Kinetic and Stoichiometric Analysis for the Binding of Escherichia coli Ribonuclease HI to RNA-DNA Hybrids Using Surface Plasmon Resonance

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To understand how ribonucleases H recognize RNA-DNA hybrid substrates, we analyzed kinetic parameters of binding of Escherichia coli RNase HI to RNA-DNA hybrids ranging in length from 18 to 36 base pairs (bp) using surface plasmon resonance (BIACore®). The $k_{on}$ and $k_{off}$ values for the binding of the enzyme to the 36-bp substrate were $1.5 \times 10^6$ $\text{m}^{-1}$ $\text{s}^{-1}$ and $3.2 \times 10^{-2}$ $\text{s}^{-1}$, respectively. Similar values were obtained with the shorter substrates. Using uncleavable 2'-O-methylated RNA-DNA substrates, values for $k_{on}$ and $k_{off}$ were $2.1 \times 10^6$ $\text{m}^{-1}$ $\text{s}^{-1}$ and $1.3 \times 10^{-1}$ $\text{s}^{-1}$ in the absence of Mg$^{2+}$ that were further reduced in the presence of Mg$^{2+}$ to 7.4 $\times 10^2$ $\text{m}^{-1}$ $\text{s}^{-1}$ and 2.6 $\times 10^{-2}$ $\text{s}^{-1}$. Kinetic parameters similar to the wild-type enzyme were obtained using an active-site mutant enzyme, Asp$^{184}$ replaced by Ala, whereas a greatly reduced on-rate was observed for another inactive mutant enzyme, in which the basic protrusion is eliminated, thereby distinguishing between poor catalysis and inability to bind to the substrate. Stoichiometric analyses of RNase HI binding to substrates of 18, 24, 30, and 36 bp are consistent with previous reports suggesting that RNase HI binds to 9–10 bp of RNA-DNA hybrid.

Escherichia coli ribonuclease HI (RNase HI) degrades only the RNA strand of an RNA-DNA hybrid (1) and is composed of a single polypeptide chain of 155 amino acid residues (2). It requires divalent cations such as Mg$^{2+}$ or Mn$^{2+}$ for activity (1). The involvement of the amino acid residues Asp$^{10}$, Glu$^{48}$, Asp$^{70}$ (3), His$^{124}$ (4), and Asp$^{184}$ (5) in the catalytic function was established by site-directed mutagenesis for the catalytic function of the enzyme. Two alternative mechanisms have been proposed: one is a two-metal ion mechanism (6) and the other is a carboxyl-hydroxyl relay mechanism (4, 7–9).

X-ray crystallographic analyses of E. coli RNase HI (6, 10, 11) and the RNase H domain of HIV-1 reverse transcriptase (12) showed that these RNases H have a similar structural topology, with the exception of the presence of a handle (6) or basic protrusion region (11) in E. coli RNase HI. The importance of this region for substrate binding has been demonstrated in several studies. The RNase HI domain isolated from HIV-1 reverse transcriptase is enzymatically inactive (13, 14), whereas that from murine leukemia virus reverse transcriptase, which has a part of this region, is active (15, 16). Site-directed mutagenesis indicated that the positively charged residues in this region are important for substrate binding (17). Incorporation of the basic protrusion of E. coli RNase HI at the equivalent position of the RNase H domain of HIV-1 reverse transcriptase resulted in the production of the active HIV-1 RNase H domain (18, 19). In addition, Cys$^{18}$, Asn$^{16}$, Asn$^{44}$, Asn$^{15}$ (7), and Thr$^{43}$ (17) have been shown to be important for substrate binding in E. coli RNase HI. Thus, it seems likely that all amino acid residues that are involved in catalytic function and substrate binding have been identified.

However, it is not fully understood how RNase H binds to its substrate. A kinetic study using synthetic nucleosides with modifications of their 2'-hydroxyl groups revealed the importance of the 2'-hydroxyl group of the nucleoside on the 3'-side of the cleaved phosphodiester and that of the second nucleoside 5'- to the cleaved phosphodiester for hydrogen bonding (20). Models for the binding of the enzyme to an RNA-DNA hybrid have been proposed based upon computer docking of the structure of E. coli RNase HI (free from its substrate) with an RNA-DNA hybrid whose structure was assumed to be an A form (6, 11), was found by NMR to be an A form (7), or that was neither A nor B (21). In these models, the RNA strand upstream of the cleavage site interacts with the enzyme. None of these models assumes that either the enzyme or the substrate alters its conformation upon binding.

Recently, kinetic analyses using RNA-DNA hybrids, under conditions in which the hybrid was cleaved at a unique site (22), suggest that DNA residues complementary to the RNA residues located six or seven residues upstream of the cleavage site interact with the basic protrusion region of the enzyme. Such an interaction seems to require a conformational change in the enzyme or substrate, or in both. Determination of kinetic parameters and stoichiometry of RNase HI molecules bound to substrates of various lengths would provide additional information about the binding of enzyme to substrate.

Magnesium ions may also modulate protein-nucleic acid interactions and participate in catalysis. For example, the binding of Saccharomyces cerevisiae RNase HI to double-stranded RNA is influenced by Mg$^{2+}$ concentration, with tight binding being detected at low concentrations and little binding in the presence of 5 mM Mg$^{2+}$ (23), and the DNA binding specificity of EcoRV is increased in the presence of Mg$^{2+}$ (24). Therefore, it is of great interest to investigate the influence of magnesium ions on binding of RNase HI to RNA-DNA substrates.

In this study, we have analyzed the interaction between E. coli RNase HI and RNA-DNA hybrids using the BIACore®

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‡ The abbreviations used are: HIV, human immunodeficiency virus; RU, resonance unit; NTA, nickel nitritriacetic acid; bp, base pair(s).
system, an instrument based on surface plasmon resonance technology. This technology is useful for obtaining kinetic data on the interaction between enzyme and substrate, particularly when the enzyme is inactive. This system enabled us to distinguish between inability to bind the substrate and inability to cleave. Furthermore, utilization of a 2′-O-methylated substrate permitted analysis of the effect of the Mg\(^{2+}\) ions on substrate binding.

**MATERIALS AND METHODS**

**Instrumentation and Reagents**—The BIAcore instrument was manufactured by Pharmacia Biosensor AB (Uppsala, Sweden). Sensor chips CM5, Tween P20, and the amine coupling kit containing (25). Poly(rA) obtained from Pharmacia. Annealing was performed by mixing 100 cells of *E. coli* gal was obtained as described (16, 23). The enzyme with an N-terminal 6-His-Tag (6-His-RNase HI) wild-type protein (28) and the mutant protein RNase HI—E. coli was amplified by polymerase chain reaction using rnhA site-containing 3′-13 His-Tag (6 His-RNase HI) were obtained from Integrated DNA Technologies. All concentrations are underlined. Polymerase chain reaction was performed in 25 cycles defining 5′-amino group (Fig. 1) were obtained from Integrated DNA Technologies and Oligos, Etc. DNA oligonucleotides (Fig. 1) were obtained from Integrated DNA Technologies. All concentrations are expressed as moles of molecules and not bp. RNA and DNA oligonucleotides (1 μM) were annealed in HBS buffer by heating for 2 min and allowed to cool to room temperature to form RNA-DNA hybrids.

**Preparation of DNA-Hybrids**—The RNA oligonucleotides biotinylated on the 5′-amino group (Fig. 1) were obtained from Integrated DNA Technologies and Oligos, Etc. DNA oligonucleotides (Fig. 1) were obtained from Integrated DNA Technologies. All concentrations are expressed as moles of molecules and not bp. RNA and DNA oligonucleotides (1 μM) were annealed in HBS buffer by heating for 2 min and allowed to cool to room temperature to form RNA-DNA hybrids.

**Construction and Purification of Wild-type and Mutant RNase HI—** E. coli RNase HI wild-type protein (28) and the mutant protein D134A, in which Asp 134 is replaced by Ala (5), were prepared as described (16, 23).

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**Construction and Purification of Wild-type and Mutant RNase HI—** E. coli RNase HI wild-type protein (28) and the mutant protein D134A, in which Asp 134 is replaced by Ala (5), were prepared as described (16, 23). A group of plasmids for overproduction of the RNase HI variants with the BIAcore instrument was man-...
RESULTS

Enzymes Examined Using Plasmon Resonance—Three versions of E. coli RNase HI were examined by plasmon resonance studies as follows: wild-type enzyme, a mutant (D134A) with very low RNase H activity, and a protein whose handle or basic protrusion had been removed, presumably altering its ability to bind to RNA-DNA hybrids. This latter protein is described here for the first time.

Preparation of RNase HI Variant Missing the Basic Protrusion—By studying the binding kinetics of enzymes one can distinguish whether the lower activity of the mutant form of a protein is due to poor binding to the substrate or to a defect in catalytic activity. Previously, it has been shown that the activity of a protein derived from the RNase H domain of HIV-1 reverse transcriptase can be overcome by inserting handle region of E. coli RNase HI into the corresponding position of the HIV-1 RNase H protein. These results support the role of the handle in binding to the RNA-DNA substrate (18, 19). To further substantiate this function of the handle region, we made a deletion of amino acid residues 83–100 and replaced it with four randomly generated amino acid residues. It has previously been shown that E. coli strain MIC3001, with the rnhA-339:cat and recB270(TS) mutation, can effectively screen for genes encoding a functional RNase H molecule (35). However, no clone was obtained after deletion-substitution of the handle region of E. coli RNase HI that could support growth of MIC3001 at the restrictive temperature, suggesting that the basic protrusion of E. coli RNase HI is critical for enzyme function in vivo. We screened the clones for an RNase HI variant that can be overproduced in a soluble form. When 22 E. coli HB101 transformants bearing pJAL600 derivatives were analyzed by SDS-polyacrylamide gel electrophoresis for overproduction, only one was able to produce soluble RNase HI in amounts suitable for subsequent purification (data not shown). Plasmid DNA was isolated from this transformant, and the DNA sequence of the mutated rnhA gene was determined. The deduced amino acid sequence connecting Ile82 and Val101 was Arg-Thr-Asn-Ser. This mutant protein, BP-RNase HI, was recovered in a soluble form after sonication lysis, and the lysate was subjected to DE52 chromatography using a column equilibrated with 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA. The protein was eluted from the column by linearly increasing the NaCl concentration from 0 to 0.5 M. Fractions containing the protein were combined and applied to a Sephacryl-S300 (Pharmacia) (2.2 × 90 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 0.1 M NaCl. Fractions containing the pure protein were combined and used for further analyses. The amount of the mutant protein BP-RNase HI purified from a 1-liter culture was about 0.64 mg.

Characterization of BP-RNase HI—No enzymatic activity was detected for the mutant protein ABP-RNase HI either in the presence of the Mg2+ or Mn2+ ion, indicating that this mutant is completely inactive. CD spectra of the wild-type protein and of the mutant protein ABP-RNase HI are shown in Fig. 2. Few spectral changes were detected between these proteins in the far ultraviolet region, in which the spectra reflect the content of the secondary structure of the protein (Fig. 2a). This result suggests that the mutant protein ABP-RNase HI folds correctly. On the other hand, in the near ultraviolet region, in which the spectra reveal environments of aromatic amino acid residues, the spectra were clearly distinguishable (Fig. 2b). This change in the spectrum may be due to a local conformational change upon the deletion of the basic protrusion and/or to the loss of two tryptophan residues.

Determination of Kinetic Parameters—For the binding of RNase HI molecules to the 36-bp RNA-DNA hybrid, TBS containing RNase HI at concentrations ranging from 10 nM to 1.5 μM were passed, at a flow rate of 5 μl/min, over the surface of the sensor chip, on which the substrate had been immobilized. The slope of the ln(RU)/RU versus RU plots in each sensogram was linear (data not shown), indicating that dissociation of the enzyme from the substrate occurs in an apparently first-order reaction. The sensograms are shown in Fig. 3. When the RNase HI molecules bound to the sensor chip were dissociated by running the RNase-free buffer at a flow rate of 5 μl/min, the dissociation rate constants (koff) decreased as the initial amount of the RNase HI molecules bound to the sensor chip decreased. This change is probably due to rebinding of the protein to the RNA-DNA hybrid on the sensor chip when the hybrid is present at high densities on the surface. Increasing the flow rate to 100 μl/min increased the dissociation rate constant nearly 5-fold, but the koff values were still dependent on the initial number of RNase HI molecules bound to the sensor chip (Fig. 4). Addition of the poly(rA)·poly(dT) competing substrate to the buffer for dissociation at 39 μg/ml resulted in a 10–20-fold increase in the dissociation rate constant (Fig. 4). The koff values were nearly constant when the initial amount of the protein bound to the sensor chip increased, indicating that the competition completely abolishes the rebinding. In the presence of this competing substrate, binding of RNase HI to the immobilized hybrid was completely inhibited (data not shown).

k_on values were calculated using Equation 1. Association profiles fit satisfactorily with a mono-exponential equation for protein concentrations up to 100 nM. k_on versus C plots for 10–80 nM protein concentrations were linear (Fig. 5), indicating that, unlike the k_off values, the k_on values are not affected by RNase H concentrations in the binding buffer.

Similar sensograms were obtained when the 30-, 24-, or 18-bp RNA-DNA hybrids were used as immobilized substrates (data not shown). Kinetic parameters of the interaction be-
**Kinetic Parameters of E. coli RNase HI**

**Fig. 3. Sensorgram of the association phase and dissociation phase for binding of E. coli RNase HI to the 36-bp RNA-DNA hybrid.** RNase HI (10, 20, 40, and 100 nM from lower trace) was injected onto the surface, on which 119 RU of the hybrid had been immobilized, at a flow rate of 5 μl/min. The dissociation was started by injecting buffer lacking RNase HI as indicated by the arrow. This sensorgram is a representative of BiACore™ measurements of the interaction between E. coli RNase HI and its substrate.

**Fig. 4. Dissociation rates of E. coli RNase HI from the immobilized substrate.** Values of $k_{off}$ are plotted versus the concentration of E. coli RNase HI. Enzyme was injected at a flow rate of 5 μl/min onto a surface on which 58 RU of the 36-bp RNA-DNA hybrid had been immobilized. Dissociation was observed upon injection of buffer at a flow rate of 5 μl/min (open squares), or buffer containing 39 μg/ml poly(rA)-poly(dT) at a flow rate of 5 μl/min (closed circles). The KINJECtCommand, which causes switching of injection loops, was used for the latter two cases.

**Fig. 5. Representative plot for determination of the association constant for E. coli RNase HI interaction with the 36-bp RNA-DNA hybrid.** RNase HI (10, 20, 40, 60, and 80 nM) was injected at a flow rate of 5 μl/min onto a surface on which 58 RU of hybrid had been immobilized. $k_{on}$ values are plotted versus the concentration of E. coli RNase HI. The slope of the curve obtained by linear transformation gives the $k_{on}$ value.

Between RNase HI and these substrates were similar with, at most, a 2-fold variation between them (Table I).

**Biphasic RNase HI Binding to RNA-DNA Hybrids—**When the concentration of RNase HI in the buffer was greater than 0.1 μM, RU values showed a slow increase followed by a fast increase, never reaching a plateau (Fig. 6). This suggests that two binding phases exist, a slow phase and a fast phase. The RU$_{eq}$/C versus RU$_{eq}$ plots for the fast binding phase were obtained over the range of 25 nM to 1.5 μM by using the end points of the fast binding mode as the RU$_{eq}$ values (Fig. 7). These plots were biphasic with two different kinetic association constants, $K_a$ values derived from linearization of the plots obtained at low RNase HI concentrations ranged from 25 to 50 nM ($K_{a1}$) and at higher RNase HI concentrations from 0.4 to 1.5 μM ($K_{a2}$) (Table I). At higher RNase HI concentrations, $K_{a2}$ values were approximately one-fourth to one-seventh those calculated from data collected at lower RNase HI concentrations.

**Stoichiometry of RNase HI Bound to RNA-DNA Hybrids—**An increase in RU values upon binding of analyte on the surface of the chip is proportional to the mass of analyte. Therefore, using the relationship $\Delta RU_{proteinate} = 0.8 \times \Delta RU_{protein}$ (36), one can calculate the ratio of RNase HI and RNA-DNA hybrid from the equation $R = \Delta RU_{RNase HI}/\Delta RU_{hybrid} \times MW_{hybrid}/MW_{RNase HI}$

$\times 0.8$; where $\Delta RU_{hybrid}$ is the increase in RU value upon binding of the hybrid to the streptavidin surface, $\Delta RU_{RNase HI}$ is the increase in RU value upon binding of RNase HI to the hybrid, and $MW_{hybrid}$ and $MW_{RNase HI}$ are the molecular weights of the hybrid and RNase HI, respectively. The RU$_{max}$ values used for the determination of stoichiometry were obtained from the $x$ intercepts of the RU$_{eq}$/C versus RU$_{eq}$ plots. The stoichiometry obtained by linear transformation of the plots at lower RNase HI concentrations (25–50 nM ($n_1$)) was 1.19 ± 0.07, 1.34 ± 0.10, 2.17 ± 0.04, and 3.18 ± 0.05 for the 18-, 24-, 30-, and 36-bp RNA-DNA hybrids, respectively. The values $n_{total} - n_1$ ($n_2$) were 0.37, 0.38, 0.45, and 0.92 for 18-, 24-, 30-, and 36-bp RNA-DNA hybrids, respectively. $n_{total}$ values for the 18-, 24-, 30-, and 36-bp RNA-DNA hybrid, respectively.

**Binding of RNA-DNA Hybrid to Immobilized 6×His-RNase HI—**Kinetic measurements were also carried out using the reverse experimental system, in which RNase HI molecules are immobilized on the surface. A histidine tag fused to the N terminus of RNase HI allows the protein to be immobilized on the chip in a unique homogeneous orientation. This enables one to analyze the interactions between a single RNase HI molecule and its RNA-DNA hybrid substrate. For binding of the 36-bp RNA-DNA hybrid to RNase HI, TBS buffer (without EDTA) containing various concentrations (from 50 nM to 1.5 μM) of the 36-bp RNA-DNA hybrid were injected onto the surface of the chip, on which RNase HI had been immobilized. Immediately following injection of the hybrid, RNase HI (1 μM) was injected as a competitor to avoid rebinding of the hybrid to other RNase HI molecules on the surface of the sensor chip. Binding of the 36-bp hybrid to immobilized RNase HI gave sensorgrams similar to those obtained for the binding of RNase HI to the immobilized hybrid (Fig. 8a). The RU versus RUC plot was comprised of a dominant binding phase with high affinity and an additional binding phase with low affinity (Fig. 8b), similar to those obtained with immobilized substrate. Kinetic parameters were also similar to those determined when...
Kinetic Parameters of E. coli RNase HI

The dissociation rate constant ($k_{\text{off}}$) and association rate constant ($k_{\text{on}}$) were calculated using BIAlogue software (Pharmacia Biosensor). The $k_{\text{on}}$ values were determined from $k_{\text{on}}$ versus C plot for the concentrations of RNase HI from 25 to 60 nm. The association constants were calculated from the ratio $k_{\text{on}}/k_{\text{off}}$ ($K_{A}$) or were determined by measuring equilibrium sensor responses and subjecting them to RU versus RU/C plot analysis ($K_{Aeq}^1$). $K_{Aeq}^1$ and $K_{Aeq}^2$ values were determined from the plots for lower RNase HI concentrations from 0.4 to 1.5 μM, respectively. $k_{\text{on}}$ is the average and S.D. of measurements from the dissociation phase from three cycles where 25, 30, and 35 nm RNase HI were injected at 5 μM/min at 25 °C in TBS.

| Hybrid | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_{A}$ | $K_{Aeq}^1$ | $K_{Aeq}^2$ |
|--------|----------------|----------------|--------|-----------|-----------|
| 36 bp  | 3.16 ± 0.07    | 1.47 ± 0.11    | 4.65   | 4.57 ± 1.35 | 1.23 ± 0.16 |
| 30 bp  | 3.13 ± 0.08    | 1.72 ± 0.11    | 5.50   | 6.08 ± 0.29 | 1.08 ± 0.38 |
| 24 bp  | 3.10 ± 0.26    | 1.57 ± 0.20    | 5.06   | 5.54 ± 2.34 | 0.804 ± 0.093 |
| 18 bp  | 4.61 ± 0.35    | 1.15           | 2.49   | 4.84 ± 0.62 | 0.983 ± 0.087 |

* $k_{\text{on}}$ and $K_{Aeq}^1$ are the average of three experiments with S.D. $K_{Aeq}^2$ is the average of four experiments with S.D.

* $k_{\text{on}}$, $K_{Aeq}^1$, and $K_{Aeq}^2$ are the average of two experiments with S.D.

* $k_{\text{on}}$ is from single experiment. $K_{Aeq}^1$ and $K_{Aeq}^2$ are the average of two experiments with S.D.

FIG. 6. Sensorgrams showing binding of E. coli RNase HI at high concentrations. Sensorgrams of the interaction between E. coli RNase HI at 0.1, 0.2, 0.4, 0.5, 1.0, and 1.5 μM (from the lower trace) and the 36-bp RNA-DNA hybrid (58 RU) are shown. The end point of the fast binding mode (A) and the starting point of dissociation (B) are indicated by arrows. The RU values at A were used for equilibrium analysis.

FIG. 7. Plot for the determination of equilibrium association constants $K_{A}$ using equilibrium sensor responses (RU$_{eq}^1$). The RU values at A in Fig. 6 were used as RU$_{eq}^1$ values. The RU$_{eq}^1$/C values were plotted versus RU$_{eq}^1$ values for the binding of RNase HI (25 nm to 1.5 μM) to immobilized 36-bp RNA-DNA hybrid. Linear transformation of the plot over the lower range of concentrations (25–50 nm) and higher range of concentrations (0.4–1.5 μM), indicated by a solid line and a broken line, respectively, gives association constants $K_{Aeq}^1$ and $K_{Aeq}^2$, respectively.

the substrate was immobilized (Table II).

Effect of 2-O-methylation of RNA on Binding of RNase HI to RNA-DNA Hybrids—When binding of RNase HI to the 36-bp 2′-O-methylated substrate was analyzed by BIAcore®, a rapid increase of the RU value was observed, which was followed by a slow increase in RU value (Fig. 9a). Such biphasic binding is similar to that observed for the binding to unmodified substrate. However, the slow phase observed in binding to the modified substrate was more pronounced than that observed for the normal substrate. For the rapid binding phase, the $k_{\text{on}}$ value for the modified substrate was one-seventh of that for the unmodified substrate, whereas the $k_{\text{on}}$ value for the modified substrate was 4-fold higher than that for the unmodified substrate (Table III). These results indicate that 2-O-methylation of the substrate considerably impairs interaction between enzyme and substrate. When the RU$_{eq}^1$/C values were plotted versus RU$_{eq}^1$ over a range of 0.1–1.5 μM for the rapid binding phase, a linear relationship was observed. This indicates that

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TABLE I

Kinetic and association constants for the binding of E. coli RNase HI to immobilized RNA-DNA hybrids

| Hybrid | $k_{\text{on}}$ | $k_{\text{off}}$ | $K_{A}$ | $K_{Aeq}^1$ | $K_{Aeq}^2$ |
|--------|----------------|----------------|--------|-----------|-----------|
| 36 bp  | 3.16 ± 0.07    | 1.47 ± 0.11    | 4.65   | 4.57 ± 1.35 | 1.23 ± 0.16 |
| 30 bp  | 3.13 ± 0.08    | 1.72 ± 0.11    | 5.50   | 6.08 ± 0.29 | 1.08 ± 0.38 |
| 24 bp  | 3.10 ± 0.26    | 1.57 ± 0.20    | 5.06   | 5.54 ± 2.34 | 0.804 ± 0.093 |
| 18 bp  | 4.61 ± 0.35    | 1.15           | 2.49   | 4.84 ± 0.62 | 0.983 ± 0.087 |

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Kinetic Parameters of E. coli RNase HI

Table II

| Interaction                  | Interaction constants | Kinetic values |
|------------------------------|-----------------------|----------------|
|                              | $k_{on}$ | $k_{off}$ | $K_a$ |
| Wild type $\rightarrow$ hybrid$^a$ | $3.16 \pm 0.07$ | $1.47 \pm 0.11$ | $4.65$ |
| Hybrid $\rightarrow$ 6xHis-RNase HI$^b$ | $2.53 \pm 0.17$ | $0.83$ | $3.27$ |

$^a$ Data from Table I. $^b$ $k_{eq}$ is the average and S.D. of measurements from the dissociation phase from three cycles where 0.1, 0.25, or 0.5 μM of the 36-bp hybrid were injected at 5 μl/min at 25 °C in TBS. $k_{on}$ and $k_{off}$ were determined from $k_{on}$ versus C plots and RU versus RU/C plots from a single experiment, respectively, for the concentrations of 0.1, 0.25, and 0.5 μM 36-bp hybrid.

RU$_{eq}$ plots. The number of the RNase HI molecules bound to the modified substrate in the presence of 10 mM MgCl$_2$ was estimated from the increase in the RU value at 1.5 μM to be 2.9 ± 0.11.

Effect of Asp$^{134}$ to Ala Mutation on the Binding of RNase HI to an RNA-DNA Hybrid—Kinetic parameters for binding of the mutant protein D134A to the 36-bp unmodified substrate are shown in Table IV. The $k_{on}$ value was similar to that obtained for the wild-type enzyme, whereas the $k_{off}$ value was about half that of the wild-type enzyme. Consequently, the $K_a$ value for the mutant protein D134A is slightly higher than that of the wild-type protein. This result indicates that the Asp$^{134}$ to Ala mutation does not seriously affect the binding of RNase HI to the substrate. Kinetic analysis of the enzymatic activity has shown that the Asp$^{134}$ to Glu, Gln, Ser, and Thr mutations, which greatly reduce the enzymatic activity, do not seriously affect the $K_m$ value (5). However, this finding could not completely exclude the possibility that the Asp$^{134}$ to Ala mutation almost fully inactivates the enzyme by reducing the binding affinity of the enzyme to the substrate, because no kinetic data were available for the mutant protein D134A. The current study excludes such a possibility.

Binding of ΔBP-RNase HI to RNA-DNA Hybrid—When the binding of ΔBP-RNase HI (9.2 μM) to the 36-bp RNA-DNA hybrid was analyzed by BIAcore$^{20}$, a slight increase in RU value was observed, which corresponds to that observed for 2.5 mM wild-type protein (Fig. 11). From the increase of the RU level, the affinity of the ΔBP-RNase HI for the 36-bp RNA-DNA hybrid was estimated to be 0.025% that of the wild-type protein for the same substrate. This result suggests that the basic protrusion is the major contributor to the interaction between enzyme and substrate.

**DISCUSSION**

Nature of Binding Properties of RNase HI to RNA-DNA Hybrid—In this study, association and dissociation of RNase HI with RNA-DNA hybrid substrates were monitored in real time using the BIAcore$^{20}$ system. Kinetic parameters for binding of RNase HI to hybrids were constant for concentrations up to 0.1 μM. When RNaseHI was injected at this concentration, we calculated that 0.9 and 2.8 RNase HI molecules were bound to the 18- and 36-bp hybrids, respectively. Similar kinetic parameters were also obtained for binding of the 36-bp hybrid to immobilized RNase HI which binds only a single substrate molecule. These results suggest that multiple RNase HI molecules bind to the substrate simultaneously and independently with little or no cooperativity. $K_a$ values obtained in this study ($2 \times 10^{-8} \text{M}$) agree well with those obtained from the titration of RNA-DNA hybrids with RNase HI using data collected from changes in CD spectra upon binding to the substrate ($10^{-8} - 10^{-9} \text{M}$) (37).

We observed two phases of binding (with fast and slow rates) when the concentration of RNase HI was greater than 0.1 μM (Fig. 6). Of the total protein bound to the substrate at 1.5 μM, −10% bound at a slower “on” rate and 90% bound at a faster
Kinetic Parameters of E. coli RNase HI

TABLE III
Kinetic and association constants for the binding of E. coli RNase HI to an immobilized 36-bp 2′-O-methylated RNA-DNA hybrid

| Substrate | $k_{\text{on}} \text{ s}^{-1}$ | $K_a \text{ M}^{-1}$ | $K_{\text{eq}} \text{ M}$ |
|-----------|-----------------|-----------------|-----------------|
| Unmodified | $3.16 \pm 0.07 \times 10^{-2}$ | $1.47 \pm 0.11 \times 10^6$ | $4.65 \times 10^7$ |
| Methylated (−Mg$^+$) | $1.31 \pm 0.08 \times 10^{-1}$ | $2.09 \pm 0.19 \times 10^7$ | $1.60 \times 10^8$ |
| Methylated (+Mg$^+$) | $2.60 \pm 0.18 \times 10^{-2}$ | $7.39 \pm 0.73 \times 10^6$ | $2.84 \times 10^5$ |

$^a$ Data from Table I.  
$^b$ $k_{\text{on}}$ is the average and S.D. of measurements from the dissociation phase from three cycles, where 0.3, 0.4, or 0.5 μM RNase HI were injected at 5 μL/min at 25 °C in TBS. $K_a$ and $K_{\text{eq}}$ are the average of three experiments with S.D.  
$^c$ $k_{\text{on}}$ is the average and S.D. of measurements from the dissociation phase from three cycles where 0.5, 0.75, or 1.0 μM RNase HI were injected at 5 μL/min at 25 °C in TBS containing 10 mM MgCl$_2$. $K_{\text{on}}$ values are the average of three experiments with S.D.  
$^d$ Not determined.

FIG. 10. Binding of E. coli RNase HI to 2′-O-methylated RNA-DNA hybrid in the presence of Mg$^+$.

Sensorgrams of the interaction between E. coli RNase HI at 0.3, 0.4, 0.5, 0.75, 1.0, and 1.5 μM (from the lower trace) and 2′-O-methylated 36-bp RNA-DNA hybrid (81 RU) in the presence of 10 mM MgCl$_2$ are shown. RNase HI was injected onto the surface at a flow rate of 5 μL/min.

TABLE IV
Comparison of kinetic and association constants for the binding of wild-type and D134A mutant E. coli RNase HI to immobilized 36-bp RNA-DNA hybrid

| Enzyme | $k_{\text{eff}} \text{ s}^{-1}$ | $k_{\text{on}} \text{ s}^{-1}$ | $K_a \text{ M}^{-1}$ | $K_{\text{eq}} \text{ M}$ |
|--------|-----------------|-----------------|-----------------|-----------------|
| Wild-type$^a$ | $3.16 \pm 0.07$ | $1.47 \pm 0.11$ | $4.65 \pm 0.135$ | $4.57 \pm 1.35$ |
| D134A$^b$ | $1.67 \pm 0.14$ | $1.20 \pm 0.04$ | $7.19 \pm 1.76$ | $7.16 \pm 1.76$ |

$^a$ Data from Table I.  
$^b$ $k_{\text{eff}}$ is the average and S.D. of measurements from the dissociation phase from three cycles, where 25, 30, or 35 mM RNase HI were injected at 5 μL/min at 25 °C in TBS, $k_{\text{on}}$ is the average of two experiments with range. $K_{\text{eq}}$ is from a single experiment.

rate. The phase with the faster on rate consisted of two parts, 70% with high and 20% low association constants, as indicated by the biphasic RU versus RUC plot (Fig. 5). It is likely that the binding with the higher association constant is predominant under physiological conditions under which the concentration of RNase HI is expected to be low. Therefore, it also seems likely that binding with higher affinity is productive binding, with additional, nonspecific binding. One explanation for the secondary interactions is that free RNase HI may interact with RNase HI molecules already bound to the substrate. However, multiphasic binding was also observed when RNase HI was immobilized, and the 36-bp RNA-DNA hybrid was in the mobile phase. Taken together, these results suggest an additional weaker interaction between RNase HI and the substrate that is separate from the normal binding site.

Length of the RNA-DNA Hybrid Interacts with RNase HI—The numbers of RNase HI molecules bound to the hybrids with high association constants are ~1.2 for the 18-bp hybrid, ~1.3 for the 24-bp hybrid, ~2.2 for the 30-bp hybrid, and ~3.2 for the 36-bp hybrid. Kinetic analyses, under conditions in which the RNA-DNA hybrid was cleaved at a unique site (22), or using RNA-DNA hybrids with 2′-O-methyl nucleosides, which limits the cleavage at a single site (38), suggest that RNase HI interacts with 9–10 bp of RNA-DNA hybrid. Using these values, one can expect the maximum number of RNase HI molecules able to bind to the 18- or 24-, 30-, or 36-bp hybrid to be 1–2, 2, 3, and 3–4, respectively. However, to completely saturate these relatively short RNA-DNA hybrids, each RNase HI molecule(s) would need to bind in such a manner as to permit the maximum number of other RNase HI molecules to bind. For example, if one RNase HI molecule were to bind to the middle of the 18-bp RNA-DNA hybrid, there would be only 4 bp at either end of the hybrid accessible to other RNase HI molecules. Thus, the observed values will always be lower than the maximum values. Therefore, our current results are consistent with a 9–10-bp binding size (22, 38).

Effect of 2′-O-Methylation of RNA in Hybrid on Binding of RNase HI—It has been reported that the conformations of 2′-O-methyl RNA-DNA hybrids are similar to those of normal RNA-DNA hybrids (39). Therefore, a 2′-O-methyl RNA-DNA hybrid can provide a good model with which to investigate the interaction between RNase HI and the substrate. Our data show that the association constant between RNase HI and the 2′-O-methylated substrate was one-thirtieth that between enzyme and the substrate. Methylation of 2′-OH group at the cleavage site, which greatly reduces catalytic efficiency, also slightly reduces the affinity between the enzyme and the substrate (a 5-fold difference in the $K_m$ value), possibly due to steric hindrance (9). It is reported that the 2′-OH group of the nucleoside adjacent to the 3′-side of the cleaved phosphodiester bond acts as a proton donor and acceptor and that the second nucleoside from the 5′-side of the cleaved phosphodiester bond acts as a proton acceptor (20). Therefore, the reduced affinity
consequent to 2′-O-methylation may be due to steric hindrance by the methyl groups and/or loss of the hydrogen bonds. These results support an interaction of 2′-OH group and RNAse HI. However, the current results cannot exclude the possibility that methylation of the 2′-OH group of the hybrid creates a substrate that binds to the enzyme in a different manner (e.g. in the opposite orientation or with different spacing).

**Effect of Magnesium Ions on the Binding of RNAse HI to a 2′-O-Methyl RNA-DNA Hybrid**—The presence of Mg$^{2+}$ may affect the RNAse HI/RNA-DNA interaction in two ways as follows: first, Mg$^{2+}$ may neutralize the charge of the phosphates and decrease the ionic interaction between the basic protrusion and the RNA-DNA hybrid; and second, Mg$^{2+}$ binds to the acidic amino acid residues at the catalytic center of the enzyme, a process necessary for cleavage. We find that, in the presence of Mg$^{2+}$, the affinity between RNAse HI and 2′-O-methyl RNA-DNA hybrid was reduced by 83% (Fig. 9 and Table III). A large reduction in affinity (3–4 orders of magnitude) in the presence of Mg$^{2+}$ has been reported for the nonspecific interaction between EcoRV and DNA (24), a reduction that was interpreted as due to the displacement by the enzyme of Mg$^{2+}$-bound to DNA. Using ΔBP-RNase HI, a protein lacking the basic protrusion, we found a drastic reduction in substrate binding (Fig. 11). Therefore, we believe the reduction in affinity in the presence of Mg$^{2+}$ results from the displacement of Mg$^{2+}$-bound to the RNA-DNA hybrid by the basic protrusion. In contrast, the specific interaction between EcoRV and DNA is not affected by Mg$^{2+}$ ions (24). Thus, the effect of the Mg$^{2+}$ ions seems to depend on its mode of interaction. Such a difference in the effect of the Mg$^{2+}$ ions could account for a moderate reduction in affinity of RNAse HI for its substrate as compared with that of EcoRV for DNA. However, a 20% reduction in the “off” rate counteracted, in part, this reduction in affinity. In the absence of the Mg$^{2+}$ ion, electrostatic repulsion between the RNA-DNA hybrid and the acidic amino acid residues in the catalytic center of RNAse HI (Asp$^{10}$, Glu$^{48}$, Asp$^{70}$, and Asp$^{124}$) would be expected to decrease affinity. The catalytic Mg$^{2+}$ ion would eliminate the negative charge repulsion at the active site of the enzyme, resulting in less release of the enzyme from the substrate.

**Enzymatic Activity and Tertiary Structure of ΔBP-RNase HI**—Keck and Marquese (40) have recently shown that the basic protrusion of E. coli RNAse HI is not essential for activity. They reported that the mutant protein, in which the amino acid residues 83–95 are replaced by six glycine residues, exhibited RNAse HI activity in the presence of the Mn$^{2+}$ ion but not in the presence of the Mg$^{2+}$ ion. In contrast, we showed that ΔBP-RNase HI, in which residues 83–100 are replaced by Arg-Thr-Asn-Ser, exhibited little RNAse H activity either in the presence of the Mg$^{2+}$ or Mn$^{2+}$ ion. This apparent discrepancy in the enzymatic activity of the mutant protein, which lacks the basic protrusion, may result from the difference in the size of the deleted region or in the flexibility of the linker which is substituted for the basic protrusion.

In this report, we discussed the importance of the basic protrusion for the interaction with the substrate, on an assumption that ΔBP-RNase HI has basically the same folding topology as that of the wild-type protein. The possibility that the deletion of the basic protrusion causes a gross structural change may not be excluded, because ΔBP-RNase HI has little enzymatic activity. However, the similarity in the far UV CD spectra between ΔBP-RNase HI and the wild-type protein and the fact that both the RNAse H domain of HIV-1 reverse transcriptase and the e. coli RNAse HI variant with the deletion of the basic protrusion (40) correctly suggest that ΔBP-RNase HI also folds correctly.

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