Biochemical characterization of RNase HII from *Aeropyrum pernix*

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*Aeropyrum pernix* contains one homolog of ribonuclease H (RNase H), *A. pernix* RNase HII (Ape-RNase HII). Activity characterization showed that Ape-RNase HII exhibited the highest activity in the presence of 5 mM Mn$^{2+}$, 1 mM Co$^{2+}$, or 10 mM Mg$^{2+}$, respectively; however, its cleavage efficiencies at different cleavage sites for Mn$^{2+}$ and Mg$^{2+}$ were different. Ape-RNase HII cleaved 12-bp RNA/DNA substrates at multiple sites and the optimum pH value was 11.0. Moreover, 16-bp DNA-r4-DNA/DNA and 13-bp DNA-r1-DNA/DNA chimeric substrates were cleaved at DNA–RNA junction. Ape-RNase HII was thermostable and the stabilization was enhanced with increased salt concentration. This work is believed to be the first in vitro functional study of Ape-RNase HII and the results should contribute to the analysis of RNase H of other archaeal species.

**Keywords** RNase HII; *Aeropyrum pernix*; thermostable; RNA/DNA hybrid

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**Introduction**

Ribonuclease H (RNase H) is a ribonucleotide-specific endonuclease that specifically degrades the RNA strand of RNA/DNA heteroduplex. RNase H activity has been suggested to be involved in DNA replication, DNA repair, transcription, development in eukarya, and antiviral immune response in humans [1–5]. Based on the differences in the amino acid sequences, RNase Hs are classified into two major families, type 1 and type 2 RNases H, which are evolutionarily unrelated [6,7]. RNase H genes have been cloned from various organisms [1–7]. Most organisms have two RNase H genes with various combinations of RNases HI/1, HI/2, and III which are encoded by *rnhA*, *rnhB*, and *rnhC* in prokaryotes, respectively [6,7]. For example, *Escherichia coli* cells contain *rnhA* and *rnhB*, and *Bacillus subtilis* and *Bacillus stearothermophilus* cells contain *rnhB* and *rnhC*. In contrast, the archaeal genomes whose DNA sequences have been completely determined contain *rnhB* gene, and *rnhA* or *rnhC* gene may be involved or not [8]. RNase H requires divalent cations, such as Mg$^{2+}$ or Mn$^{2+}$, for activity with different metal ion preference. *Escherichia coli* RNase HI, *B. subtilis* RNase HII, and *B. stearothermophilus* RNase HIII prefer Mg$^{2+}$ over Mn$^{2+}$ for activity; *E. coli* RNase HII, *B. subtilis* RNase HIII, and *B. stearothermophilus* RNase HII prefer Mn$^{2+}$ over Mg$^{2+}$ for activity [7,9,10]; whereas *Archaeoglobus fulgidus* RNase HIII shows a broad metal tolerance with a preference for Mg$^{2+}$ and Mn$^{2+}$ [4].

Although the active site and suggested key structural elements necessary for RNA primer removal have been recognized through structural analysis of RNase HII from *A. fulgidus*, *Methanococcus jannaschii*, and *Thermococcus kodakaraensis* [11–13], researches on biochemical functions of archaeal RNase HII are still much limited. To provide further biochemical evidence of archaeal DNA replication, much more work about biochemical characterization of archaeal RNase HII still remains to be done.

*Aeropyrum pernix* K1 is a strictly aerobic hyperthermophile, which grows optimally >90°C [14]. The complete genome sequence of *A. pernix* K1 offers us a lot of information about the biology of this microbe [15]. From this archaeon, only one *rnhB* gene, but no additional RNase H genes, was isolated through genome analysis. In this study, RNase H from *A. pernix* K1 was overproduced in *E. coli*, purified, and biochemically characterized. We show that, like other archaeal RNases HII, *A. pernix* RNase HII (Ape-RNase HII) exhibited activity in the presence of Mn$^{2+}$ or Mg$^{2+}$, but the RNase H’s cleavage efficiencies at different cleavage sites were very different for Mn$^{2+}$ and Mg$^{2+}$. These efforts offer us a basis for further study on the structure–function relationship, the mechanism of type 2 RNase H enzymes, and the biological importance of Ape-RNase HII.

**Materials and Methods**

**Materials**

T4-DNA ligase, *Pyrobest*™ DNA polymerase, restriction endonucleases, T4 polynucleotide kinase, ribonuclease
inhibitor, and oligonucleotides were obtained from TaKaRa (Dalian, China). A pET expression kit was purchased from Novagen (North Ryde, Australia). *Aeropyrum pernix* KI genomic DNAs were purchased from Japan Collection of Microorganisms (2_1 Hirosawa, Wako, Saitama 351-0198, Japan).

**Construction of expression vector**

Based on the *rnhB* sequence of *A. pernix*, the amplification primers were synthesized. The forward primer was 5'-CCCGGGGAATTCGTCGCGCATGCTGTCGGCG-3', and the reversed primer was 5'-CCCGGGGTCTGGACCTAA CCTCCAGAAGACTCGT-3'. The *rnhB* gene encoding Ape-RNase HII was amplified from *A. pernix* K1 genomic DNA by polymerase chain reaction (PCR) using Pyrobest™ DNA polymerase with primers mentioned above. The PCR products were inserted into pET-28b vectors between EcoRI and *salI* after restriction enzyme digestion, producing a recombinant expression plasmid pET28-Ape-RNaseHII. DNA sequencing was used to confirm the constructed plasmid.

**Expression and purification of recombinant Ape-RNase HII**

*Escherichia coli* BL21 (DE3) harboring pET28-Ape-RNaseHII plasmid was cultured in Luria–Bertani media containing 25 μg/ml kanamycin at 37°C until OD₆₀₀ reached 0.8. Ape-RNase HII was induced with 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 30°C for 6 h to express the recombinant protein. Harvested cells were used to purify dissolvable Ape-RNaseHII by Ni²⁺–nitrilotriacetate resin column according to the procedure recommended by Novagen. The purified proteins were stored in small aliquots at −20°C in storage buffer (20 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.5 mM EDTA, 1 mM DTT, and 50% glycerol).

**Enzymatic activity**

The substrates were hydrolyzed at 30°C for 1–10 min by specified amounts of Ape-RNase HII. Unless specified requirements otherwise, assays were performed in reaction buffer containing 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1 mM β-mercaptoethanol, 10 mM MgCl₂, and 10 μg/ml bovine serum albumin (BSA). Reactions were stopped by adding an equal volume of stopping buffer (100 mM EDTA, 8 M urea, and 0.1% xylene cyanol) and analyzed by electrophoresis on 20% polyacrylamide gels (with 8 M urea), followed by measurements with an FLA-5100 phosphorimager (Fujifilm, Tokyo, Japan). The products were identified by comparing their patterns of migration on the gel with those of the oligonucleotides generated by partial digestion of the labeled strand with snake venom phosphodiesterase (VPDase) [16]. The initial cleavage rate (*V₀*) was obtained from the slope (nM RNA cleaved/min) of the best-fit line for the linear portion of the plot, which comprised, in general <30% of the total reaction and data from at least five time points. One unit was defined as the amount of enzyme producing 1 μmole of acid-soluble material/min at 30°C. The specific activity was defined as the enzymatic activity/mg protein.

**Kinetic parameters**

For determining kinetic parameters, the concentration of the substrate varied from 0.05 to 5 μM. The reaction was performed at 30°C and stopped by adding stop buffer at different time points. The amount of enzyme was controlled such that the amount of the hydrolyzed substrate at the last time point did not exceed 30% of the total. The hydrolysis of the substrate with the enzyme followed Michaelis–Menten kinetics and the kinetic parameters, *Kₘ* and *Vₘₐₓ*, were determined from the Lineweaver–Burk plot.

**Effect of metal ions on the enzymatic activity**

Enzymatic reactions were performed in the reaction mixture (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1 mM β-mercaptoethanol, 10 μg/ml BSA, 0.5 μM of 12-bp RNA/DNA duplex, and 12 nM recombinant Ape-RNase HII) containing different concentrations of divalent ions, Mg²⁺, Mn²⁺, Ca²⁺, Zn²⁺, or Ni²⁺ (from 0.01 to 100 mM).

**pH effect**

pH effects were determined by performing the assay as above with 12 nM recombinant Ape-RNase HII and 0.5 μM of 12-bp RNA/DNA duplex except that the reaction buffer was substituted with 10 mM imidazole-HCl (pH 5.0–6.0), 10 mM Tris-HCl (pH 7.0–9.0), or 10 mM glycine-NaOH (pH 9.0–12.0).

**Substrate specificity**

A 12-bp RNA/DNA (5'-FAM-cggagauagcg-3', 5'-CGCTCTCCCG-3'), a 13-bp DNA-r1-DNA/DNA (5'-FAM-CG TCCCaCCGTGC-3', 5'-GCACGGTTTTGGGACG-3'), and a 16-bp DNA-r4-DNA/DNA (5'-FAM-CGTCCCAaaaCC GTGC-3', 5'-GCACGGTTTTGGGACG-3') were used as substrates. Ribonucleotides are shown by lower-case letters in the substrates mentioned above, while the deoxyribonucleotides are shown by upper-case letters. The 5' end of the substrates was labeled with 6-FAM. The double-stranded substrates were prepared by hybridizing the fluorescently labeled oligonucleotides with a 2 molar equivalent of their complementary oligonucleotides. The cleavage assays were performed in reaction mixture containing 0.5 μM of different substrates and specified amounts of Ape-RNase HII.
Optimum reaction temperature assay
The optimum reaction temperature was determined by performing the assays as above with Ape-RNase HII at 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, and 90°C. A 39-bp DNA–rN1-DNA (5′-FAM-CCGGGCCGATCGTCAAGAT CCAGCTCGACGATCGGCCGCG-3′) was used as substrate, which could form stable stem–loop structure at 30–90°C.

Thermostability of Ape-RNase HII
Recombinant Ape-RNase HII (12 nM) was dissolved in the buffer (10 mM Tris–HCl, pH 9.0, 1 mM β-ME, 10 μg/ml BSA, and various concentrations of KCl and MgCl2) and treated at 70°C for different time periods; the temperature effects were analyzed by determining the remaining enzyme activities with 0.5 μM of 12-bp RNA/DNA duplex at 30°C.

Results

Gene cloning and Ape-RNase HII purification
rnhB (APE0496.1) gene fragment was amplified from genomic DNA by PCR, and the amplified fragments were inserted into EcoRI–SalI site of pET28b vector to express the N-terminal His-tagged Ape-RNase HII protein. After induction of expression with IPTG, the recombinant Ape-RNase HII was purified by Ni2+-affinity chromatography. The prepared Ape-RNase HII protein appeared as a single band on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) (Fig. 1), indicating that it is electrophoretically pure. About 2 mg of preparations was obtained from one liter of culture.

Amino acid residues conservation
According to the BLAST search results, only one rnhB gene was presumed to encode Ape-RNase HII. The amino acid sequences of Ape-RNase HII deduced from the nucleotide sequences were aligned with those of other bacterial and archaeal RNase HII in Fig. 2. Ape-RNase HII showed amino acid sequence identity of 35% to Pfu-RNase HII, 33% to Afu-RNase HII, 33% to Tko-RNase HII, and 28% to Mja-RNase HII. According to Ape-RNase HII, three conserved motifs of type 2 RNase H (G-X-D-E-X-G-X-G, D-S-K-X-L and V/I-A/S-A-A-S-I-X-A-K, where X represents any amino acid residue) corresponded to Gly6–Gly13, Asp41–Leu45, and Val142–Arg150. In addition, four conserved amino acid residues, which were expected to form the active site of RNase H2, were also fully conserved in the Ape-RNase HII sequence (Asp8, Gly9, Asp107, and Asp136), suggesting that Ape-RNase HII structurally and functionally resemble to other RNase HII.

Cleavage-site specificity and substrate specificity
Three different substrates were examined for cleavage by Ape-RNase HII. Ape-RNase HII cleaved 12-bp RNA/DNA at multiple sites between the 3rd and 11th residues, but most preferentially at c10–g11 and less preferentially at g5–a6 and u7–g8 [Fig. 3(A)]. Ape-RNase HII cleaved 16-bp DNA-r4-DNA/DNA at a9–a10 [Fig. 3(B)]. Thirteen-base pair DNA-r1-DNA/DNA containing one single ribonucleotide could be cleaved at C6–a7 junction [Fig. 3(C)]. The amount of the enzyme required for 50% cleavage of the 16-bp DNA-r4-DNA/DNA was roughly at least 2 folds lower than that of the 12-bp RNA/DNA, and 10 folds lower than that of the 13-bp DNA-r1-DNA/DNA. It seemed that DNA-r4-DNA/DNA duplexes were more suitable for cleavage by Ape-RNase HII than RNA/DNA and DNA-r1-DNA/DNA duplexes.

Metal ion and pH effect
To analyze the enzyme properties for 12-bp RNA/DNA, the kinetic parameters of Ape-RNase HII were first determined. The Km and Vmax values of Ape-RNase HII were 0.361 μM and 0.196 μM/min, respectively. The specific activity was 0.2256 U/mg. The Km, Vmax, and activity values were an average of three independent measurements. Errors were <10% of the reported value.

Ape-RNase HII exhibited enzymatic activity in the presence of MnCl2, MgCl2, and CoCl2, but not CaCl2 and ZnCl2 [Fig. 4(A)]. The highest Mn2+-, Mg2+-, and Co2+-dependent activities were exhibited in the presence of 5 mM MnCl2, 10 mM MgCl2, and 1 mM CoCl2, respectively [Fig. 4(A)]. The specific activity of Ape-RNase HII determined with 5 mM MnCl2 was slightly higher than that determined with 10 mM MgCl2, and about 2 folds higher than that determined with 1 mM CoCl2, indicating Ape-RNase HII shows preference for Mn2+ for activity. For 12-bp RNA/DNA substrates, the cleavage pattern of Mg2+-dependent activity was the same as that of Co2+-dependent activity, but different from that of

Figure 1 SDS–PAGE of Ape-RNase HII overproduced in E. coli cells
Samples were subjected to 15% SDS–PAGE and stained with Coomassie brilliant blue. Lane 1, low-molecular-weight marker; lane 2, purified protein. Numbers beside the gel represent the molecular weights of individual standard proteins.
Mn$^{2+}$-dependent activity [Fig. 4(B)]. The activity values at different ion concentrations were an average of three independent measurements.

The enzymatic activities were determined at pH values from 7.0 to 12.0. Ape-RNase HII exhibited the highest activity at around pH 11.0 (Fig. 5). The activity values at different pH were an average of three independent measurements.

However, we measured the enzymatic activity at pH 9.0, because solubility of the metal ion decreased as pH increased, deposit had a tendency to be produced after 24 h in 10× reaction buffer, although 1× reaction buffer was stable. Moreover, both substrate and enzyme might be unstable at a highly alkaline pH, which was not suitable for all of tests we needed to do.

**Optimum reaction temperature**

Ape-RNase HII was produced from hyperthermophilic *A. pernix* archaeon. In order to study the increased activity and stability at high temperatures, the optimal enzymatic reaction temperature was determined. Thirty-nine-base pair DNA-rN1-DNA substrates were cleaved at various temperatures from 30°C to 90°C. Ape-RNase HII exhibited the highest activity at 70°C (Fig. 6). Although the activity of Ape-RNase HII decreased slightly >70°C, it still remained 7 folds higher than those at 30°C.

**Stability**

The stability of Ape-RNase HII against heat inactivation was analyzed by incubating the enzyme with various concentrations of KCl and MgCl$_2$ at 70°C for 10 min, and then measuring their residual activity. The activity values at different conditions were an average of three independent measurements. It was noted that Ape-RNase HII was fully stable at 70°C for at least 10 min under the assay condition containing 100 mM MgCl$_2$ (Table 1). Along with the reduction of MgCl$_2$ concentration, the activity of the Ape-RNase HII reduced heavily. Under the assay condition containing 10 mM MgCl$_2$, little activity could be detected. The stability of enzyme was also related with the concentration of KCl. The increased KCl concentration was benefit to the stability of Ape-RNase HII (Table 1). At the presence of 250 mM KCl and 50 mM MgCl$_2$, the activity...
of Ape-RNase HII being heated at 70°C for 10, 20, 30, 40, 50, and 60 min, respectively, was measured and the ratio $V_0$ was 0.978, 0.971, 0.968, 0.959, 0.951, and 0.947. Thus, the stability of Ape-RNase HII was related with the ion concentration in the assay. The higher salt concentration, the more stable the enzyme.

**Discussion**

We have shown that the *A. pernix* genome contained the *rnhB* gene encoding RNase HII. From this bacterium only *rnhB* gene, and no additional RNase H genes, was isolated through genome analysis [15]. Like Tht-RNases HII, Ape-RNase HII can cleave DNA–RNA–DNA/DNA. Furthermore, it can also cleave RNA/DNA, which is the same as Tht-RNase HI, but different from Tht-RNases HII. It seems that Ape-RNase HII has the common characteristic of Tht-RNases HI and Tht-RNases HII, which is consistent with the suggestion that type 2 enzymes are the more universal type [6].

Ape-RNase HII was shown to be strictly metal-dependent nucleases. Ape-RNase HII exhibited activity in
the presence of Mg$^{2+}$, Mn$^{2+}$, or Co$^{2+}$, and the maximal activities with them were insignificantly different [Fig. 4(A)]. Differential activities with different concentration have also been observed for other RNase H. Eco-RNase HII, Bsu-RNase HII, and Bst-RNase HII all show a preference for Mn$^{2+}$ [7,9,10], RNase HII from hyperthermophilic archaeon *Pyrococcus kodakaraensis* prefers Co$^{2+}$ for activity and can also tolerate Mg$^{2+}$, Mn$^{2+}$, and Ni$^{2+}$. Afu-RNase HII prefers Mg$^{2+}$ and Mn$^{2+}$, and can also tolerate Co$^{2+}$ and Ni$^{2+}$. Metal ions play an important role for RNase HII in hydrolysis of substrate. The rather broad metal ion specificity of archaeal RNase HII may be benefited for the survival of archaeon. Although Ape-RNase HII could utilize both Mg$^{2+}$ and Mn$^{2+}$ as cofactors, there was drastic difference in the cleavage efficiency at each cleavage site [Fig. 4(B)]. There are no other RNases H having this characteristic at present except *Halobacterium* sp. NRC-1 RNase HI [17]. It has been shown that minor alteration to the RNase H primer could have a dramatic effect on enzyme positioning [18]. The phenomenon that the cleavage efficiencies were different at cleavage sites with Mg$^{2+}$ or Mn$^{2+}$ might be caused by the alteration of Ape-RNase HII geometry which affected the position of the RNase H active site on a static enzyme as well as the modifying critical contacts between Ape-RNase HII and the RNA/DNA hybrid. More work needs to be done to explain this phenomenon.

Ape-RNase HII exhibited activity at an alkaline pH (Fig. 5), which was consistent with other RNases H [6,7,9,10,19]. However, Ape-RNase HII displayed maximum cleavage efficiency at pH 11.0 (Fig. 5). This behavior differs markedly from other ribonuclease H, such as Eco-RNase HII and Cpn-RNase HII, which display a maximum initial rate at pH 9.8 and 9.0, respectively, in the pH-dependent range [9,19]. The pH effect results suggest that Ape-RNase HII shows preference to alkaline more than other RNases HII being studied. Although the pH effect of RNase HII from *Aeropyrum pernix*, *Methanobacterium jannaschii*, *Pyrococcus Furiosus*, and *T. kodakaraensis* are still not determined, our results mentioned above might give some suggestion about the preference to alkaline for other archaeal RNase HII. The tolerance to the high alkaline by Ape-RNase HII may be a survival strategy of an organism, indicating the role of RNase H in DNA replication.

During DNA replication, lagging strand DNA synthesis results in the production of 100–200 bp Okazaki fragments, proceeded by a 7–12 nucleotide RNA primer. Thus, 12-bp RNA/DNA, which provides a model of an RNA primer flanked by Okazaki fragments, has been used as the substrate of RNase H in many previous researches [7,19–21]. In order to compare the activity and cleavage specificity of Ape-RNase HII with other RNase H, we also chose 12-bp RNA/DNA as one of substrates. In our study, the cleavage activity of 16-bp DNA-r4-DNA/DNA was better than that of 12-bp RNA/DNA, and much better than that of 13-bp DNA-r1-DNA/DNA by Ape-RH2. It has been demonstrated that 2’-OH is very important in the binding of RNase H-substrate interaction and the cleavage

### Table 1 Thermostability of Ape-RNase HII

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<th>MgCl$_2$ (mM)</th>
<th>Ratio $V_0^a$</th>
<th>KCl (mM)</th>
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Ape-RNase HII was pre-incubated in 10 mm Tris-HCl (pH 9.0) containing 1 mM EDTA, 10% glycerol, 0.1 mg/L BSA, various concentrations of KCl and MgCl$_2$ at 70°C for 10 min before the activity assay.

*a* Ape-RNase HII was treated with various concentrations of MgCl$_2$ in the presence of 50 mM KCl.

*b* Ape-RNase HII was treated with various concentrations of KCl in the presence of 10 mM MgCl$_2$. $V_0$ means the initial cleavage rate of the untreated enzyme to the treated enzyme.

### Figure 5 pH effect on Ape-RNase HII

The assay was performed in reaction buffer with pH values varied from 7.0 to 12.0 in the absence or presence of Ape-RNase HII. The initial rates of Ape-RNase HII activity were plotted.

### Figure 6 Optimum reaction temperature of Ape-RNase HII

The 0.5 µM 39-bp DNA-rN1-DNA was cleaved at different temperature from 30°C to 90°C by 60 nM Ape-RNase HII. The initial rates of Ape-RNase HII activity were plotted.
of DNA–RNA–DNA/DNA duplex by type 1 RNase H required minimal four riboses [22]. Maybe the better cleavage activities of 16-bp DNA–r4–DNA/DNA and 12-bp RNA/DNA attribute to the better binding with enzyme. Okaraki fragment and DNA–RNA–DNA/DNA duplex can be formed in DNA replication. It is believed that RNase H cleaves RNA/DNA or DNA–RNA–DNA/DNA duplex in order to obtain genome DNA [23]. Rydberg and Game [24] demonstrated that type 2 RNase H was involved in excising a single ribonucleotide from DNA, strengthening a proposed role of this enzyme in the repair of misincorporated ribonucleotides. *Aeropyrum pernix* has only one *rrnhB* expressing Ape-RNaseHII, suggesting the important role of this enzyme not only in DNA replication, but also in DNA repair. Moreover, the cleavage activities of 16-bp DNA–r4–DNA/DNA and 12-bp RNA/DNA were much better than those of 13-bp DNA–r1–DNA/DNA, proposing that Ape-RNaseHII was more involved in DNA replication.

Hyperthermophiles adapt to high temperatures by producing heat-adapted enzymes, which are characterized by increased activity and stability at high temperatures compared with their mesophilic counterparts [25]. In hyperthermophilic proteins, amino acid composition and intrinsic propensity, disulfide bridges, hydrophobic interactions, hydrogen bonds, salt bonds, metal binding, post-translational modifications, and so on, have been thought to be correlated to thermostability of hyperthermophilic enzymes [26]. According to salt bonds, increased salt concentration is not benefit to the stability of protein because of the demolition of the salt bonds [26–28]. In our research, increased KCl concentration contributed to the stability of Ape-RNase HII (Table 1), which was contradictory with previous results [27,28]. This suggests that the salt bonds are not important to the stabilization of Ape-RNase HII. The increased manganese ion was also good to the stabilization of Ape-RNase HII (Table 1), which might be induced by the coordination with the active center of enzyme to form the more stable conformation. Most of thermophilic enzymes from thermophilic archaea can be expressed in *E. coli* and their activity and thermostability are similar with wild-type proteins. Very small number of enzymes was subjected to post-translational modification to obtain high thermostability. We report that Ape-RNaseHII purified from *E. coli* remained high activity at 70–90°C, and the increased salt concentration was a benefit to the stability. It seemed that the post-translational modification was not the main stabilization mechanism of Ape-RNase HII. Moreover, it has been reported that the buried hydrophobic residues were strongly responsible for the hyperstability of Tko-RNase HII from hyperthermophilic archaean [29]. It will be informative to examine whether Ape-RNase HII can be stabilized by similar mechanisms. Studies of proteins from hyperthermophiles may provide general or additional insights into forces stabilizing the native conformation of proteins.

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