Catalysis by *Escherichia coli* Ribonuclease HI Is Facilitated by a Phosphate Group of the Substrate

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ABSTRACT: To investigate the role of the phosphate group 3′ to the scissile phosphodiester bond of the substrate in the catalytic mechanism of *Escherichia coli* ribonuclease HI (RNase HI), we have used modified RNA−DNA hybrid substrates carrying a phosphorothioate substitution at this position or lacking this phosphate group for the cleavage reaction. Kinetic parameters of the H124A mutant enzyme, in which His^{124} was substituted with Ala, as well as those of the wild-type RNase HI, were determined. Substitution of the *pro*-R_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond of the substrate with sulfur reduced the k_{cat} value of the wild-type RNase HI by 6.9-fold and that of the H124A mutant enzyme by only 1.9-fold. In contrast, substitution of the *pro*-S_p-oxygen of the phosphate group at this position with sulfur had little effect on the k_{cat} value of the wild-type and H124A mutant enzymes. The results obtained for the substrate lacking this phosphate group were consistent with those obtained for the substrates with the phosphorothioate substitutions. In addition, a severalfold increase in the K_m value was observed by the substitution of the *pro*-R_p-oxygen of the substrate with sulfur or by the substitution of His^{124} of the enzyme with Ala, suggesting that a hydrogen bond is formed between the *pro*-R_p-oxygen and His^{124}. These results allow us to propose that the *pro*-R_p-oxygen contributes to orient His^{124} to the best position for the catalytic function through the formation of a hydrogen bond.

Ribonucleases H (RNases H) hydrolyze the P−O3′ bond of the RNA strand of an RNA−DNA hybrid duplex in the presence of divalent cations such as Mg^{2+} or Mn^{2+} (*1*). These enzymes are widely distributed in various organisms. RNase HI of *Escherichia coli* is structurally homologous to the RNase H domain of human immunodeficiency virus 1 (HIV-1) reverse transcriptase (*2*). The RNase H domain of HIV-1 reverse transcriptase is regarded as a putative target for anti-HIV therapies. Furthermore, inactivation of mRNA by antisense oligonucleotides depends on the RNase H activity (*3, 4*). Therefore, understanding of the mechanisms of catalytic function and substrate recognition for RNase HI will be helpful for these applications. *E. coli* RNase HI is most extensively studied for structure−function relationships (*2, 5*−*7*). The X-ray crystal structure of *E. coli* RNase HI has also been determined (*8*−*10*). However, the tertiary structure of the complex between *E. coli* RNase HI and its substrate has not been determined. On the basis of NMR and site-directed mutagenesis studies that identified the amino acid residues interacting with the substrate (*11, 12*), a model for the enzyme−substrate complex has been proposed (*11*).

For the catalytic function of *E. coli* RNase HI, two alternative mechanisms have been proposed. One is a two-metal-ion mechanism (*10*), where one of the two metal ions activates the attacking hydroxide ion, and the other is a general acid−base mechanism (*11, 13*−*18*), where an amino acid residue, instead of the metal ion, activates the attacking hydroxide ion. The two-metal-ion mechanism is based on the finding that Mn^{2+} binds at two sites in the RNase H domain of HIV-1 reverse transcriptase (*19*). In contrast, the general acid−base mechanism is based on the identification of a single divalent cation binding site in *E. coli* RNase HI by X-ray analysis (*15*). Furthermore, NMR (*20*) and kinetic (*21*) studies suggest that only one metal ion binds to the substrate-free enzyme. The Mg^{2+} ion does not interact with the phosphate group of the RNA, but interacts with the 2′-hydroxyl group of the cleavage site through the formation of an outer-sphere complex with a water molecule (*17, 22*). Activation of *E. coli* RNase HI by a single metal ion and inhibition of the enzyme by subsequent binding of a second metal ion has also been suggested (*23*). These studies make the general acid−base mechanism more plausible than the two-metal-ion mechanism, though further analyses will be required to verify the catalytic mechanism of the enzyme. According to the latest general acid−base mechanism (*13*), Asp^{10} and Asp^{70} are responsible for the binding of the Mg^{2+} ion to the correct position in the active site of the enzyme; His^{124} accepts a proton from the attacking water molecule, which acts as a general base; Asp^{134} holds this water molecule; Glu^{48} anchors the water molecule that acts as a general acid.

An active role of the substrate in catalytic function has been proposed for type II restriction endonucleases. *Eco*RI

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[1] Abbreviations: RNase H, ribonuclease H.

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and *Eco*RV cleave modified oligonucleotides, in which the phosphate group 3′ to the scissile phosphodiester bond is substituted with an H-phosphonate group or is missing, with a greatly reduced rate (*24*). *Eco*RV cannot cleave the substrate with the substitution of the *pro*-S_p-oxygen of the phosphate group with sulfur (*25*). On the basis of these observations, as well as the cocrystal structure of the *Eco*RV T93A mutant complexed with DNA and Ca^{2+} ions (*26*), a substrate-assisted catalysis model has been proposed (*24*−*27*). It has also been suggested that the substrate-assisted catalysis is widely employed among type II restriction endonucleases (*28*). In the model for *Eco*RV, the *pro*-R_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond assists Lys^{92}, which has been proposed to stabilize and orient the attacking hydroxide ion generated by a divalent metal ion, through the formation of a hydrogen bond (*26*). *Eco*RV and *E. coli* RNase HI share a common characteristic in the catalytic function. Both enzymes require a divalent metal ion for catalytic activity, contain a cluster of the acidic amino acid residues providing the metal binding site, and hydrolyze the P−O3′ bond. Therefore, it is of interest whether the phosphate group functions similarly for *E. coli* RNase HI as for *Eco*RV. His^{124} and Asp^{134} of *E. coli* RNase HI are the candidates for the residue assisted by the phosphate group, because these residues have been proposed to interact with the attacking hydroxide ion as does Lys^{92} in *Eco*RV (*18*).

To investigate whether the phosphate group 3′ to the scissile phosphodiester bond of the substrate plays a similar role for *E. coli* RNase HI as that for EcoRV, we have analyzed the effect of modification of the substrate on the enzymatic activity of the wild-type enzyme. We have analyzed this effect on the enzymatic activity of the H124A mutant enzyme, in which His^{124} is replaced by Ala, as well to provide the information on the interaction between the phosphate group and His^{124}. We have not analyzed this effect for the enzymatic activity of the D134A mutant protein, in which Asp^{134} is replaced by Ala, because this mutant protein does not exhibit any RNase H activity (*29*). Here we demonstrate that the interaction between the phosphate group of the substrate and His^{124} of the enzyme contributes to efficient catalysis.

EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP (>5000 Ci/mmol) was obtained from Amersham. Oligonucleotides were prepared and purified as described previously (*17*). The absolute configuration of the phosphorothioate diester was determined as previously described (*30*). *Crotalus durissus* phosphodiesterase was from Boehringer Mannheim. Other chemicals were of reagent grade. *E. coli* RNase HI wild-type enzyme (*31*) and the H124A mutant enzyme carrying the His^{124} → Ala mutation (*18*) were prepared as described.

Cleavage of the Substrate with RNase HI. The 9-bp and 18-bp hybrid duplexes, in which the RNA strands were ^{32}P-labeled at the 5′-end, were constructed. The 9-bp hybrid duplexes were prepared by hybridizing the ^{32}P-labeled 9-base RNA (10 pmol) with the complementary 9-base strand (15 pmol). The 18-bp hybrid duplex, in which the RNA strand is discontinuous, was prepared by hybridizing the ^{32}P-labeled front half 9-base RNA (10 pmol) and the unlabeled back half 9-base RNA (20 pmol) with the complementary 18-base strand (15 pmol). The labeled oligonucleotide duplexes (10 pmol) were mixed with *E. coli* RNase HI in a solution (10 μL) of 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 10 μg/mL bovine serum albumin and were incubated at 30 °C for 10 min. The hydrolysates were separated on a 20% polyacrylamide gel containing 7 M urea and were analyzed with Instant Imager (Packard). These hydrolysates were identified by comparing their migrations on the gel with those of the oligonucleotides generated by the partial digestion of the ^{32}P-labeled oligoribonucleotides with snake venom phosphodiesterase (*32*). To obtain the kinetic parameters, the enzyme reactions were performed under conditions in which the substrate was present in an excess amount relative to the enzyme. The substrate concentrations were varied from 0.1 to 1 μM. The kinetic parameters were determined by a least-squares fit of the data obtained from Lineweaver−Burk plots.

RESULTS

Design of Oligonucleotide Substrates. An RNA−DNA hybrid is not suitable for kinetic analyses of RNase HI, because this enzyme does not cleave the RNA strand of the substrate in a site-specific manner. In the present study, the cleavage was limited at a single site by using the chimeric oligonucleotides composed of oligo(2′-*O*-methylribonucleotide) with a tetradeoxyribonucleotide gap as the strand complementary to the RNA sequence. When the chimeric oligonucleotide was used for the substrate, the cleavage site was restricted to the phosphodiester linkage in the RNA strand 3′-adjacent to the RNA−DNA hybrid region (*33*). This method has been applied to analyze the interactions between the active site of *E. coli* RNase HI and the cleavage site of the substrate. It has been shown that the Mg^{2+} ion interacts with the 2′-hydroxyl group of the nucleotide 5′ to the scissile linkage by forming an outer-sphere complex (*17*), that the 2′-hydroxyl group of the nucleoside on the 3′-side of the scissile linkage acts as both a proton donor and an acceptor, and that the 2′-hydroxyl group of the second nucleoside 5′ to the scissile linkage acts as a proton acceptor (*34*).

To investigate the role of phosphate group 3′ to the scissile phosphodiester bond of the substrate, we used oligonucleotide hybrid duplexes, in which this phosphate was replaced with a phosphorothioate group (Figure 1a). Stereospecific phosphorothioate substitutions have been applied to studies on substrate recognition by the *Eco*RI endonuclease (*35*, *36*) and the *Eco*RV endonuclease (*25*) and to the elucidation of the catalytic mechanism of the *Eco*RV endonuclease (*25*). In this study, a modified RNA, 5′-r(GGAGAUG$_{ps}$AC)-3′, containing a phosphorothioate linkage (Figure 1a) (indicated by $_{ps}$) was used as the RNA strand of the hybrid duplex. The R_p and S_p isomers of the modified RNA were labeled with ^{32}P at the 5′-ends and were annealed with a chimeric oligonucleotide, 5′-$G_mU_mC_m$d(ATCT)C_mC_m-3′, where G_m, C_m, and U_m represent the 2′-*O*-methylribonucleosides, to form the R_p and S_p substrates, respectively. A hybrid duplex containing an unmodified RNA with the same sequence was also constructed (PD substrate).

Although the phosphorothioate is a useful modification, the possibility that the substitution of the oxygen with sulfur perturbs the catalytic function through steric hindrance cannot be excluded. Therefore, we also used an RNA−DNA hybrid substrate lacking the phosphate group 3′ to the scissile

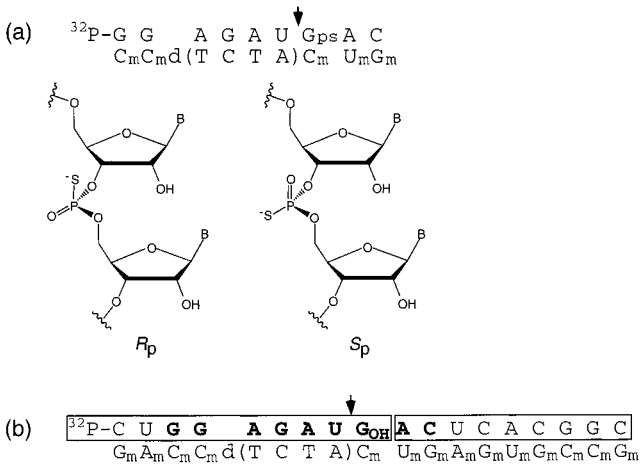

FIGURE 1: Oligomeric RNA−DNA substrates. The (a) 9-bp and (b) 18-bp hybrid substrates, in which the upper sequence represents the RNA and the lower sequence represents the chimeric oligonucleotide, are shown. A_m, G_m, C_m, and U_m represent the 2′-*O*-methylribonucleosides. The position of the phosphorothioate is indicated by ps within the 9-bp substrate. Structures of the R_p (left) and S_p (right) phosphorothioate linkages are shown below the 9-bp substrate. Each component of the discontinuous RNA strand of the 18-bp substrate is boxed. The RNA sequence of the 18-bp substrate common to that of the 9-bp substrates is designated in boldface type. The arrow indicates the cleavage site.

phosphodiester bond. This substrate (MP substrate) was prepared by annealing 5′-r(CUGGAGAUG)-3′ (RNA1), which was labeled with ^{32}P at the 5′-end, and 5′-r(ACUCACGGC)-3′ (RNA2), in which the phosphate group at the 5′-end was lacking, with a chimeric oligonucleotide, 5′-$G_mC_mC_mG_mU_mG_mA_mG_mU_mC_m$d(ATCT)$C_mC_mA_mG_m$-3′, where A_m, G_m, C_m, and U_m represent the 2′-*O*-methylribonucleosides (Figure 1b). It has previously been shown that RNA is cleaved at an additional site as well as the expected site when the cleavage site is designed to be the phosphodiester bond immediately preceding the 3′-end residue of the RNA strand with a chimeric oligonucleotide like RNA1(*33*). Thus, the duplex was extended on the 3′-side of RNA1 with RNA2 to avoid such an additional cleavage.

Cleavage of the Substrates Containing Phosphorothioate by the Wild-Type Enzyme. The R_p, S_p, and PD substrates were treated with the wild-type enzyme. The results are shown in Figure 2. These substrates were cleaved at the expected sites. The wild-type enzyme showed significantly lower cleavage efficiency on the R_p substrate than on the S_p and PD substrates (Figure 2a). To further analyze the cleavage reaction in detail, the kinetic parameters of the wild-type enzyme were determined (Table 1). The k_{cat} value of the wild-type enzyme on the R_p substrate was 6.9-fold lower than that on the PD substrate, whereas the k_{cat} value on the S_p substrate was rather slightly higher than that on the PD substrate. These results suggest that the phosphate group 3′ to the scissile phosphodiester bond of the substrate contributes to efficient catalysis in a stereospecific manner. The K_m value on the R_p substrate was 3.5-fold higher than that on the PD substrate, whereas the K_m value of the wild-type enzyme on the S_p substrate was rather slightly lower than that on the PD substrate, suggesting a stereospecific interaction between the phosphate group and the enzyme.

It is unlikely that the difference in the stability of the substrate affects the kinetic parameters, because similar T_m values have been reported for the R_p and S_p substrates of the 9-bp hybrid duplex, in which the scissile phosphate group was replaced with a phosphorothioate group (*17*).

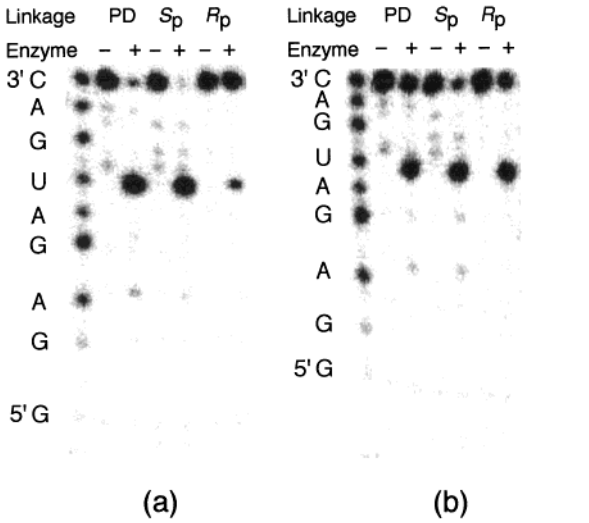

FIGURE 2: Cleavage of the 9-bp substrates by *E. coli* RNase HI. The hydrolyses of the 5′-^{32}P-labeled 9-bp substrates by the wild-type enzyme (a) and H124A mutant enzyme (b) were carried out at 30 °C for 10 min and the hydrolysates were separated on a 20% polyacrylamide gel containing 7 M urea, as described under Experimental Procedures. The concentrations are 1.0 μM for the substrates, 0.7 nM for the wild-type enzyme, and 28 nM for the H124A mutant enzyme. PD, S_p, and R_p are the same as indicated in Table 1.

Table 1: Kinetic Parameters of the Wild-Type and H124A Mutant Enzymes for the Hydrolysis of Hybrid Duplex[a]

	wild type			H124A		
substrate	K_m (μM)	k_{cat} (min^{-1})	relative k_{cat} (%)	K_m (μM)	k_{cat} (min^{-1})	relative k_{cat} (%)
PD	0.197	191	100	0.959	0.897	100
S_p	0.140	288	151	0.633	1.05	117
R_p	0.698	27.7	14.5	0.485	0.482	53.7
MP	0.808	3.02		0.861	0.135	

[a] The hydrolysis of substrate with the enzyme was carried out at 30 °C for 10 min in 10 μL of 10 mM Tris-HCl (pH 8.0) containing 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 10 μg/mL bovine serum albumin. Kinetic parameters were determined by a least-squares fit of the data obtained from Lineweaver−Burk plots. Errors, which represent the 67% confidence limits, are within 10% of the values reported. PD, S_p, and R_p represent the substrates containing phosphodiester, S_p-phosphorothioate, and R_p-phosphorothioate linkages, respectively, at the position designated by ps in Figure 1a. MP represents the missing-phosphate substrate lacking the phosphate group at the position designated by OH in Figure 1b.

Cleavage of the Substrates Containing Phosphorothioate by the H124A Mutant Enzyme. To examine whether the phosphate group 3′ to the scissile phosphodiester bond of the substrate interacts with His^{124}, we have determined the kinetic parameters of the H124A mutant enzyme, which retains 1% of the activity of the wild-type enzyme (*18*). If the phosphate group assists the catalytic function of His^{124}, the catalytic activity of the H124A mutant enzyme will be insensitive to the modification of the phosphate group. The H124A mutant enzyme cleaved all these substrates with a comparable efficiency (Figure 2b). The k_{cat} value of the H124A mutant enzyme on the R_p substrate was only 1.9-fold lower than that on the PD substrate, whereas the k_{cat} value on the S_p substrate was rather slightly higher than that

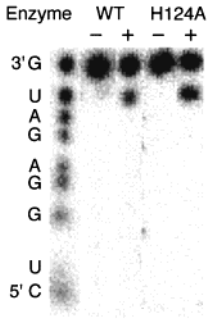

FIGURE 3: Cleavage of the missing-phosphate substrate by *E. coli* RNase HI. The hydrolyses of the 5′-^{32}P-labeled 18-bp substrate, in which the RNA strand was composed of two discontinuous RNA fragments, by the wild-type enzyme and H124A mutant enzyme were carried out at 30 °C for 10 min and the hydrolysates were separated on a 20% polyacrylamide gel containing 7 M urea, as described under Experimental Procedures. The concentrations are 1.0 μM for the substrates, 0.7 nM for the wild-type enzyme, and 28 nM for the H124A mutant enzyme.

on the PD substrate. The significantly smaller effect of the phosphorothioate substitution on the k_{cat} value of the H124A mutant enzyme than that of the wild-type enzyme suggests that the contribution of the phosphate group to the catalytic function is mediated by His^{124}. As shown in Table 1, the His^{124} → Ala mutation reduced the k_{cat} value of the enzyme by 200∼300-fold when the PD or S_p substrate was used as a substrate but reduced it by only 57-fold when the R_p substrate was used as a substrate. This implies that the catalytic function of His^{124} on the R_p substrate is attenuated as compared to that on the PD substrate and thereby suggests that the phosphate group is required to make His^{124} fully functional. Likewise, the His^{124} → Ala mutation increased the K_m value of the enzyme by ∼4−5-fold when the PD or S_p substrate was used as a substrate but rather slightly reduced it when the R_p substrate was used. This result suggests that His^{124} directly interacts with the *pro*-R_p-oxygen of the phosphate group, probably through the formation of a hydrogen bond. The increase in the K_m value on the PD or S_p substrate by this mutation is consistent with the previous result that the K_m value of the H124A mutant enzyme on M13 RNA−DNA hybrid was higher than that of the wild-type enzyme by 2.3-fold (*16*).

Cleavage of the Missing-Phosphate Substrate. The substrate lacking the phosphate in question was cleaved by the wild-type and H124A mutant enzymes at the expected site (Figure 3). The kinetic parameters are shown in Table 1. The His^{124} → Ala mutation reduced the k_{cat} value of the enzyme by 210-fold when the PD substrate was used as a substrate but reduced it by only 22-fold when the MP substrate was used as a substrate. This result is consistent with those obtained for the substrates with the phosphorothioate substitutions. The His^{124} → Ala mutation hardly affected the K_m value of the enzyme when the MP substrate was used as a substrate. This result also supports the existence of the interaction between the phosphate group and His^{124}.

DISCUSSION

In the present paper, we showed that substitution of the *pro*-R_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond with sulfur significantly reduces the k_{cat} value of the wild-type RNase HI, whereas it has little effect on that of the H124A mutant enzyme. Furthermore, we used an RNA−DNA hybrid substrate lacking this phosphate group and obtained results consistent with those for the substrate containing phosphorothioate. These results suggest that this phosphate group functions cooperatively with His^{124} for catalysis.

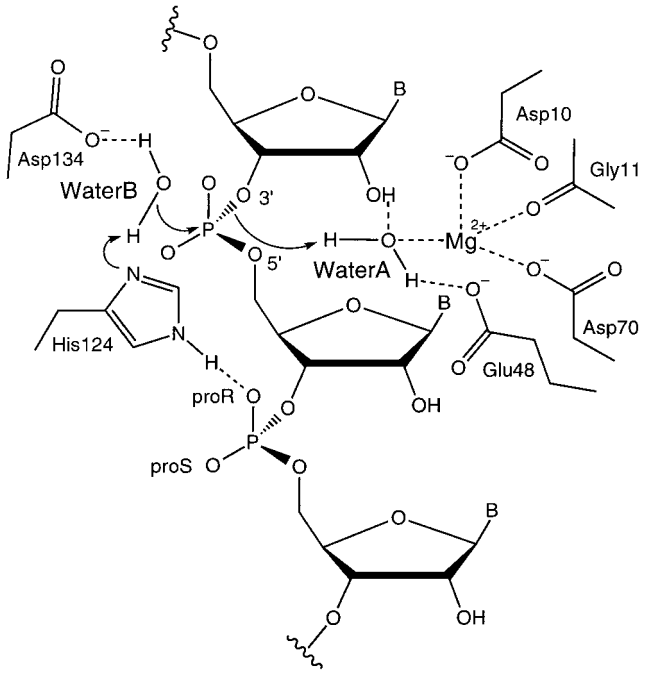

FIGURE 4: Proposed catalytic mechanism of *E. coli* RNase HI. The possible involvement of the phosphate group 3′ to the scissile phosphodiester bond in a general acid−base mechanism for the hydrolysis of the P−O3′ bond of RNA by *E. coli* RNase HI is represented schematically. Water B and Water A represent the water molecules that act as a general base and general acid, respectively.

It has been proposed that His^{124} functions as a general base. Thus, a possible role of the phosphate group is to enhance the catalytic efficiency by removing a proton from His^{124}. Substitution of the *pro*-R_p-oxygen of the phosphate group with sulfur results in an uncharged double-bonded oxygen at the S_p-position (*37*). This uncharged oxygen cannot abstract a proton, explaining the reduced activity by the R_p substitution. However, the pK_a of a nonbridging phosphate oxygen is too low to efficiently accept a proton from His^{124}, as previously pointed out for *Eco*RV (*25*, *26*). The phosphate group is also not likely to operate as a general base by directly deprotonating a water molecule for the same reason. Therefore, we propose that the phosphate group contributes to orient His^{124} to the best position for the activation of the attacking hydroxide ion through the formation of a hydrogen bond between the imino proton of His^{124} and the *pro*-R_p-oxygen (Figure 4). This model well accounts for the reduction in the k_{cat} values by the substitution of the *pro*-R_p-oxygen of the phosphate group with sulfur, because a sulfur cannot form a hydrogen bond. This hydrogen bond could also elevate the pK_a of His^{124}, thereby facilitating the generation of the attacking hydroxide ion. The increase in the K_m value by the substitution of His^{124} with Ala or the substitution of the *pro*-R_p-oxygen with sulfur is consistent with the prediction that a hydrogen bond is formed between His^{124} and the *pro*-R_p-oxygen. Other than a hydrogen bond,

an electrostatic interaction between His^{124} and the *pro*-S_p-oxygen could also be possible to fix the position of His^{124}, because a loss of charge of the double-bonded oxygen at the S_p-position by the substitution of the *pro*-R_p-oxygen of the phosphate group with sulfur can also account for the reduction in the k_{cat} value and the increase in the K_m value. However, this possibility is unlikely, because His^{124} is mostly deprotonated at pH 8 (*16*).

Two types of the crystal structure of *E. coli* RNase HI, in which the locations of His^{124} are different from each other, have been determined. In the crystal structure of Katayanagi et al. (*8*, *9*), the imidazole group of His^{124} is not located close enough to interact with the active-site residues. In contrast, it is located within 4 Å of the Asp^{10} and Asp^{70} carboxylates in the crystal structure of Yang et al. (*10*). These results, as well as the ^{15}N NMR relaxation analysis (*38*) and the normal-mode analysis (*39*), suggest that the loop containing His^{124} is flexible. This flexibility of His^{124} has been proposed to modulate the catalytic activity of *E. coli* RNase HI (*23*). According to this proposal, inhibition of the catalytic activity of *E. coli* RNase HI at high Mn^{2+} ion concentrations is due to a shift of His^{124} to an improper position for the catalytic function by binding of a second metal ion to Asp^{134}. In addition, a disordered structure of the loop containing His^{539} in the RNase H domain of HIV-1 reverse transcriptase (*19*) also supports flexibility of the loop containing His^{124}. Therefore, this loop probably changes its conformation upon binding of the substrate so that His^{124} can locate close to the catalytic site. During this process, the *pro*-R_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond may recruit His^{124} to the best position for catalysis and thereby enhance the catalytic reaction.

It has been suggested that *E. coli* RNase HI makes contact with its substrate duplex in the minor groove and interacts with *pro*-S_p-oxygen of the scissile phosphodiester bond, which is accessible from the minor-groove side (*17*). Similarly, not the *pro*-R_p-oxygen but the *pro*-S_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond is accessible from the minor-groove side. However, an RNA−DNA hybrid structure determined by NMR has shown that the *pro*-R_p-oxygen is oriented toward the scissile phosphodiester bond, whereas the *pro*-S_p-oxygen is oriented opposite to the scissile phosphodiester bond (*40*). Moreover, our general acid−base mechanism requires His^{124} to be located in the vicinity of the scissile phosphodiester bond. Therefore, the interaction between His^{124} and the *pro*-R_p-oxygen rather than the *pro*-S_p-oxygen is rationalized also from the structural aspect.

In the substrate-assisted catalysis model for *Eco*RV, the *pro*-R_p-oxygen of the phosphate group 3′ to the scissile phosphodiester bond assists Lys^{92}, which functions to stabilize and orient the attacking hydroxide ion, through the formation of a hydrogen bond (*26*). In this model, three metal ions are involved in the catalytic function, one of which generates the attacking hydroxide ion. However, a substrate-assisted catalysis model for *Eco*RV, in which only one metal ion is involved in the catalytic function, has also been proposed (*24*, *27*). Therefore, the current result by itself cannot exclude the two-metal-ion mechanism of *E. coli* RNase HI. In any case, the role of the phosphate group in the catalytic reaction of *E. coli* RNase HI seems to resemble that of *Eco*RV in assisting a residue involved in supplying the attacking hydroxide ion. However, the effect by the substitution or removal of the phosphate group on the catalytic reaction in *E. coli* RNase HI is much smaller than that in *Eco*RV. The greater significance of Lys^{92} in the *Eco*RV function than that of His^{124} in the *E. coli* RNase HI function probably causes this difference. Replacement of Lys^{92} of *Eco*RV with Ala or Gln reduces cleavage activity by 10^4-fold or greater (*41*), whereas replacement of His^{124} in *E. coli* RNase HI with Ala, Lys, Gln, or Glu reduces cleavage activity by only ∼30−100-fold (*16*). Even higher activity was retained by the substitution of the phosphate group 3′ to the scissile phosphodiester bond of the substrate with the R_p-phosphorothioate than by the substitution of His^{124} of the enzyme. This suggests that His^{124} is placed on an approximately proper position of the active site upon binding of the substrate even in the absence of the phosphate group and that fine-tuning of the position of His^{124} is achieved by the phosphate group. Such a role of the phosphate group might be associated with the cleavage site preference, because the relative position of the phosphate group to the active site may depend on the local structure of the RNA−DNA hybrid, which has been shown to be varied with the sequence (*40*).

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SUPPORTING INFORMATION AVAILABLE

Detailed experimental procedures for preparation of oligonucleotides, determination of the absolute configuration of the phosphorothioate diester, and preparation of *E. coli* RNase HI wild-type and H124A mutant enzymes. This material is available free of charge via the Internet at http://pubs.acs.org.

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