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Crystal structure of apo and copper bound HP0894 toxin from *Helicobacter pylori* 26695 and insight into mRNase activity

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ABSTRACT

The toxin–antitoxin (TA) systems widely spread among bacteria and archaea are important for antibiotic resistance and microorganism virulence. The bacterial kingdom uses TA systems to adjust the global level of gene expression and translation through RNA degradation. In *Helicobacter pylori*, only two TA systems are known thus far. Our previous studies showed that HP0894–HP0895 acts as a TA system and that HP0894 exhibits intrinsic RNase activity. However, the precise molecular basis for interaction with substrate or antitoxin and the mechanism of mRNA cleavage remain unclear. Therefore, in an attempt to shed some light on the mechanism behind the TA system of HP0894–HP0895, here we present the crystal structures of apo- and metal-bound *H. pylori* 0894 at 1.28 Å and 1.89 Å, respectively. Through the combined approach of structural analysis and structural homology search, the amino acids involved in mRNase active site were monitored and the reorientations of different residues were discussed in detail. In the mRNase active site of HP0894 toxin, His84 acts as a catalytic residue and reorients itself to exhibit this type of activity, acting as a general acid in an acid–base catalysis reaction, while His47 and His60 stabilize the transition state. Lys52, Glu58, Asp64 and Arg80 have phosphate binding and specific sequence recognition. Glu58 also acts as a general base, and substrate reorientation is caused by Phe88. Based on experimental findings, a model for antitoxin binding could be suggested.

1. Introduction

Bacteria and archaea containing genetic elements encoding toxin–antitoxin (TA) pairs, known as addiction modules [1], were discovered about three decades ago on the mini-F plasmid of *Escherichia coli*. This plasmid called the *ccd* operon, acts by killing the cells that become plasmid-free [2]. This behaviour of *ccd* operon is referred to as 'plasmid addiction', as the *ccd* operon makes the cell 'addicted' to the plasmid [3]. These TA pairs have become attractive to many biochemists and microbiologist owing to their significant and diverse involvement at the gene transcriptional level [4,5].

Bacterial TA operons consist of a stable toxin and a proteolytically labile or unstable antitoxin [4,6–8]. The two open reading frames for both of these proteins, generally with the antitoxin being upstream of the toxin, are always found within the same TA operon [9]. Under normal conditions, both proteins form a tight complex in which antitoxin neutralizes the effect of toxin with the complex remaining in the dormant stage [10,11]. In most known cases, the antitoxin acts as a repressor of the TA operon transcription [12,13]. However, under stress conditions such as oxidative stress, a change in temperature, starvation due to lack of amino acids and nutrition, or even the addition of an antibiotic, the release of a specific protease causes the degradation of the proteolytically labile antitoxin more rapidly than the toxin [6,7,13,14]. Thus, the amount or concentration of toxin and antitoxin present in the cell causes an imbalance and the free toxin in the cell inhibits cellular processes, inhibiting the growth and leading to cell death [6,7,13,14]. Due to this stringent mechanism, known as negative autoregulation by a feedback mechanism, a steady-state level of antitoxin is required to form a stable toxin–antitoxin complex [11].

Bacteria use TA systems to adapt to stress conditions by modulating their global levels of biological processes such as translation and DNA synthesis [4,6,7,15–18]. The preliminary role of these TA systems may be to arrest cell growth and to enable the survival under unfavourable stress conditions. However, many other possible functions of TA systems have been reported, examples being the stabilization of genomic parasites, gene regulation, growth control, persistence, and programmed cell death [4,6,19].

Thus far, three broad classes of TA pairs based on the functions of these TA systems have been identified. Class I TA systems are known for their sequence-specific endoribonuclease activity which functions independently of the ribosome. Examples include MazE–MazF and ChpBI–ChpBK pairs. Class II TA systems are codon-dependent

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endoribonucleases [1], such as RelB–RelE, DinJ–YafQ, and YefM–YoeB pairs. In class III TA systems, the inhibition of the toxin activity is directly driven by the interaction of RNA molecules [20], such as YafN–YafO module [21–25]. However, opinion differs as to whether YafN–YafO belong to class II or class III TA system [1,10,11]. Classification on the basis of the function of antitoxin classifies TA systems in three types as well. Type I and type III TA systems include small RNAs which works as an antitoxin that inhibits either level of expression of toxin (Type I) or activity of toxin (type III) [26–28]. In type I, expression of toxin gene is regulated by an antisense RNA, which is transcribed from the same toxin–antitoxin module but in a reverse orientation. The antisense RNA interrupts the translation of toxin mRNA [29,30]. In a type III TA system, an RNA antitoxin forms a complex with the toxin protein, resulting in neutralization of toxicity [29,31]. In type II system, toxin and antitoxin proteins form a strong toxin–antitoxin complex, playing a role in plasmid maintenance known as post-segregational killing [26,29].

Bacterial and archaeal chromosomes consist of several identified families of TA modules, mostly identified on the *E. coli* K-12 chromosome, which codes for at least 19 different type II TA systems [29], including *relBE*, *yefM–yoeB*, *mazEF*, *dinJ–yafQ*, *yafNO*, *hipBA*, *chpBK*, *hicA*, *mqsAR*, *prlF–yhaV*, *ygjNM*, and *ygiUT* [4,10,16,25,32–36]. Most of these are endoribonucleases that exhibit mRNase activity with different specificities, while some type II TA systems inhibit replication by interacting with DNA gyrase and affecting peptidoglycan biosynthesis [37].

Among all of these TA systems, *RelBE*, *MazEF*, *YefM–YoeB*, *dinJ–yafQ* are widely studied [1,10,11,35,38,39]. MazF is independent of the ribosome in its expression of its functionality, which is to cleave mRNA at ACA sequences by mimicking ssRNA [40–42]. However, some researchers have shown that mazF behaves like RelE at ribosomal A-site [43].

The RelE superfamily of bacterial toxins includes RelE, YafQ, YoeB, HigB and YhaV toxins [18,23,44–47]. All of these inhibit translation through related, but distinct, mechanisms. RelE and YafQ are both ribosome-associated endoribonucleases. RelE is active when it associates with the ribosomal A-site [48], where mRNA sequence specificity plays a significant role in cleavage efficiency, with pyrimidines in the first position and purines in second and third positions of the codon [49]. This mechanism requires three steps to be performed. The 2′-OH of RNA must be activated by abstraction of the proton by a general base, allowing it to act as a nucleophile and the RNA to orient itself for an inline attack at the phosphate. Then, a negatively charged trigonal bipyramidal transition state must be stabilized [48], and the leaving 5′-OH group should be stabilized through the donation of a proton by a general acid [48]. YafQ is a ribosome-associated endoribonuclease, which upon overexpression, inhibits translation process by interrupting cellular mRNA at specific sequences [10]. It cleaves RNA in frame AAA codons followed by A or G in subsequent codon [46], preferably the 5′ position to A between the second and third nucleotides in the codon [10]. The activity of YafQ toxin is neutralized by DinJ antitoxin through the formation of a strong protein–protein complex [10]. In contrast, ribosome associated YoeB toxin inhibits translation by destabilization of the initiation complex, with marginal mRNA cleavage activity [50].

These TA systems have been targeted for the development of alternative antibiotics against nosocomial infections by multidrug-resistant bacteria [1,23,51], biofilm formation, and multidrug tolerance [10,52,53]. As mentioned earlier, *E. coli* has many TA systems, but *Helicobacter pylori* have only two known TA systems thus far [38,39]. Therefore, more detailed information is needed on these TA systems to target *H. pylori* for the development of alternative antibiotics.

In our earlier studies, we found that the HP0894 toxin, a member of RelE superfamily, binds with HP0895 antitoxin of the *hp0894–hp0895* TA operon of *H. pylori* 26695 [38,39,54]. The intrinsic RNase activity and the bacterial cell growth-arresting activity of HP0894 were also established. HP0894, a member of RelE superfamily, may also follow the mRNase mechanism, as shown by RelE, but the general acid, general base and a trigonal bipyramidal transition state, which are required for an efficient RNase activity, have not been discussed in detail thus far for HP0894. Here, we report the structure of apo- and copper-bound HP0894 toxin, suggest its mechanism of RNase activity of HP0894, and discuss the substrate binding residues that are involved in.

2. Materials and methods

2.1. Cloning and expression of HP0894

The gene encoding HP0894 was amplified by PCR using *H. pylori* genomic DNA as a template. The following primer pair was used: HP0894Fwd (5′-CATATGTTGAAGCTCAATCTTAAAAAATC) and HP0894Rev (5′-GGATCCTTAAAACAGCTCGCTATGAC); the enzyme restriction sites used for cloning are underlined. The PCR products were purified, digested with *Nde*I and *BamH*I enzymes (NEB, UK) and ligated with the pET-15b vector (Novagen Inc.). The construct resulted in an N-terminus hexahistidine tag and a thrombin cleavage site to facilitate purification. The recombinant plasmid was then overexpressed in *E. coli* BL21(DE3) competent cells (Novagen Inc.).

2.1.1. Purification of HP0894

The cells containing the recombinant plasmid were allowed to grow at 37 °C until the OD_{600} reached 0.5. Protein overexpression was induced by addition of isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a concentration of 0.5 mM and the cells were grown for additional 4 h. The cells were harvested by centrifugation at 4293 ×*g* for 10 min. The harvested cells were resuspended in lysis buffer (20 mM Tris–HCl, 500 mM NaCl, pH 7.9 and 10% v/v glycerol) and lysed at 4 °C using an ultrasonic processor (Cole Parmer Inc.) for 10 min, after which the lysate was centrifuged at 6708 ×*g* for 1 h. The supernatant was passed through 0.45 μm pore size membrane (Millex-HV filter unit, Millipore, USA) to remove any insoluble particles. The flow-through was loaded on an open Ni^{2+}-NTA column (Qiagen, USA; 3 ml of resin per litre of cell culture) pre-equilibrated with lysis buffer. The column was washed with a 5× excess volume of loading buffer containing 40 mM imidazole and the protein of interest, HP0894 was then eluted with a 0–1 M imidazole gradient. The isolated fraction was dialyzed using a cellulose membrane with a 3000 molecular weight cut off (MWCO) against 20 mM Tris, 500 mM NaCl at pH 7.9, after which thrombin digestion was done at 20 °C for 12 h to remove the N-terminal hexahistidine tag. The buffer change and purity were achieved using gel filtration column (Superdex 75 (10/300GL) from GE Healthcare Sciences, Germany). The buffer used for gel filtration column was 20 mM MES, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM β-mercaptoethanol (BME), and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at pH 6.0. The thrombin cleavage resulted in three non-native amino acids (GSH) attached to the N-terminal of HP0894. The purity of the protein was judged to be more than 95% as shown by a SDS-PAGE analysis. A protein concentration of 39 mg/mL was used for crystal screening.

2.1.2. Selenomethionine labelling of HP0894

Selenomethionine (SeMet) labelling was done as described in an earlier study [55]. *E. coli* BL21(DE3) strain carrying the recombinant pET-15b vector with HP0894 was grown overnight in 10 mL of M9 medium and then used to inoculate 1 L M9 media supplemented with magnesium chloride, glucose, vitamins and trace metals. The culture was allowed to grow at 37 °C until the OD_{600} reached to 0.5, after which it was further supplemented with 100 mg/L each of phenylalanine, threonine, lysine, and 50 mg/L each of leucine, isoleucine, valine, proline and selenomethionine to promote the shutdown of methionine biosynthesis and selenomethionine uptake by *E. coli* cells. HP0894 overexpression was induced by an addition of 0.5 mM IPTG and the cells were allowed to grow for an additional 4 h. Isolation and purification of the SeMet-HP0894 protein were carried out as described above.