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Crystal Structure and Structure-based Mutational Analyses of RNase HIII from *Bacillus stearothermophilus*: A New Type 2 RNase H with TBP-like Substrate-binding Domain at the N Terminus

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Ribonuclease HIII (Bst-RNase HIII) from the moderate thermophile *Bacillus stearothermophilus* is a type 2 RNase H but shows poor amino acid sequence identity with another type 2 RNase H, RNase HII. It is composed of 310 amino acid residues and acts as a monomer. Bst-RNase HIII has a large N-terminal extension with unknown function and a unique active-site motif (DEDE), both of which are characteristics common to RNases HIII. To understand the role of these N-terminal extension and active-site residues, the crystal structure of Bst-RNase HIII was determined in both metal-free and metal-bound forms at 2.1–2.6 Å resolutions. According to these structures, Bst-RNase HIII consists of the N-terminal domain and C-terminal RNase H domain. The structures of the N and C-terminal domains were similar to those of TATA-box binding proteins and archaeal RNases HII, respectively. The steric configurations of the four conserved active-site residues were very similar to those of other type 1 and type 2 RNases H. Single Mn and Mg ions were coordinated with Asp97, Glu98, and Asp202, which correspond to Asp10, Glu48, and Asp70 of *Escherichia coli* RNase HI, respectively. The mutational studies indicated that the replacement of either one of these residues with Ala resulted in a great reduction of the enzymatic activity. Overproduction, purification, and characterization of the Bst-RNase HIII derivatives with N and/or C-terminal truncations indicated that the N-terminal domain and C-terminal helix are involved in substrate binding, but the former contributes to substrate binding more greatly than the latter.

Keywords: RNase HIII; *Bacillus stearothermophilus*; crystal structure; TATA-box binding protein; substrate binding domain

Introduction

Ribonuclease H (RNase H) endonucleolytically hydrolyzes the RNA strand of RNA/DNA hybrids in the presence of divalent cations.[1] The enzyme is widely present in all three kingdoms of living organisms, including bacteria, archaea, and eukaryotes.[2] RNase H is involved in DNA replication, repair, and/or transcription.[3–6] An RNase H1 knockout mouse is embryonically lethal, because mitochondrial DNA is not synthesized.[7] RNase H also plays a crucial role in the pharmacology of DNA-like antisense drugs.[8] RNase H is also present in retroviruses as a C-terminal domain of reverse transcriptase (RT). This activity is required for proliferation of HIV-1 and is therefore regarded as one of the targets for AIDS therapy.[9]

RNases H are classified into two evolutionarily unrelated families, type 1 and type 2 RNase H.[2] According to the crystal structures of type 1[10–14] and type 2[15–17] RNase H, these enzymes share a main-chain fold and steric configurations of

Abbreviations used: RNase H, ribonuclease H; TBP, TATA-box binding protein; RT, reverse transcriptase; Bst-RNase HIII, RNase HIII from *Bacillus stearothermophilus*; Tk-RNase HII, RNase HII from *Thermococcus kodakaraensis*; ts, temperature-sensitive.

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the four acidic active-site residues, suggesting that their catalytic mechanisms are basically identical. It has been controversial for a long time whether the enzyme requires one[18–21] or two[11,13] metal ions for activity. However, recent observation that two metal ions bind to the active site of a type 1 RNase H under conditions in which the enzyme exhibits the activity, only when the enzyme–substrate complex is formed,[22] indicates that RNase H adopts a two-metal mechanism. According to this mechanism, one metal ion is required for activation of an attacking water molecule and the other is required for stabilization of the penta-covalent intermediate.

Many organisms contain both type 1 and type 2 RNase H in the single cells.[2] For example, *Escherichia coli* cells contain RNases HI and HII, and human cells contain RNases H1 and H2. RNases HI and H1 represent type 1 RNases H, and RNases HII and H2 represent type 2 RNases H. However, several bacteria, such as *Bacillus subtilis*, *B. stearothermophilus*, *Streptococcus pneumoniae*, *Chlamydia trachomatis*, and *Aquifex aeolicus*, have two different type 2 RNases H, RNases HII and HIII.[2] RNases HIII are characterized by the presence of a large (70–90 residues) N-terminal extension with unknown function. Eukaryotic RNases H1 contain a double-stranded, RNA-binding motif (40 residues) at the N-terminal non-RNase H domain.[23,24] Likewise, *B. subtilis* and *B. stearothermophilus* RNases HII have a large (71 residues) N-terminal extension, which has been shown to be important for substrate binding.[25] However, N-terminal extensions of RNases HIII, which show a weak but significant sequence similarity with one another, are different from those of eukaryotic RNases H1 and bacterial RNases HII in amino acid sequence. In addition, RNases HIII are different from other RNases H in kind of the active-site residues. The active site of RNase H is formed by four acidic residues, which are generally conserved as Asp, Glu, Asp, and Asp (DEDD motif) from the N to C termini in the primary structure. In the RNase HIII sequences; however, the fourth aspartate residue is replaced by Glu. In order to understand the role of the N-terminal extension and a unique active-site residue of RNase HIII, structural and mutational studies will be required.

We have recently cloned the gene encoding RNase HIII (Bst-RNase HIII) from the moderate thermophile *B. stearothermophilus* and characterized the enzymatic properties of the recombinant protein.[26] We have also crystallized this protein and performed preliminary X-ray diffraction studies.[27] Bst-RNase HIII is composed of 310 amino acid residues and shows the amino acid sequence identities of 47.1%, 34.8%, 25.3%, and 22.6% with RNase HIII from *B. subtilis*, *S. pneumoniae*, *C. trachomatis*, and *A. aeolicus*, respectively. It shows the poor amino acid sequence identity of 15% with Bst-RNase HII. It has been reported that *B. subtilis* RNase HIII is more closely related to *E. coli* RNase HI than to *B. subtilis* RNase HII and *E. coli* RNase HII in enzymatic properties, such as metal ion specificity, specific activity, and cleavage site specificity, despite its more distant evolutionary relationship to *E. coli* RNase HI than to other two enzymes.[28] Bst-RNase HIII has similar enzymatic properties to *B. subtilis* RNase HIII, although it prefers Mg^{2+} to Mn^{2+} much more weakly than *B. subtilis* RNase HIII. These results suggest Bst-RNase HIII is also more closely related to RNase HI than to RNase HII.

Here, we determined the crystal structure of Bst-RNase HIII in metal-free, Mg^{2+}-bound, and Mn^{2+}-bound forms at 2.1–2.6 Å resolutions. These structures, which highly resemble one another, represent the first RNase HIII structure. Bst-RNase HIII was shown to consist of the N-terminal domain with a TBP-like structure and the C-terminal RNase H domain. Based on this structure, as well as the structure-based mutational analyses of Bst-RNase HIII, we discuss the role of these domains in substrate recognition and catalysis.

Results

Crystallization and data collection

Preliminary X-ray diffraction data of the native crystal at 2.8 Å have been reported.[27] These data were collected using one of the single crystals separated from plate-like overlapping poly-crystals. In this study, we further separated several additional single crystals for structural analyses and obtained X-ray diffraction data of the native crystal at 2.3 Å resolution. Bst-RNase HIII crystals were also grown in the presence of 50 mM $MgCl_2$. The Mg^{2+}-bound crystal, which diffracted to 2.1 Å at a synchrotron source, was equal to the native crystal in the space group and unit-cell parameters. We further obtained Mn^{2+}-bound crystals by a soaking method, which diffracted to 2.6 Å at a synchrotron source. The heavy-atom derivatives were also prepared by the soaking method. All of these crystals contain one protein molecule per asymmetric unit. Data collection statistics are summarized in Table 1.

Overall structure

The crystal structure of Bst-RNase HIII was determined to 2.3 Å for the metal-free form, 2.1 Å for the Mg^{2+}-bound form and 2.6 Å for the Mn^{2+}-bound form. The refinement statistics are summarized in Table 1. No significant difference was observed among these structures with root-mean-square deviations (RMSDs) of 0.27 Å between the metal-free and Mg^{2+}-bound proteins, 0.38 Å between the metal-free and Mn^{2+}-bound proteins, and 0.27 Å between the Mg^{2+}-bound and Mn^{2+}-bound proteins.

The structure of Bst-RNase HIII is composed of two distinct domains, the N-terminal domain (residues 2–90) and C-terminal domain (residues 91–309) (Figure 1). The space-filling model indicates