 1. *Stereochemical consequences of RNase-H-assisted degradation of (R_P)-**R3** hybridised to a complementary oligodeoxyribonucleotide, which forces single-site cleavage at the $A_{PS}C$ step.*

tandem treatment with adenylate kinase and pyruvate kinase into cytidine 5′-α-[^{18}O]thiotriphosphate of S_P configuration (**11**), which was finally used as the substrate for terminal deoxyribonucleotidyl transferase (TdT). The TdT-catalysed extension of an oligonucleotide primer **12** gave the final $n+1$ oligonucleotide **13** with a newly formed internucleotide phosphorothioate linkage of R_P configuration.[11, 12] MALDI-TOF analysis of this product provided evidence of the absolute configuration of **9**, because the lack of [^{18}O]oxygen isotope in the oligonucleotide **13** indicates the R_P configuration for **9** and shows that the RNase-H-assisted hydrolysis proceeds with inversion of configuration at the phosphorus atom. This result is discussed in the context of the architecture of the active site of the enzyme.

Results

Design of the target oligonucleotides

In attempts to find a DNA/RNA substrate suitable for stereochemical studies on RNase H, we synthesised the **R-1** oligoribonucleotide and numerous complementary DNA pentadecamers containing incorporated 2′-OMe nucleosides (Table 1). The [^{32}P]-labelled **R-1** was hybridised to the complementary DNA fragments **D-5** – **D-8**, and the resulting duplexes were treated with *E. coli* RNase H as described in the Experimental Section.

In our preliminary studies we identified cleavage sites for each of the DNA/RNA duplexes listed in Table 1 and we selected the RNA substrate suitable for assessment of the stereochemistry of RNA cleavage. The position of the scissile bond in the RNA strand and the extent of its degradation differed in the oligomers **5** – **8** depending on the location and size of the oligodeoxyribonucleotide gap, and only one of these oligomers, namely **R-1**/**D-6**, was digested with significant efficiency, to furnish as the product the CAUCU pentamer with the terminal 5′-phosphate group. In order to investigate the stereochemical course of *E. coli* RNase H, we synthesised the oligoribonucleotides **R-2** – **R-4**, each of which contains a single internucleotide phosphorothioate linkage between A and C, and separated them into the R_P and the S_P diastereomers by ion-exchange chromatography. The stereochemistry at the phosphorothioate phosphorus atom was assigned by exhaustive digestion with stereoselective nucleases: nuclease P 1 and snake venom phosphodiesterase (svPDE). The R_P isomer of the **R-2** oligoribonucleotide in hybridisation with **D-6** was cleaved by RNase H at the modified internucleotide bond, with the formation of the 5′-phosphorothioylated pentaribonucleotide psCAUCU. However, further ligation experiments (see below) showed that the oligomer intended for enzymatic ligation has to be longer than a pentamer. We therefore also constructed the RNA hexadecamer **R-3**, which incorporates one more U nucleotide at the 3′-end of the chain than in the **R-1** and **R-2** oligomers. We did not extend the **R-3** oligoribonucleotide by the next nucleotides since we were aware that *E. coli* RNase HI can digest the single-stranded RNA fragment adjacent to the RNA/DNA duplex.[13] The **R-3**/**D-6** duplex was cleaved at the expected site (Table 1 B), which provided hexamer CAUCUU with the 5′-phosphorothioate group as one of the products (see below).

RNase-H-catalysed hydrolysis

The products of hydrolysis of the **R-3**/**D-6** duplex were isolated and collected by RP-HPLC. MALDI-TOF mass spectrometric analysis confirmed the presence of the two expected products: the AGAGCUCAAA decamer (*m/z* 3180) and the CAUCUU hexamer with the 5′-phosphorothioate group (*m/z* 1889.35; Figure 1 A). The same procedure was repeated with the use of [^{18}O] water (finally 52 atom % ^{18}O). The $_{18OPS}$CAUCUU hexamer **9** (*m/z* 1891.25, Figure 1 B), crucial for the intended stereochemical analysis, was isolated by RP-HPLC.

To investigate the role of the phosphate group 5′-adjacent to the scissile bond of the RNA substrate we also synthesised the **R-4** oligoribonucleotide containing two internucleotide phosphorothioate linkages (Table 1 B). This oligonucleotide was separated by ion-exchange chromatography into four diastereomers with absolute configurations as follows: (R_P,R_P)-**R-4 A**, (R_P,S_P)-**R-4 B**, (S_P,R_P)-**R-4 C** and (S_P,S_P)-**R-4 D**.

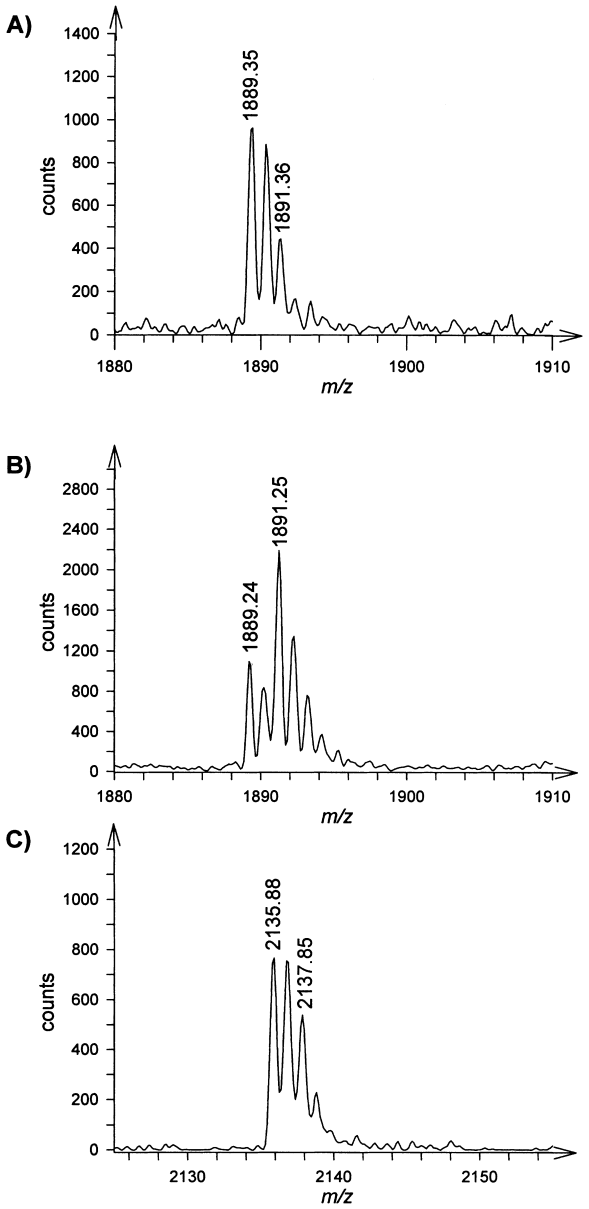


Figure 1. *Results of MALDI-TOF mass spectrometric analysis of the products of degradation of* ***R-3/D-6*** *by RNase H in [^{16}O]water: A) the hexamer $_{PS16O}$CAUCUU; B) the analogous product after cleavage in [^{18}O]water, $_{PS18O}$CAUCUU; C) the final product of TdT-assisted elongation of d(A_SA) with [^{18}O]CTPαS, the heptamer d[A_SA]$_{PS}$(rC).*

The **R-4** oligoribonucleotides were labelled with ^{32}P at their 5′-ends, annealed with the chimeric **D-6** oligodeoxyribonucleotide and incubated with RNase H. The (R_P,R_P)-**R-4 A** and (S_P,R_P)-**R-4 C** oligonucleotides were cleaved at the expected sites with comparable efficiencies, although **R-4 A** was degraded 20 % more efficiently than its counterpart **R-4 C** (data not shown). The **R-4 B** and **R-4 D** oligoribonucleotides were not cleaved. This result indicates that one of the nonbridging oxygen atoms of the 5′-phosphate group adjacent to the scissile phosphodiester bond of the RNA substrate may be involved in contacts with the enzyme, whereas its modification by sulfur substitution makes this contact weaker.

Attempted enzymatic ligation for stereochemical studies of RNase H

Towards our goal of determining the stereochemistry of RNase H action, we attempted to use the method developed earlier by Mizuuchi et al.,[9] in which the crucial step for the assignment of stereochemistry of an endonuclease of interest was enzymatic ligation by T4 DNA ligase. That approach seemed to be feasible, since it had been shown earlier that T4 DNA ligase is able not only to join oligodeoxyribonucleotides but also long RNA fragments.[14,15] In our preliminary experiments we used this enzyme for ligation of short RNA fragments (hexa- and octaribonucleotides bearing the terminal 5′-phosphate group) to the 3′-end of the 44-base "hairpin" oligodeoxyribonucleotide template d[CGCATCTC*AAAGATG*AGAAGAGGGCCCT$_4$GGGCCC-TCTTCT], which possesses the single-stranded 5′-end fragment complementary to these oligoribonucleotides. These oligoribonucleotides were joined by the T4 DNA ligase. In this case, the efficiency of ligation was 20 % (data not shown). In the case of the $_{18OPS}$CAUCUU hexamer bearing the terminal 5′-phosphorothioate group, however, the efficiency of the ligation reaction with the same template was much lower and provided the ligated product in a yield of around 4 % (estimated after polyacrylamide gel electrophoresis, data not shown). We have also used T4 RNA ligase,[16] but no ligation either of the $_{18OPS}$-CAUCU pentamer or of the $_{18OPS}$CAUCUU hexamer (**9**) was observed. In control experiments we found that this enzyme was very efficient in ligation of short oligoribonucleotides bearing unmodified 5′-phosphate groups (data not shown). Interestingly, in experiments with the T4 RNA ligase, instead of the expected product of ligation, an A_{P4}A dinucleotide appeared as the only product. This result, confirmed by MALDI-TOF MS, is in agreement with earlier reports that A_{P4}A can be nonspecifically synthesised by RNA ligase.[17] Not even the use of some agents that are known to increase efficiency of enzymatic ligation (bovine serum albumin, polyethylene glycol or Ficoll 70)[18] could increase the efficiency of this reaction. Further attempts to elucidate the stereochemistry of RNase H cleavage by the ligation approach were therefore abandoned.

Stereochemical course of RNase H

The lack of success of attempts to assign the RNase H stereochemistry by the method developed by Mizuuchi, based on enzymatic ligation of the $_{18OPS}$CAUCUU hexamer (**9**), led us to apply another method recently developed for the assignment of the sense of chirality at the phosphorus atom in isotopomeric adenosine 5′-[^{18}O]phosphorothioate.[11]

The hexamer $_{18OPS}$CAUCUU (**9**, *m/z* 1891.25, Figure 1B), obtained from the RNase-H-catalysed cleavage of the phosphorothioate RNA **R3**, was digested by nuclease P1 to provide a mixture of cytidine 5′-phosphorothioate (**10**, both CMPS[^{18}O] and CMPS[^{16}O]) and three other mononucleotides (uridine 5′-phosphate, adenine 5′-phosphate and cytidine 5′-phosphate; UMP, AMP and CMP, respectively). The degradation was carried out in the presence of $H_2{}^{16}O$, and since nuclease P1 did not remove terminal 5′-phosphate or 5′-phosphorothioate, we could reasonably expect that the ^{18}O isotope incorporated into CMPS would be solely the result of the action of RNase H. After HPLC isolation, CMPS[^{18}O] **10** in the presence of adenylate kinase (AK) and pyruvate kinase (PK) was converted into the corresponding triphosphate (CTPαS[^{18}O], **11**) with the [^{18}O] oxygen atom located at either the nonbridging (path **a**) or the bridging (path **b**) position, respectively (Scheme 1). The stereochemistry of reactions catalysed by these two enzymes is well documented.[19, 20] Although AK has generally been viewed as highly specific towards adenosine 5′-mononucleotides, and the method of enzymatic phosphorylation based on the use of AK has been applied to adenosine or deoxyadenosine 5′-phosphorothioates,[21] it has also been reported that the enzyme is able to phosphorylate CMP.[22] Here we have shown that CMPS can also be phosphorylated by AK, although the efficiency of the reaction with CMPS is lower than that with adenosine 5′-phosphorothioate (AMPS). It should be underlined that AK-catalysed phosphorylation of cytidine 5′-phosphorothioate required the use of special conditions, namely the exhaustive dialysis of commercially available AK preparation and higher concentrations of the enzyme than used for AMPS phosphorylation (see the Experimental Section). We have also observed phosphorylation of dCMPS and UMP under the same conditions.

The CTPαS[^{18}O] was used as the substrate for the TdT-catalysed primer elongation reaction.[23] We applied the d[A_5A] hexamer (**12**) as a primer. We intended to elongate the primer by only one nucleotide, therefore we used a concentration of CTPαS lower than that of the primer and only a one-hour incubation of the reaction mixture. Under these conditions, the primer was elongated by one nucleotide, with the formation of the d[A_5A]$_{PS}$rC heptamer (**13**). The product **13** was isolated by RP-HPLC and was then analysed by MALDI-TOF MS (Figure 1C). The signal at *m/z* 2135.88 corresponds to the oligonucleotide **13b** without ^{18}O isotope, and the pattern of peaks in the MALDI-TOF mass spectrum clearly indicates the loss of the ^{18}O label (see panel C in Figure 1). We have therefore shown that the RNase-H-catalysed hydrolysis proceeds with inversion of configuration at phosphorus (Scheme 1, pathway **b**).

Discussion

The prerequisites for the determination of the stereochemical course of enzymatic reactions at phosphorus are P-chiral substrates and products, both of known sense of P-chirality.[24–26] The absolute configurations at the phosphorus atom of internucleotide phosphorothioate functions of oligoribonucleotides labelled with phosphorothioate at the scissile bond and used as substrates for RNase H were determined with the aid of enzymes of known stereoselectivity.[27–29] Hydrolysis of (R_P)-phosphorothioate oligonucleotides **R-2** and **R-3** in [^{18}O]water with assistance of RNase H provided P-chiral isotopomeric products, such as the short oligonucleotide 5′-[^{18}O]phosphorothioate **9**. Digestion of this oligonucleotide with nuclease P1 furnished cytidine 5′-[^{18}O]phosphorothioate, and stereochemical analysis of this product by the method presented above and depicted in Scheme 1 showed that the RNase-H-catalysed reaction proceeds with inversion of configuration at phosphorus. This result supports the claim that a one-step, S_N2-type mode of enzyme action takes place. This means that direct in-line attack of the hydroxide ion opposite to the leaving 3′-O group occurs, and that the reaction involves the pentacoordinate transition state without participation of a covalent enzyme–substrate intermediate. This observed inversion of configuration at phosphorus can be taken as evidence that RNase H belongs to the polynucleotide transferases,[30] the superfamily of proteins that includes resolvase,[31] integrase,[30, 32] transposase[33] and exonuclease III.[34] It has previously been shown that reactions catalysed by integrase and transposase proceed with inversion of configuration.[9] These enzymes have homologous active sites, and are therefore likely to share a common mechanism for catalysis. Analysis of catalytic strategies used by members of the superfamily should be helpful for understanding the RNase H mechanism.

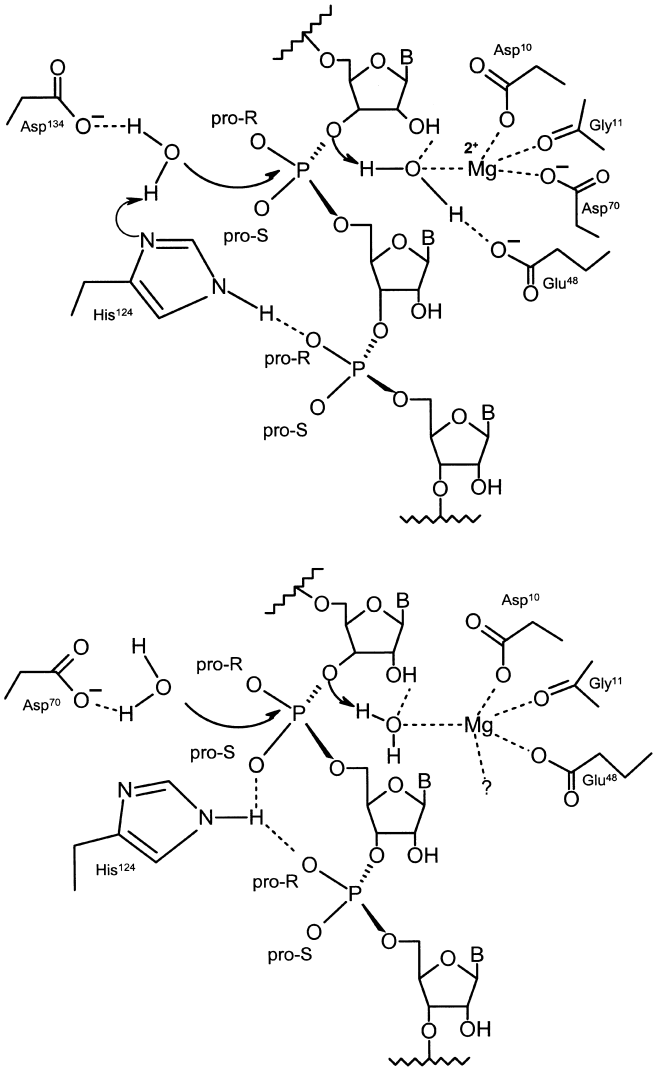
Although several aspects of the mechanism of RNase H action have been studied, the manner in which the enzyme degrades RNA strands into RNA/DNA hybrids is still a matter of debate. RNases H show conservation of the key active site residues Asp10, Glu48, Asp70 and Asp134 (*E. coli* numbering).[35, 36] Initially, alternative mechanisms were proposed for catalytic action of *E. coli* RNase H. The first, based on the identification of a single divalent cation-binding site in the enzyme by X-ray analysis[37] and NMR studies,[38, 39] was the *general acid–base* mechanism. Kinetic studies also suggested that only one metal ion binds to substrate-free enzyme.[40] The Mg^{2+} ion interacts with the 2′-hydroxy group of ribose at the cleavage site through formation of an outer-sphere complex with a water molecule.[41] On the other hand, two Mn^{2+} cations at distances of less than 4 Å were observed in the crystal structure of RNase H domain HIV-1 reverse transcriptase,[42] which is structurally homologous to the RNase HI of *E. coli*.[2] For these reasons the *two-metal-ion* mechanism, similar to that suggested for Klenow 3′-exonuclease, has been proposed.[36] Here, one of two metal ions activates the attacking hydroxide ion.

Keck et al.[43] recently proposed an *activation–attenuation* model of catalysis. The enzyme is optimally active with one bound divalent metal ion, which stabilises the pentacovalent

intermediate, and can be inhibited by a second metal anchored by the substrate and Asp134, which is involved in the neutralisation of His124. Such a possibility has been confirmed by the crystal structure of *E. coli* RNase H, which shows two Mn^{2+} ions in the active site.[44] These studies make the general acid-base mechanism more probable than the two-metal-ion mechanism. According to the latest version of the *one-metal-ion* mechanism, Asp10 and Glu48 are responsible for the binding of an Mg^{2+} ion to the correct position in the active site of the enzyme. Generation of a nucleophilic hydroxyl ion is suggested to occur by deprotonation of water, with Asp70 acting as a general base. His124 is viewed as the proton sink for Asp70 that causes its reactivation.[43] Mutations of the carboxylic aminoacids (Asp10, Glu48 and Asp70) significantly reduced the catalytic efficiency of the mutated enzymes, while mutations of the conserved His124 and Asp134 had less profound effects on catalysis.[45–47] However, according to Keck et al. the mutation of His124 makes deprotonation and reactivation of Asp70 impossible, so direct proton transfer from the aspartate residue to the bulk solvent may occur. This effect would result in the residual activity of the enzyme with substituted His124.[43]

The use of oligoribonucleotide substrates modified by a single internucleotide phosphorothiate linkage revealed that the non-bridging oxygen atoms of the scissile bond and the 3′-adjacent phosphate group also participate in the formation of a tight interface between the enzyme and its substrate. Results reported by Uchiyama,[7] as well as our own studies, have shown that RNase H is stereoselective towards internucleotide phosphorothioate linkages of R_P configuration, and that the enzyme is unable to degrade phosphorothioate bonds of S_P configuration. This observation indicates the importance of the *pro-S* oxygen atom of the scissile bond and suggests that the substitution of the *pro-*S_P oxygen atom by a sulfur atom strongly disturbs the contacts of the scissile bond with the active site of the enzyme. However, the manner in which the RNase H binds to the scissile phosphate still remains obscure. It has been proposed that the *pro-*S_P oxygen atom of this phosphate group may contact His124 and orient it in a suitable position for its catalytic function. The contacts of this histidine residue with the RNA/DNA duplex are also emphasised in recent work by Sarafianos et al.,[48] who analysed the crystal structure of a complex between HIV-1 reverse transcriptase and an RNA/DNA heteroduplex containing a polypurine tract. This is the first work to present a crystal structure of HIV-1 RT in complexation with a RNA/DNA heteroduplex and show contacts of the RNase H domain of HIV-1 RT with its RNA substrate. The HIV-1 RNase H domain has a structure very similar to that of the RNase HI of *E. coli* and of *Thermus thermophilus*, but none of the RNases H has been cocrystalised with RNA/DNA. The results of Sarafianos et al. are therefore valuable with regard to bacterial RNase H. In the structure reported by Sarafianos, hydrogen bonding between His539 of the RNase H domain of HIV RT (equivalent to His124 in *E. coli* RNase H) and one of the nonbridging oxygen atoms of the scissile phosphate group has been indicated. The use of stereodefined phosphorothioate oligoribonucleotides allowed the oxygen atom to be identified as *pro-*S_P oriented.

At this point it seems appropriate to emphasise that all mechanisms so far proposed, which include very recent work by Yazbeck et al.,[49] have assumed that the phosphorus atom is attacked by an activated water molecule in-line with the phosphodiester bond to be cleaved (Scheme 2 A), but this has,



Scheme 2. *Proposed mechanisms for the cleavage of RNA assisted by E. coli RNase H: involvement of His124 in contacts with the pro-*R_P *oxygen atom of the 3′-adjacent internucleotide linkage (according to Haruki et al.[50] and reported recently by Yazbeck et al.[49]). A) Interaction of His124 through a bifurcated hydrogen bond with both the pro-*S_P *oxygen atom of the scissile bond and the pro-*R_P *oxygen atom of the 3′-adjacent internucleotide linkage (based on the model proposed by Keck et al.[43]). B) This alternative is discussed in detail in the text.*

however, never been shown. Moreover, such a schematic representation of the architecture of the active site of RNase H interacting with oligoribonucleotide does not emphasise the stereoselective involvement of nonbridging oxygen atoms of the scissile phosphodiester bond. Our results presented here reconfirm the involvement of the *pro-*S_P oxygen atom in interaction with the active site component of the enzyme and provide evidence that RNase H operates by an in-line mechanism, with water attacking opposite the scissile bond. Haruki

et al. have suggested that His124 interacts with the phosphate group 3′-adjacent to the scissile phosphodiester bond.[50] The authors observed that substitution of the *pro*-R_P oxygen atom of this phosphate group by a sulfur atom significantly decreased the rate of hydrolysis of the scissile bond. According to their model, the *pro*-R_P oxygen of this internucleotide linkage forms a hydrogen bond with His124. From our findings, it cannot be ruled out that His124 could interact with both these oxygen atoms (i.e., the S_P oxygen atom of the scissile bond and the R_P oxygen atom of the 3′-adjacent phosphate) in different steps of the catalytic process. In two crystal structures of *E. coli* RNase H obtained in the absence of substrate, the locations of His124 are different from each other,[35, 36] and the flexibility of the His124-containing loop has also been confirmed by other studies.[47, 51] This loop probably changes its conformation upon binding of the substrate. The location of His124 may also depend on the local structure of the RNA/DNA hybrid. In view of a degree of flexibility of His124, we propose that the same amino acid residue binds the *pro*-S_P oxygen atom of the scissile bond and/or the *pro*-R_P oxygen atom of the 3′-adjacent phosphate group through a "bifurcated" hydrogen bond (Scheme 2B). However, we do realise that such an "extended" model of the catalytic mechanism of *E. coli* RNase H, in which at least two internucleotide phosphodiester bonds of an RNA substrate are involved in the enzyme action (Scheme 2B), is still a working hypothesis, in which the anchoring of the *pro*-S oxygen atom of scissile phosphate group exposes the phosphorus atom to attack by water from the side opposite to the cleaved P—O^3 bond. As proposed earlier, Asp70 acts in this model as a general base and His124 functions as a proton shuttle.[7, 43] The pro-S_P oxygen atom of the scissile bond and the pro-R_P oxygen atom of the phosphate group 3′-adjacent to the former linkage are postulated to interact with His124 through bifurcated hydrogen bonds. Moreover, the nonbridging oxygen atoms of the phosphate group 5′-adjacent to the scissile bond are also involved in contacts with the enzyme. The **R-4A** oligoribonucleotidem which contains two internucleotide phosphorothioate bonds of R_P configuration, was a slightly better substrate than its counterpart **R-4C**, which contains an internucleotide linkage of S_P configuration at the 5′-adjacent position. Katayanagi et al.[35] and Sarafianos et al.[48] suggested that Gln72 in *E. coli* RNase H and Gln500 in the RNase H domain of HIV-1 RT (equivalent to Gln72) interact with the phosphate group 5′-adjacent to the scissile bond. Substitution of Ala for Gln72 by site-directed mutagenesis was found to reduce the affinity of RNase H for its substrate.[5]

Therefore, like many other sequence-specific[52–54] and sequence nonspecific nucleases[6, 55] RNase H interacts not only with the scissile phosphate group, but also with the 3′- and/or 5′-adjacent internucleotide bonds. This observation is in agreement with our very early results, which indicated that modification of oligonucleotides with internucleotide phosphorothioates at positions adjacent to or even remote from scissile bonds cleaved by restriction endonucleases influence the efficiency of the cleavage process.[56]

On the other hand, the studies of Sarafianos et al. suggest that not only His539 but also Asn474 (equivalent to Asn44 in *E. coli* RNase H) are close to the scissile phosphate group. The final explanation of which one of these two amino acids is involved in hydrogen bonding with the pro-S_P oxygen atom of the scissile bond remains to be found by use either of appropriate mutants of RNase H for stereochemical studies or by determination of the crystal structure of the complex between bacterial RNase HI and RNA/DNA duplex.

Experimental Section

Enzymes and chemicals: *E. coli* RNase H (EC. 3.1.26.4), T4 RNA ligase (EC. 6.5.1.3), T4 DNA ligase (EC. 6.5.1.1), T4 polynucleotide kinase (EC. 2.7.1.78) and terminal deoxynucleotidyl transferase (EC. 2.7.7.31) were purchased from Amersham (Buckinghamshire, UK). Snake venom phosphorodiesterase (EC. 3.1.15.1) was supplied by Boehringer Mannheim (Mannheim, Germany). Nuclease P1 (EC. 3.1.30.1) was obtained from Pharmacia LKB (Uppsala, Sweden). Adenylate kinase (EC. 2.7.4.3) and pyruvate kinase (EC. 2.7.1.40) were purchased from Sigma (St. Louis, MO). The adenylate kinase was supplied as an $(NH_4)_2SO_4$ suspension and was dialysed extensively against tris(hydroxymethyl)aminomethane chloride (Tris-Cl, 50 mM, pH 7.5), EDTA (0.1 mM) and dithiothreitol (0.1 mM) prior to use. [^{18}O]Water was purchased from ICON Isotopes (Summit, NJ)

Chemical synthesis of oligonucleotides: The synthesis of the oligonucleotides listed in Table 1 was performed on an ABI 394 DNA synthesiser (Applied Biosystems, Inc., Foster City, CA) on a 1-μmol scale by standard phosphoramidite or phosphoramidite/sulfurisation procedures. The solid-phase synthesis of RNAs was carried out by the same procedure, except that the coupling waiting time step was increased to 10 min. Empty columns (Applied Biosystems, Warrington, GB) were packed with 2′-*O*-TBDMS-5′-*O*-dimethoxytrityl ribonucleoside-derivatised controlled pore glass (ChemGenes, Ashland, MA). 2′-*O*-TBDMS-5′-*O*-dimethoxytrityl ribonucleoside 3′-*O*-phosphoramidites (ChemGenes) were dissolved to 0.1 M in an anhydrous acetonitrile (Baker, Phillipsburg, NJ). Assembly was carried out by using the dimethoxytrityl (DMT) off mode, and oligonucleotides were cleaved from the support with a solution of ethanol saturated with ammonia. All details of the further deprotection procedure are given elsewhere.[57]

All unmodified oligodeoxyribonucleotides and 2′-OMe-modified oligoribonucleotides were purified by two-step RP-HPLC (DMT-on and DMT-off, ODS Hypersil column). The phosphorothioate oligoribonucleotides were purified and separated into pure isomers by ion-exchange chromatography (DNAPack PA 100 column, Dionex, Austin, TX) with the following buffers and buffer gradient: 0–10% B (5 min), 10–30% B (25 min), 30–35% B (30 min), 35–100% B (40 min), at a flow rate of 1 mL min^{-1} and temperature of 42 °C (buffer A: 10 mM $NaClO_4$, 1 mM Tris-Cl, pH 9.17; buffer B: 0,4 M $NaClO_4$, 1 mM Tris-Cl, pH 9.17). The isomers of the **R-3** oligonucleotide, for example, were eluted as follows: the R_P isomer at 24.29 min and the S_P isomer at 24.86 min. Their absolute phosphorus atom configurations in the internucleotide phosphorothioates were determined by independent digestion with R_P-specific snake venom phosphodiesterase[27, 28] and S_P-specific nuclease P1.[29] The resulting dinucleoside phosphorothioates were analysed by MALDI-TOF mass spectrometry.

The purities and identities of all oligonucleotides were assessed by polyacrylamide gel electrophoresis and/or MALDI-TOF mass spectrometry.

RNase-H-catalysed hydrolysis:

a) The 5′-end-^{32}P-labeled oligoribonucleotide **R-1** (or one of its analogues **R-2** – **R-4** containing internucleotide phosphorothioate(s), 17 pmol) was mixed with one of the corresponding complementary oligodeoxyribonucleotides **D-5** – **D-8** (50 pmol) in a solution (10 μL) of Tris-Cl (20 mM, pH 8.0) that contained KCl (100 mM), $MgCl_2$ (10 mM) and dithiothretiol (1 mM). The hybridisation mixture was heated to 85 °C and then slowly cooled to 20 °C (2 h). RNase H (5 units) was then diluted in the same buffer and the resulting solution was added to the hybridisation mixture to give a final volume of 20 μL. After 1 h incubation at 37 °C, a stop solution (10 mM ethylenediaminetetraacetic acid (EDTA), formamide, bromophenol blue and xylene cyanol, 20 μL) was added to the incubation mixture. An aliquot (10 μL) was then loaded onto 20% polyacrylamide/7 M urea gel. The products of the reaction were analysed by autoradiography.

b) Large-scale hydrolysis: The RNA/DNA duplex was prepared by hybridisation of the (R_P)-**R-3** oligomer (6.2 nmol) with the corresponding complementary 15-mer **D-6** (9.41 nmol) in a solution (245 μL) of Tris-Cl (20 mM, pH 8.0) that contained KCl (100 mM), $MgCl_2$ (10 mM) and dithiothreitol (1 mM). The hybridisation mixture, which included either [^{16}O]water or [^{18}O]water (75% ^{18}O atom) was heated to 85 °C and then slowly cooled to 20 °C (2 h). Next, RNase H (945 units) in the same buffer and the [^{18}O]water were added to give a final volume of 680 μL (the level of ^{18}O atoms was now 52%). After 1 h incubation at 37 °C the products were isolated by RP-HPLC (ODS Hypersil column, 5 μm) with a linear gradient of 0 – 33% CH_3CN/0.1 M triethylammonium bicarbonate, pH 7.4, 0.85% min^{-1} at a flow rate of 1 mL min^{-1}. Under these conditions, the 5′-phosphorothioylated $_{18OPS}$CAUCUU hexamer (**9**; 6.17 nmol) was eluted at 26.69 min. Large-scale hydrolysis was carried out twice, and 13.54 nmol $_{18OPS}$CAUCUU hexamer was finally obtained.

Nuclease-P1-catalysed degradation: Degradation of the $_{18OPS}$CAUCUU hexamer (**9**; 13.54 nmol) by nuclease P1 was carried out in the presence of the enzyme (4 μg) in a buffer that contained Tris-Cl (100 mM, pH 7.2) and $ZnCl_2$ (1 mM, 100 μL). The reaction mixture was incubated at 37 °C for 4 h. After quenching of the reaction by heating at 95 °C for 2 min, the resulting CMPS[^{18}O] (**10**; 11 nmol, 0.08 optical density units (OD)) was isolated by RP-HPLC under the conditions described above (retention time = 10.67 min).

Conversion of CMPS[^{18}O] into (S_P)-CTPαS[^{18}O]: The CMPS[^{18}O] (**10**; 11 nmol, 0.08 OD) obtained after nuclease-P1-catalysed hydrolysis of the hexamer **9** was dissolved in a solution (10 μL) of Tris-Cl (50 mM, pH 7.5) that contained KCl (50 mM), $MgCl_2$ (10 mM), adenosine 5′-triphosphate (ATP, 0.8 mM) and phosphoenolpyruvate (10 mM). Adenylate kinase (18 units) and pyruvate kinase (5 μg) were added and the mixture was incubated at 37 °C for 6 h. After quenching of the reaction by heating at 95 °C for 2 min, the sample was analysed by RP-HPLC and the resulting (S_P)-CTPαS[^{18}O] (**11**; 2.88 nmol, 0.021 OD) was isolated with a retention time of 19.37 min under the conditions described above.

Terminal deoxyribonucleotidyl transferase assay: The assay mixture (30 μL) containing (S_P)-CTPαS[^{18}O] (**11**; 1.65 nmol, 0.012 OD), the d[A_5A] primer (**12**; 2 nmol) and TdT in sodium cacodylate (100 mM, pH 7.2), $MgCl_2$ (10 mM) and $CoCl_2$ (1 mM, 20 units) was incubated at 37 °C for 1 hour. Then, after the mixture had been heated at 95 °C for 3 min, the resulting products were isolated by RP-HPLC under the conditions specified above (retention times for d[A_6] and d[A_5A]$_{PS}$(rC) were 33.58 and 34.71 min, respectively).

Mass spectrometry: Mass spectrometry analyses were performed as described elsewhere.[6]

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