The Putative Substrate Recognition Loop of Escherichia coli Ribonuclease H Is Not Essential for Activity*

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The RNase H family of enzymes catalyzes the hydrolysis of RNA from RNA-DNA hybrids in a divalent metal-dependent fashion. To date, structure/function studies have focused on two members of this family: Escherichia coli RNase H1, a small monomeric protein; and human immunodeficiency virus, type I (HIV) RNase H, a domain of HIV reverse transcriptase. The isolated RNase H domain from HIV reverse transcriptase can be expressed independently and shares significant structural homology with its E. coli homologue; however, unlike the bacterial protein, it is inactive. The most notable difference between the inactive domain from HIV and the active E. coli protein is a basic helix/loop sequence, present in E. coli but absent from the HIV homologue. Substitution of this basic region into the HIV domain partially restores its activity and increases its thermodynamic stability. By deleting the basic helix/loop region, we have modeled the structural difference between these two polypeptides onto the E. coli homologue. Surprisingly, the resulting mutant protein is active in Mn²⁺-dependent fashion. Therefore, the basic helix/loop is not required for RNase H activity.

RNase H, a family of structurally homologous enzymes from both prokaryotes and eukaryotes, selectively hydrolyzes the RNA strand of RNA-DNA hybrids in a divalent cation-dependent fashion. The best characterized members of this family are Escherichia coli RNase H1 and the human immunodeficiency virus, type I (HIV)² RNase H domain (for review, see Ref. 1). E. coli RNase H1 is a small (155 residues) single domain protein. In HIV, however, this essential activity is carried out by a domain of the much larger heterodimeric protein, reverse transcriptase (1, 2). In spite of the structural homology between the two (Fig. 1), the HIV domain is inactive when expressed independently of the rest of reverse transcriptase. The structural basis for this lack of activity is unclear.

A structural comparison of these two homologues reveals two primary differences that may contribute to the inactivity of the isolated retroviral domain. (i) Whereas the E. coli RNase H structure is well ordered throughout the protein, the COOH-terminal region of the HIV RNase H is dynamic and disordered as shown by NMR (3, 4) and crystallographic (5) studies. This region includes a loop with a conserved histidine and the COOH-terminal-most helix (helix E). Mutational studies in both homologues demonstrate the importance of this region for activity (6–10). (ii) Comparison of the sequences and structures of these two homologues also reveals a highly basic helix/loop region that is present in E. coli RNase H but missing from the HIV RNase H (5, 11). Site-directed mutagenesis studies on the bacterial protein have demonstrated that this basic helix/loop region is important for the Mg²⁺-dependent activity of the enzyme (12): neutralizing mutations generally result in an increase in Michaelis constant (Kₘ). This observation has led to the belief that this region is important for substrate binding and that the inactivity of the isolated HIV RNase H domain is due to poor substrate recognition (5, 12). Indeed, on transplanation of this region (residues 79 to 102) into the isolated domain from HIV, a Mn²⁺-dependent RNase H activity is restored (13, 14). In addition to restoring activity, insertion of this E. coli basic helix/loop stabilizes the HIV RNase H domain (14). The molecular basis for this restoration of activity is unclear: has the basic helix/loop improved the substrate-binding affinity of the domain or its stability?

We have created a variant protein that removes the majority of this basic helix/loop from E. coli RNase H* (RNase H* is a cysteine-free version of E. coli RNase H1 (15)). In essence, our deletion mutant models the structural features of HIV RNase H onto the E. coli homologue. This was accomplished by substituting 13 of the 16 residues comprising the basic helix/loop (including 5 basic residues) with a short linker of 6 glycines. The resulting variant protein folds and maintains a Mn²⁺-dependent RNase H activity. Hence, the putative substrate-binding loop of E. coli RNase H1 is not essential for activity. Comparisons of the metal requirements for RNase H activity have revealed an unexpected and complex Mn²⁺-dependent activity. These activity studies help to determine whether this putative substrate recognition loop is essential for RNase H activity, and they further our understanding of the basis for inactivity in HIV RNase H.

EXPERIMENTAL PROCEDURES

Materials—All buffer components were from Sigma unless otherwise specified: ribonucleotides (Boehringer Mannheim); acetylated bovine serum albumin (BSA) (U. S. Biochemical Corp.); and heparin-Sepharose (Pharmacia Biotech Inc.). Restriction endonucleases and T4 ligase (New England Biolabs, Beverly, MA) were used as directed by the suppliers’ recommendations. All synthetic oligonucleotides were made on an Applied Biosystems 392 RNA/DNA synthesizer. The HIV RNase H domain was purified as described previously (14). E. coli RNA polymerase was kindly provided by Michael Chamberlin (University of California, Berkeley). Purified E. coli RNase H* (E. coli RNase H1 with alanine replacing the 3 free cysteines) was obtained as a gift from Jonathan Dabora (15).

Creation of pJM101—pJM101, a gift from Jonathan Dabora, was created by cassette mutagenesis of pSM101, a T7 expression plasmid that encodes for E. coli RNase H* (15). pJM101 is the product of ligating the BamHI-BglII fragment of pSM101 with a synthetic cassette of two complementary oligonucleotides with BamHI and BglII ends, following standard cloning protocols (16). The resulting plasmid
(pJ MD101) encodes for a variant of E. coli RNase H* (RNH110) in which residues 83-95 are substituted by 6 glycine residues (Fig. 1; details of plasmid sequence available upon request). This deletion constitutes the majority of the basic helix/loop (residues 84-99) as defined by Kanaya et al. (12). The sequence of pJ MD101 was confirmed by standard sequencing techniques.

Purification of RNH110—E. coli BL21 (DE3) cells transformed with pLysS (Novagen, Madison, WI) and pJMD101 were grown at 37°C in Luria-Bertani medium (16) with 100 µg/ml ampicillin and 25 µg/ml chloramphenicol. Cells at mid-logarithmic phase (A600 = 0.5-0.6) were induced to overexpress RNH110 with 1 mM isopropyl β-D-thiogalactopyranoside and were harvested by centrifugation after 3 h of growth. Cell pellets were resuspended in 50 mM Tris, pH 8.0, 20 mM NaCl, 0.5 mM EDTA and lysed by sonication. RNH110 was found in the insoluble fraction of the sonicate (as determined by polyacrylamide gel electrophoresis), presumably in inclusion bodies. Sonication pellets were re-suspended in a membrane solubilization buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1% (w/v) Nonidet P-40, 1% (w/v) deoxycholic acid) and sonicated further. Insoluble material (including RNH110) was pelleted by centrifugation and then resuspended in a protein solubilization buffer (200 mM Tris, pH 8.0, 6 mM guanidine HCl (GdnHCl)). Solubilized RNH110 was refolded by dialysis against low salt buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM NaCl) and purified by centrifugation at 50000 x g for 1 h to remove debris. RNase assays were carried out at 37°C in a standard RNase H reaction buffer (14) (50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM divalent cation (MgCl2 or MnCl2), 1.5 µM BSA, 1 µM (base pairs) RNA-DNA hybrid), unless otherwise specified. In addition, 1 µl of a saturated solution of polyacrylamide absorbent gel was added per 20 µl of reaction to help maintain substrate solubility in high MnCl2 concentrations (final concentration, ~0.1 mM, used only with 1 mM MnCl2 reactions). Enzymes were diluted from concentrated stocks in 50 mM Tris, pH 8.0, 100 mM NaCl to their final stock concentrations in 50 mM Tris, pH 8.0, 100 mM NaCl, 1.5 µM BSA, 50% glycerol just before assaying them for activity. At indicated time intervals, 20 µl reaction aliquots were stopped by adding 50 mM EDTA, 200 µg/ml Torula RNA (final concentration) on ice. The remaining substrate was precipitated with the addition of 5% (w/v) trichloroacetic acid, 20 mM sodium pyrophosphate (final concentration), followed by incubation on ice for 10 min. Precipitated substrate was pelleted for 10 min in a microcentrifuge, and the acid-soluble radioactivity of 90% of the supernatant was determined by liquid scintillation counting.

RNAse H Activity Assays—Synthesis and purification of the RNA-DNA hybrid substrate were performed as described previously (17). RNAse H assays were carried out at 37°C in 10 mM Tris, pH 8.0, 6 mM guanidine HCl (GdnHCl) (18). RNase H* at 15–40 µg/ml in 50 mM potassium phosphate, pH 8.0, 50 mM KCl with the dA280 at 1 mg/ml = 1.80.

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Results

Construction of a Variant of E. coli RNase H* Lacking the Basic Helix Loop Region—A plasmid encoding a deletion mutant of E. coli RNase H* with its basic helix/loop region replaced by a glycin linker was created by cassette mutagenesis of pSM101 (see “Experimental Procedures”). The resulting plasmid (pJ MD101) encodes the variant protein RNH110, which is composed of the E. coli RNase H* sequence from Met-1 to Ile-82, a Gly6 linker, and then E. coli RNase H* from Lys-96 to Val-155. Essentially, we have removed the basic helix/loop of the enzyme (residues 83-95 (Fig. 1)) and replaced it with a flexible glycine linker. Six glycine residues were judged to be sufficient as a flexible linker after molecular modeling using INSIGHT (Biosym Technologies, San Diego, CA). The deletion mutant was modeled to resemble the RNase H domain of HIV which lacks the basic helix/loop region (Fig. 1). The coding region of pJ MD101 was confirmed by standard DNA sequencing techniques (16), and purification of the overexpressed protein RNH110 to apparent homogeneity is described under “Experimental Procedures.”

The Deletion Protein, RNH110, Is Folded and Stable in Solution—Since the recombinant protein, RNH110, was isolated from inclusion bodies and purified under denaturing conditions, it is especially important to evaluate its efficiency of renaturation. Far-UV CD spectropolarimetry was used to assay for refolding of RNH110 (Fig. 2). The secondary structure of the RNase H domain (16) is shown below the sequences, and active site residues are indicated by asterisks above the residues (17). The basic helix/loop region as defined by Kanaya et al. (12) is boxed. Residues shown in boldface result in at least a 2-fold increase in Mg2+ dependent Km on mutation to alanine (12). Italicized amino acids indicate the 3 cysteine to alanine mutations present in E. coli RNase H*.

A

B

Fig. 1. Structural comparison of E. coli RNase H and the isolated HIV RNase H domain (A) and design of RNH110, an E. coli RNase H deletion mutant (B). A, ribbon diagrams of the crystal structures of E. coli RNase H1 (28, 29) and the isolated HIV-1 RNase H domain (5) (drawn with Mscrit (20)). The dashed line on the HIV RNase H ribbon represents a region of the crystal structure for which no electron density was observed (5). B, amino acid sequence comparison of E. coli RNase H* and RNH110, a deletion mutant of E. coli RNase H*. The secondary structure of E. coli RNase H1 (as determined in Ref. 29) is shown below the sequences, and active site residues are indicated by asterisks above the residues (17). The basic helix/loop region as defined by Kanaya et al. (12) is boxed. Residues shown in boldface result in at least a 2-fold increase in Mg2+ dependent Km on mutation to alanine (12). Italicized amino acids indicate the 3 cysteine to alanine mutations present in E. coli RNase H*.
RNH110 are consistent with an ($\alpha + \beta$)-like fold (19), very similar to that of the E. coli RNase HI (28, 29).

The relative thermodynamic stability of the deletion mutant was determined by both thermal and chemical denaturation. Table I compares these results for the deletion mutant, E. coli RNase H*, and the HIV RNase H domain after which the deletion was modeled. By all criteria ($T_{m}$, $C_{m}$, and $\Delta G_{m}$ (H$_2$O)), the deletion mutant displays intermediate stability; it is less stable than E. coli RNase H* and more stable than the isolated HIV RNase H domain.

RNH110 has a Mn$^{2+}$-dependent RNase H activity—RNH110, E. coli RNase H*, and the HIV RNase H domain were all assayed for activity using the soluble RNase H activity assay described under “Experimental Procedures.” Using Mg$^{2+}$ as the divalent cation (1–50 mM enzyme, 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM MgCl$_2$, 1 mM RNA-DNA base pairs), only RNase H* was found to be active (data not shown). Neither the HIV RNase H domain nor RNH110 display Mg$^{2+}$-dependent RNase H activity, even at 100 mM enzyme concentration. When MnCl$_2$ is substituted for MgCl$_2$, however, the results are quite different (Fig. 3). E. coli RNase H* is active in 1 mM MnCl$_2$, but with a reduction of 100-fold in activity relative to 1 mM MgCl$_2$ (data not shown). Surprisingly, the deletion mutant, RNH110, is also active in the presence of MnCl$_2$, in spite of its lack of Mg$^{2+}$-dependent activity. At 1 mM MnCl$_2$, the specific activity of RNH110 is similar to that of its E. coli RNase H* parent in the same conditions. This is surprising in light of the known importance of the deleted region for substrate binding (12) and the inability to detect Mg$^{2+}$-dependent activity. As noted previously (14), the isolated HIV RNase H domain remained inactive under all of our experimental conditions.

Given the similar Mn$^{2+}$-dependent activity observed for RNH110 and E. coli RNase H*, we investigated the divalent cation dependence of both enzymes (Fig. 4). The activity of the helix/loop deletion protein depends on both the concentration and identity of the divalent cation used. Activity was observed only in MnCl$_2$-containing reactions and only at concentrations above ~50 $\mu$M divalent cation. No activity was observed for this mutant under any MgCl$_2$ conditions (Fig. 4A). The activity of RNase H* is also dependent on divalent cation concentration and identity. The Mg$^{2+}$-dependent activity of RNase H* plateaus to a maximum at ~1 mM Mg$^{2+}$. Activation of the enzyme with Mn$^{2+}$ is quite different, with maximal

| Protein               | $T_m$ | $C_m$ | $\Delta G_{m}$ (H$_2$O) |
|-----------------------|-------|-------|------------------------|
| HIV RNase H domain    | ND    | 1.18  | 3.9                    |
| RNH110                | 48    | 1.41  | 5.8                    |
| E. coli RNase H*      | 55    | 1.60  | 7.2                    |

**Fig. 2.** Far-UV CD spectra of E. coli RNase H* (○) and RNH110 (●). The spectra were taken at 25°C using a 1-cm path length strain-free cuvette (RNH110 at 1.8 $\mu$M concentration, 5 mM potassium phosphate, pH 8.0, 5 mM KCl and E. coli RNase H* at 8.17 $\mu$M concentration, 2 mM potassium phosphate, pH 8.0, 5 mM KF (15)).

**Fig. 3.** Manganese-dependent RNase H activity as shown by the release of precipitable counts/min (radiolabeled RNA in RNA-DNA hybrids) as a function of time. Reactions were carried out in 50 mM Tris, pH 8.0, 50 mM NaCl, 1 mM MnCl$_2$, 1.5 mM acetylated BSA, 1 mM (base pairs) RNA-DNA hybrid, 0.1 mg/ml polyacrylamide absorbent gel, 50 mM enzyme at 37°C. ○, E. coli RNase H*; ●, RNH110; ▲, HIV RNase H domain; ×, no-enzyme control. Data points are the average ± S.D. (bars) of two points.

**DISCUSSION**

In vitro studies of HIV RNase H activity are hampered by the fact that the RNase H domain is inactive when expressed independently of the rest of reverse transcriptase. Substitution of the basic helix/loop region from E. coli RNase HI into the RNase H domain from HIV has been shown to restore Mn$^{2+}$-dependent RNase H activity (13, 14). In addition to activating the domain, this substitution creates a chimeric domain with increased thermodynamic stability compared with the inactive HIV domain (14). Whether the role of the basic helix/loop in activating the HIV domain is stability, substrate affinity, or both is therefore unclear. In an attempt to dissect the potential roles of the basic helix/loop, we performed the converse experiment and modeled the structural characteristics of HIV RNase H onto the active E. coli RNase H* by creating a mutant form of the E. coli enzyme with its basic helix/loop region replaced by a short glycine linker. Our deletion mutant (RNH110) was expected to display the instability and inactivity that are observed in the isolated HIV RNase H domain. Instead we found that RNH110 retained a Mn$^{2+}$-dependent RNase H activity and was more stable than the HIV domain.

The obligate Mn$^{2+}$ dependence of the deletion protein is
H* varies as a function of both the identity and the concentrations of Mn2+

RNase H*, RNH110 shows no Mg2+-dependent activity but still maintains a weakened Mn2+-dependent activity (22). A histidine-tagged version of the HIV RNase H domain displays Mn2+-dependent activity (23, 24). A recent study has shown that a series of mutations in the Moloney murine leukemia virus reverse transcriptase RNase H domain abolish its Mg2+-dependent activity but allow a Mn2+-dependent activity (25). As already described, substitution of a basic helix/loop in the HIV domain restores a Mn2+-dependent activity (13, 14). Furthermore, inhibitors affect HIV reverse transcriptase Mg2+- and Mn2+-dependent RNase H activities differently, implying a difference between the two activities (26). In this report, we describe the first mutation in E. coli RNase H (removal of the basic helix/loop) that results in a strictly Mn2+-dependent activity.

The importance of the basic helix/loop region for substrate binding in E. coli RNase HI (the protease site is within the basic helix/loop) reforming an active RNase H. Furthermore, we have found two fragments of E. coli RNase H* that can refold by dialysis from GdnHCl to form an active RNase H molecule lacking the entire basic helix/loop region.2

Our observation of a Mn2+-dependent activity in RNH110 directly addresses the role of this region in the activity of E. coli RNase H. Taken together with the observations of Kanaya et al. (12, 27), our work suggest that the basic helix/loop serves two roles: substrate affinity and RNase H stability. Future studies of both the E. coli and HIV RNase H homologues should help to further elucidate this important relationship between stability and activity in the RNase H family of enzymes.

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