Recognition of 2′-hydroxyl groups by *Escherichia coli* ribonuclease HI

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Abstract In order to investigate the hydrogen-bonding interactions between *Escherichia coli* ribonuclease HI and the 2′-hydroxyl functions of the substrate, oligonucleotide duplexes containing 2′-amino-2′-deoxyuridine or 2′-fluoro-2′-deoxyuridine at a specific site were used, and their affinities for the enzyme were determined by kinetic analyses. The results indicate that the hydroxyl groups of the nucleoside 3′-adjacent to the cleaved phosphodiester linkage and the second nucleoside 5′ to the cleaved phosphodiester act as both a proton donor and an acceptor and as a proton acceptor, respectively, in the enzyme–substrate complex. A molecular model was constructed using the interactions derived from the results.

Key words: Ribonuclease H; Oligonucleotide; Interaction; Hydrogen bond; Tertiary structure

1. Introduction

Ribonuclease H (RNase H) is an enzyme that hydrolyzes the RNA strand of an RNA · DNA hybrid duplex, and it is distributed widely in various organisms [1]. RNase HI from *Escherichia coli* (*E. coli*), as well as the RNase H domain of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, has been investigated extensively by site-directed mutagenesis [2–9], X-ray crystallography [10–15], and nuclear magnetic resonance (NMR) spectroscopy [16–19]. *E. coli* RNase HI is composed of a five-stranded β-sheet and five α-helices, and its main chain folding is very similar to that of the RNase H domain of HIV-1 reverse transcriptase [10,14]. Three amino acid residues, Asp-10, Asn-44, and Glu-48, and the main chain carbonyl of Gly-11 form a binding site for a divalent metal cation, which is required for the catalytic activity [12,13]. Asp-70, His-124, and Asp-134 are also involved in the catalytic function [3,4,6,18], and the positively-charged residues in the basic protrusion are used for substrate binding [5,11]. The structures of the substrates, i.e. RNA · DNA hybrid duplexes, have been studied mainly by NMR spectroscopy [20–23], and the interactions with the enzyme have been discussed in terms of a molecular model [22,24]. However, the tertiary structure of the RNase H complex with its substrate has not been determined.

One strategy for the investigation of the interactions between proteins and nucleic acids is to use oligonucleotides modified at specific sites. Further information about the interactions can be derived by combining the results with the data obtained from the studies of the protein. Concerning RNase H, little effort has been made for this purpose. Instead, modified oligonucleotides have been used mostly to obtain the site specificity of the cleavage or the resistance to nucleases [25–30]. In our previous study, modified oligonucleotide duplexes were applied, for the first time, to the elucidation of the mechanism of the RNase H reaction [31]. The base, the phosphodiester, and the sugar moiety at the cleavage site were modified in turn, and the results suggested that a hydrogen bond is formed between the enzyme and the pro-S_P oxygen of the cleaved phosphodiester linkage and that the Mg^{2+} ion interacts with the 2′-hydroxyl function of the nucleoside 5′ to the scissile linkage by forming an outer-sphere complex.

In this study, we used modified oligonucleotide duplexes to investigate the interactions between *E. coli* RNase HI and the 2′-hydroxyl groups of the substrate, which should contribute greatly to substrate recognition by the enzyme. Either an amino group or a fluorine atom was substituted for the 2′-hydroxyl function of each nucleoside at positions −2, −1, +1, and +2 (the nucleoside 5′ to the cleaved phosphodiester was designated as 0, and the number increased in the 3′-direction) in the substrate, in which the cleavage site was restricted to a single site between positions 0 and +1 by using a complementary strand containing 2′-*O*-methylribonucleosides. Although the sugar pucker of the 2′-amino-2′-deoxynucleoside might be different from that of the ribonucleoside, the kinetic parameters showed clear differences in the enzyme affinity between the parent duplex and the substrates substituted at positions +1 and −1. Hydrogen-bonding interactions in the complex of *E. coli* RNase HI with its substrate are discussed.

2. Materials and methods

2.1. Oligonucleotide duplexes

Oligonucleotides were prepared and purified as described previously [31]. The RNA strands (2 nmol) were labeled using [γ-^{32}P]ATP (1.7 pmol, 370 kBq) and T4 polynucleotide kinase (from *E. coli* strain A19, Takara Shuzo, 6 units) in 50 mM Tris-HCl (pH 9.5), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 5% glycerol (30 μl). After incubation at 37°C for 50 min, ATP (0.1 μmol) and the kinase (6 units) were added, and the solutions were incubated at 37°C for another 10 min and then mixed with ethylenediaminetetraacetic acid (EDTA; 1 μmol). The mixtures were evaporated in vacuo, and, after the addition of the loading buffer containing 10 M urea, 50 mM Tris, 50 mM EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanol (10 μl), they were applied to a 20% polyacrylamide gel containing 7 M urea. After electrophoresis, the bands detected by ultraviolet absorption were cut out of the gel, and the products were eluted with water and passed through a NENSORB 20 column (DuPont). The labeled oligonucleotides (300 pmol) were mixed with the complementary strand (360 pmol) in water (30 μl), and the mixtures were heated at 65°C for 2 min and then cooled to room temperature.

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Abbreviations: RNase H, ribonuclease H; HIV-1, human immunodeficiency virus type 1; NMR, nuclear magnetic resonance; ATP, adenosine 5′-triphosphate; EDTA, ethylenediaminetetraacetic acid; PAGE, polyacrylamide gel electrophoresis; A_m, G_m, and C_m, 2′-*O*-methylribonucleosides; U_a, 2′-amino-2′-deoxyuridine; U_f, 2′-fluoro-2′-deoxyuridine.

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2.2. Cleavage of the substrates with RNase H

The labeled oligonucleotide duplexes (10 pmol) were mixed with *E. coli* RNase HI (provided by Dr. S. Kanaya of Protein Engineering Research Institute) in a solution (10 μl) of 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 0.01% (w/v) bovine serum albumin. These mixtures were incubated at 30°C for 20 min and subjected to 20% polyacrylamide gel electrophoresis (PAGE).

2.3. Kinetic analysis

The reaction mixtures (20 μl) included *E. coli* RNase HI and each substrate at the following concentrations: 0.01 nM and 0.5–5 μM (R9), 0.0125 nM and 0.5–5 μM (−2A and −2F), 0.4 nM and 2–20 μM (−1A), 0.1 nM and 1–10 μM (−1F), 5.0 nM and 9.5–90.5 μM (+1A), 1.25 nM and 2.5–25 μM (+1F), 0.2 nM and 2–20 μM (+2A), and 0.1 nM and 1–10 μM (+2F), respectively. These mixtures were incubated at 30°C. Aliquots (3 μl) were taken at intervals of 6 min, and were mixed with the loading buffer containing 10 M urea, 50 mM Tris, and 50 mM EDTA. The samples (ca. 1,500 cpm) were subjected to PAGE, and after the gels were dried, the radioactivity of each band was quantified on a FUJIX BAS2000 Bio Imaging Analyzer (Fuji Photo Film). The kinetic parameters were obtained from the $[S]/v$ vs. [S] plots, where the x- and y-intercepts gave $-K_m$ and K_m/V_{max}, respectively.

2.4. Model building

The model of *E. coli* RNase HI complexed with a 21-base-pair RNA · DNA hybrid duplex was constructed in a manner similar to those described previously [11,24]. The hybrid duplex structure [32] and the crystal structure of RNase HI in complex with a Mg^{2+} ion [12] were manipulated for docking on a computer graphics screen (HYDRA, Polygen). All of the inappropriate contacts were corrected through the conformation energy minimization procedure using the AMBER all-atom force field [33] with SPC waters [34].

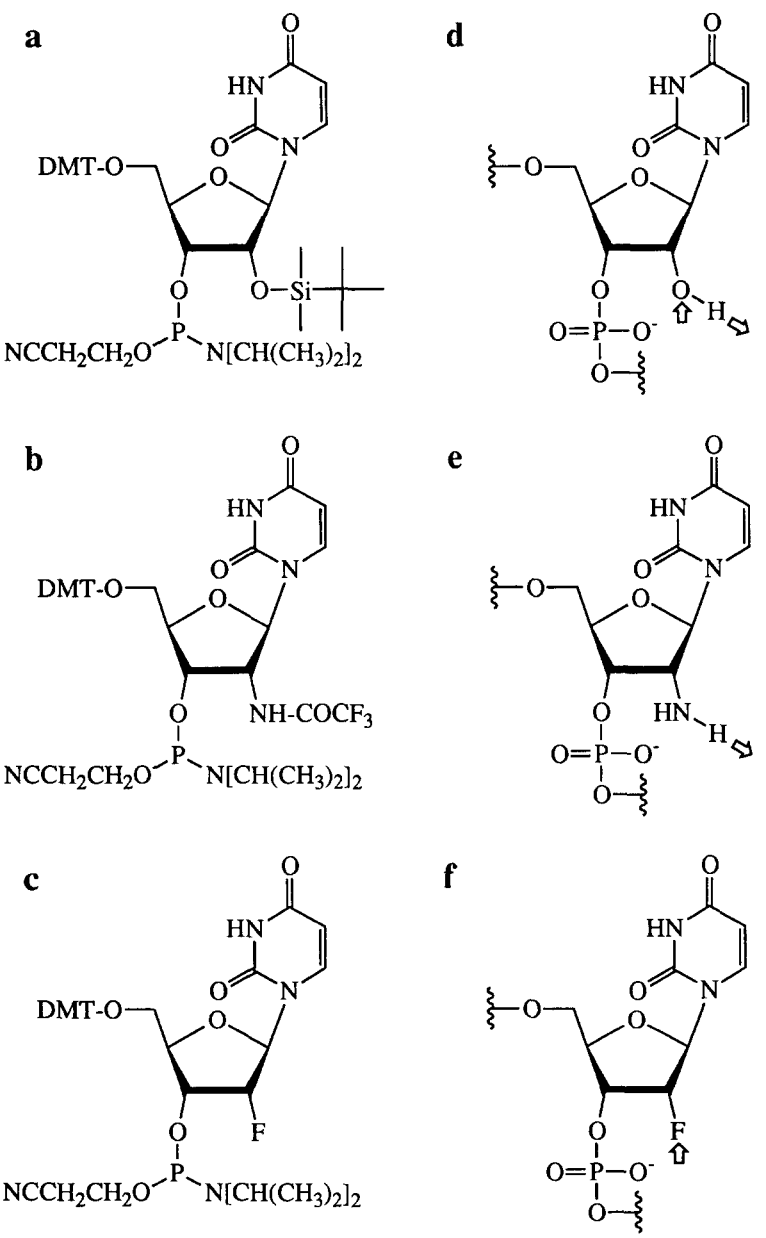

Fig. 1. (a–c) The phosphoramidite coupling units used for the oligonucleotide synthesis. DMT is the 4,4′-dimethoxytrityl group. (d–f) The structures of the modified uridines. The donor and acceptor for the hydrogen-bonding interaction are shown by arrows.

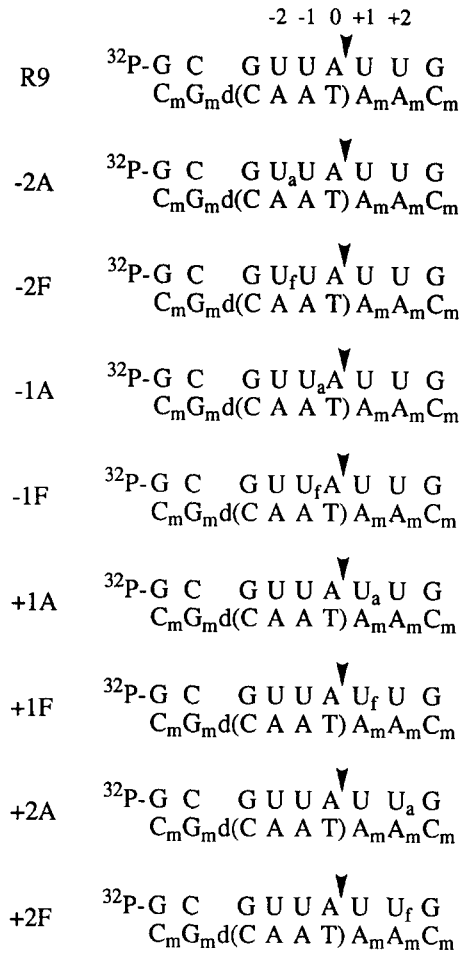

Fig. 2. The designation and the sequences of the oligonucleotide duplexes used in this study. U_a, U_f, and N_m represent 2′-amino-2′-deoxyuridine, 2′-fluoro-2′-deoxyuridine, and 2′-*O*-methylribonucleoside, respectively. The nucleotide positions are defined at the top, and the arrows indicate the RNase H cleavage site.

3. Results and discussion

3.1. Oligonucleotides modified at the 2′-position

RNase H catalyzes the hydrolysis of the phosphodiester linkages in the RNA strand of an RNA · DNA hybrid duplex, but such a substrate is not suitable for the quantitative analysis of the enzyme reaction, because this enzyme does not cleave the internucleotide linkages in a site-specific manner. The site specificity was attained by using an oligo(2′-*O*-methylribonucleotide) with a tetradeoxynucleotide gap as a splint complementary to the RNA strand, and the cleavage site was restricted, probably by the steric hindrance of the 2′-methoxy groups, to the internucleotide linkage in the RNA strand 3′-adjacent to the RNA · DNA hybrid region [25]. Using this strategy, we determined the interactions between the active site of *E. coli* RNase HI and the cleavage site of the substrate [31]. It was shown that the 2′-hydroxyl group of the nucleoside 5′ to the cleaved phosphodiester interacted with the Mg^{2+} ion by forming an outer sphere complex. In the present study, this strategy was used again, and the 2′-hydroxyl functions neighboring the cleavage site were replaced with either an amino group or a fluorine atom, in order to investigate the hydrogen-bonding interactions between *E. coli* RNase HI and its substrate. In the hydrogen bond, the amino group and the fluorine atom at the 2′-position act as a proton donor and a proton acceptor, respectively, while the hydroxyl group can be both (Fig. 1), and these substituents have a size similar to that of the hydroxyl group. Therefore, the affinity of the substituted substrate for the enzyme should be changed when the hydrogen bond is lost by the substitution at the respective sites.

An RNA · DNA hybrid containing 2′-*O*-methylribonucleosides, r(GCGUUAUUG) · $C_mA_mA_m$d(TAAC)G_mC_m (R9) where A_m, G_m, and C_m represent the 2′-*O*-methylribonucleosides, was designed as the parent duplex. Since the phosphodiester 3′ to the adenosine of the ribo-strand in this duplex was hydrolyzed by *E. coli* RNase HI, the position of this adenosine was termed 0, and from this adenosine the position numbers increased and decreased in the 3′- and 5′-directions, respectively (Fig. 2). Oligonucleotides containing either 2′-amino-2′-deoxyuridine (U_a) or 2′-fluoro-2′-deoxyuridine (U_f) at position −2, −1, +1, or +2 were synthesized using the phosphoramidite coupling units shown in Fig. 1, and were purified to homogeneity by high-performance liquid chromatography. After ^{32}P-labeling, they were annealed to the complementary strand containing 2′-*O*-methylribonucleosides. The designations and the sequences of the 2′-substituted duplexes are shown in Fig. 2.

3.2. RNase H reaction with the 2′-substituted substrates

The ^{32}P-labeled substrates were treated with *E. coli* RNase HI under certain conditions (1.0 μM substrate with either 1.0 or 10 nM enzyme at 30°C for 20 min), and the products were separated by denaturing 20% PAGE (Fig. 3). It was found in all cases that only the phosphodiester linkage between positions 0 and +1 was hydrolyzed, with varying cleavage rates. Substitution of either an amino group or a fluorine atom for the 2′-hydroxyl function at position −2 induced almost no change in the apparent cleavage rates. The largest effect was observed at position +1. Enzyme concentrations 100- and 1000-fold higher than that for R9 were required for +1F and +1A, respectively, in order to obtain similar cleavage rates (data not shown). At positions −1 and +2, the ratios of the product formed with 1.0 nM enzyme were less than 50%, and the 2′-amino substrates were cleaved less efficiently than the 2′-fluoro duplexes.

In order to investigate the effects of these substitutions in detail, kinetic analyses of the RNase H reaction with the substituted substrates were carried out. The velocities (v) at varying substrate concentrations ([S]) were measured under the conditions in which less than 10% of the substrate was converted to the product, and the kinetic parameters were obtained from the [S]/v versus [S] plots. The Gibbs free energy change ($\Delta\Delta G$) caused by the substitution was calculated using the following equation: $\Delta\Delta G = -RT\ln\{(k_{cat}/K_m)_{substituted}/(k_{cat}/K_m)_{parent}\}$, where R = the gas constant = 1.987 cal/mol and T = the absolute temperature = 303 K [35]. The $\Delta\Delta G$ values, as well as the kinetic parameters, are listed in Table 1.

The Michaelis constants (K_m) were used as indices of the enzyme affinity. When the 2′-hydroxyl function at position −2 was replaced with either an amino group (−2A) or a fluorine atom (−2F), only a small change in the K_m value, with a slightly increased k_{cat}, was observed. It has been reported that the incorporation of a 2′-amino-2′-deoxynucleoside decreases the

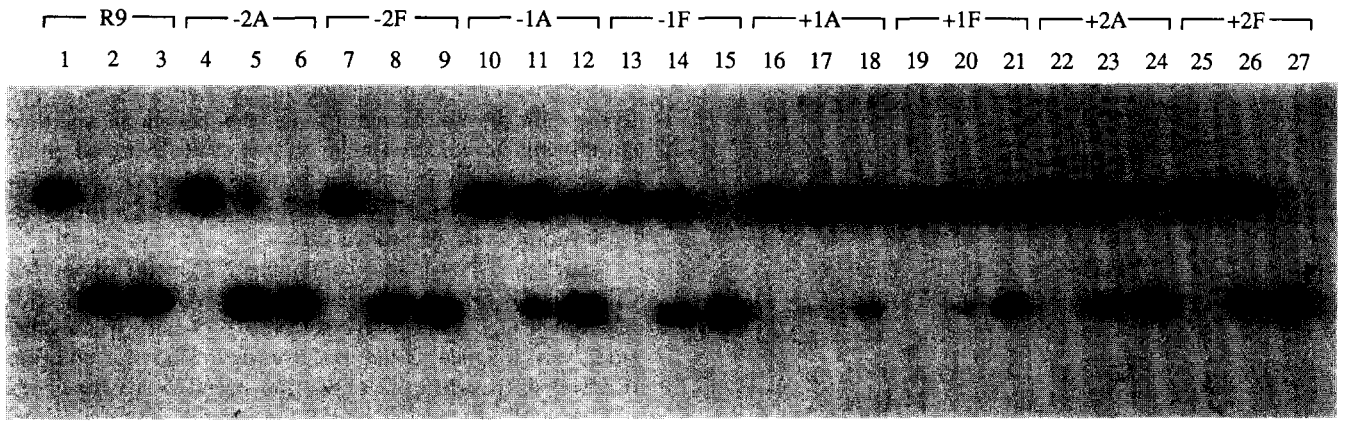

Fig. 3. Cleavage of the substrates with *E. coli* RNase HI. The enzyme concentrations were 0, 1.0 and 10 nM (from the left) for each substrate. The cleavage sites are shown in Fig. 2.

thermal stability of the duplex [36], whereas the 2′-fluoro-2′-deoxynucleoside does not affect the duplex stability [37]. Therefore, the slightly larger K_m value for the −2A substrate can be attributed to duplex destabilization caused by the amino substitution, although the experiments were performed at 30°C, at which the 9-base-pair duplexes should be formed. These substrates modified at position −2 are good controls for the evaluation of the overall effects of the substitution, including the duplex stability. At position −1, there was a great difference between the amino and fluoro substitutions. The substrate with an amino group (−1A) showed a reduced affinity for the enzyme, whereas the K_m value for the −1F substrate was similar to those of −2F and R9. This result suggests a hydrogen-bonding interaction between the 2′-hydroxyl group at this position and the enzyme, in which the 2′-hydroxyl acts as a proton acceptor. The most drastic change in K_m was observed for the substrates substituted at position +1. With either the amino or fluoro substitution, the affinity was reduced, and the free energy change of more than 3 kcal/mol revealed the loss of a hydrogen bond. This observation is in contrast to the results obtained for the substitution at position 0, where the 2′-hydroxyl group interacts with the metal ion [31]. Therefore, it is concluded that the 2′-hydroxyl group at this position acts as both a proton donor and a proton acceptor in hydrogen bonds with the enzyme. The larger K_m value for +1A than for +1F may reflect the conformational change of the sugar moiety caused by the amino substitution. At position +2, both +2A and +2F showed larger K_m values than R9, but the increases are only less than three-fold when −2A and −2F are used as controls. These differences are not large enough to attribute them to the loss of a hydrogen bond.

Table 1
Kinetic parameters for the substrates and complex destabilization by the substitution

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\mu M^{-1} \cdot min^{-1}$)	$\Delta\Delta G$ (kcal/mol)
R9	3.0	9.0×10^2	300	0
−2A	6.8	1.4×10^3	200	0.23
−2F	4.4	1.1×10^3	240	0.11
−1A	32	3.4×10^2	11	2.0
−1F	4.9	1.5×10^2	31	1.4
+1A	160	81	0.52	3.8
+1F	44	90	2.0	3.0
+2A	19	4.9×10^2	25	1.5
+2F	12	4.6×10^2	37	1.3

Substitution of the 2′-hydroxyl groups, combined with kinetic studies, was performed by Herschlag et al. to investigate their contributions to binding and catalysis by *Tetrahymena* ribozyme [38], and Lesser et al. used a similar strategy to study the recognition of the base sequence by the *Eco*RI restriction endonuclease [39]. In this study, we have shown that the hydrogen-bonding interactions between the enzyme and the 2′-hydroxyl groups of the substrate can be revealed by replacing them with an amino group and a fluorine atom.

3.3. Construction of a tertiary structure model of the enzyme–substrate complex

In view of the results described above, our tertiary structure model of *E. coli* RNase HI complexed with the substrate and Mg^{2+} [31] was examined. This model was originally constructed using the crystal structure of the Mg^{2+}-bound enzyme [12] and the active site interaction data obtained in our previous work [31]. The hydrogen-bonding interactions derived in this study were added to this model (Fig. 4).

At position −1, the glutamine at position 72 (Gln-72) is the only candidate for the hydrogen donor to the 2′-hydroxyl group, since no other amino acid residue is found at this site. This glutamine is conserved in the RNase H domain of HIV-1 reverse transcriptase [14], and its counterpart of *Thermus thermophilus* RNase H is histidine [40], which also can act as a proton donor in a hydrogen bond. The mutation of Gln-72 to alanine, which resulted in a reduced affinity for the substrate [24], also suggests its contribution to the substrate recognition. At position +1, the backbone amides of Cys-13 reside close to the 2′-hydroxyl. Since Cys-13 is located in the β-sheet structure, it is difficult to significantly change the orientation of the side chain of this cysteine, and cysteine is not conserved at this position in HIV-1 reverse transcriptase. Therefore, the backbone imino and carbonyl groups of Cys-13 can be assigned as the proton donor and the acceptor, respectively, in the hydrogen-bonding interactions with the +1 hydroxyl of the substrate.

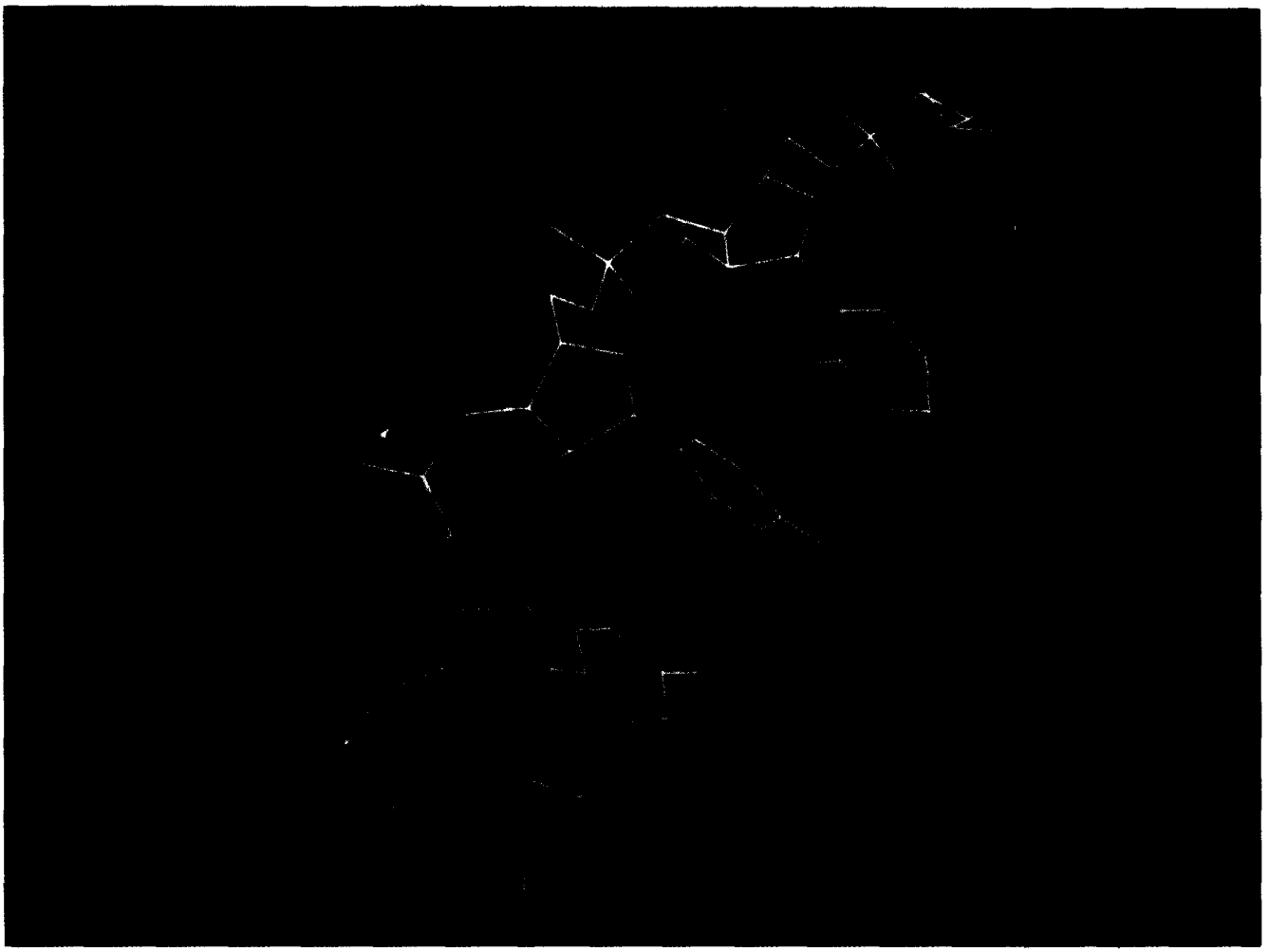

Fig. 4. A structure model showing the interactions between *E. coli* RNase HI and its substrate. The enzyme and the RNA and DNA strands are shown in blue, white, and purple, respectively. The hydrogen bonds between the amino acid residues (yellow) and the 2'-hydroxyl groups (pink) discussed in the text are indicated by broken lines. The interactions at the active site, which is composed of the amino acids shown in orange, the Mg^{2+} ion, and a water molecule, were derived in our previous work [31].

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