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Functional role of putative critical residues in *Mycobacterium tuberculosis* RNase P protein

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ABSTRACT

RNase P is involved in processing the 5′ end of pre-tRNA molecules. Bacterial RNase P contains a catalytic RNA subunit and a protein subunit. In this study, we have analyzed the residues in RNase P protein of *M. tuberculosis* that differ from the residues generally conserved in other bacterial RNase Ps. The residues investigated in the current study include the unique residues, Val27, Ala70, Arg72, Ala77, and Asp124, and also Phe23 and Arg93 which have been found to be important in the function of RNase P protein components of other bacteria. The selected residues were individually mutated either to those present in other bacterial RNase P protein components at respective positions or in some cases to alanine. The wild type and mutant *M. tuberculosis* RNase P proteins were expressed in *E. coli*, purified, used to reconstitute holoenzymes with wild type RNA component *in vitro*, and functionally characterized. The Phe23Ala and Arg93Ala mutants showed very poor catalytic activity when reconstituted with the RNA component. The catalytic activity of holoenzyme with Val27Phe, Ala70Lys, Arg72Leu and Arg72Ala was also significantly reduced, whereas with Ala77Phe and Asp124Ser the activity of holoenzyme was similar to that with the wild type protein. Although the mutants did not suffer from any binding defects, Val27Phe, Ala70Lys, Arg72Ala and Asp124Ser were less tolerant towards higher temperatures as compared to the wild type protein. The K_m of Val27Phe, Ala70Lys, Arg72Ala and Ala77Phe were >2-fold higher than that of the wild type, indicating the substituted residues to be involved in substrate interaction. The study demonstrates that residues Phe23, Val27 and Ala70 are involved in substrate interaction, while Arg72 and Arg93 interact with other residues within the protein to provide it a functional conformation.

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1. Introduction

Transfer RNAs (tRNAs) are transcribed as precursor tRNA (pre-tRNA) molecules, containing additional sequences at the 5′ and 3′ ends. Unlike in bacteria, some eukaryotic tRNA genes contain introns also (Sharp et al., 1985). The pre-tRNA molecules need to undergo processing of the 5′ and 3′ ends in order to participate in the protein synthesis events. The 3′ end processing of pre-tRNA is initiated by RNase E and carried forward by tRNA nucleotidyl-transferases (Morl and Marchfelder, 2001; Ow and Kushner, 2002). The 5′ end processing of the pre-tRNA molecule is carried out by a ubiquitous endoribonuclease named ribonuclease P (RNase P) (Guerrier-Takada et al., 1983). RNase P also cleaves other RNA molecules like the precursors to 4.5S RNA (Peck-Miller and Altman, 1991), small nucleolar RNAs (Coughlin et al., 2008), transfer-messenger RNAs, and B12 riboswitches from *E. coli* and *B. subtilis* (Komine et al., 1994).

RNase P is a ribonucleoprotein in which the RNA subunit contains the catalytic activity. Bacterial RNase P enzymes contain one protein and one RNA subunit, whereas the eukaryotic enzyme has one RNA and ten protein subunits. The protein component of RNase P forms a functional complex with the RNA subunit (Esakova and Krasilnikov, 2010). The protein subunit in RNase P holoenzyme modulates the recognition of the pre-tRNA substrate. It is shown that the *B. subtilis* holoenzyme binds pre-tRNA more tightly than tRNA, whereas reverse is true for the RNA component alone (Kurz et al., 1998). The protein component contacts the pre-tRNA leader sequence via direct and indirect interactions (Crary et al., 1998). In the presence of protein component, requirement of magnesium for efficient catalysis by the RNA component is also reduced (Kurz and Fierke, 2002). Previous chemical protection and hydroxyl radical footprinting studies with *E. coli* RNase P indicate that the protein's binding site on the RNA component is neither close to the active

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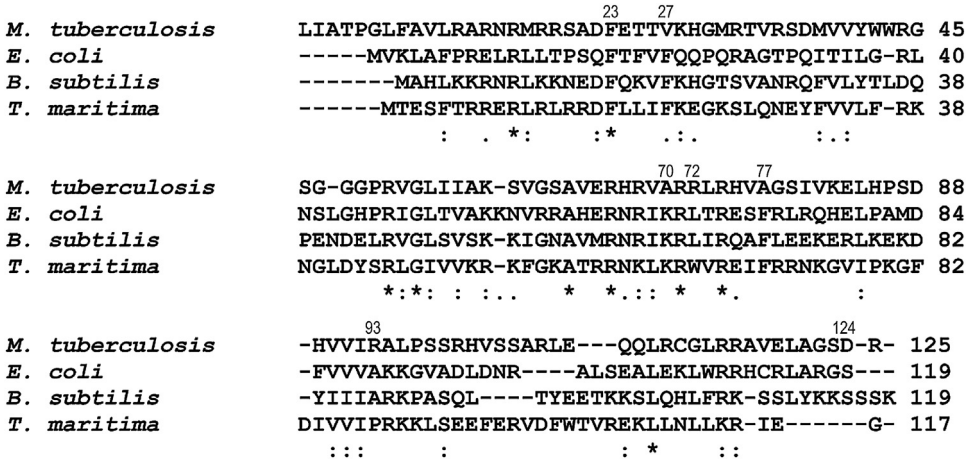

Fig. 1. Comparison of amino acid sequence of protein component of RNase P of *M. tuberculosis* with other bacterial proteins. The sequences of RNase P protein components of *M. tuberculosis, E. coli, B. subtilis* and *T. maritima* were aligned using Clustal Omega software. The positions of the residues studied have been written on the top. The (*) represent conserved residues, while (:) and (.) represent residues with strongly similar and weakly similar properties respectively.

site nor to the substrate binding site, suggesting that the enhanced activity of holoenzyme, as compared to that of RNA alone could be due to some conformational changes in RNA brought about as a result of binding of protein (Westhof et al., 1996).

The secondary structure of RNase P protein component is conserved across different bacteria; it has 3 α-helices and 4 β strands with an overall topology of αββββαβα (Kazantsev et al., 2003; Stams et al., 1998). The β strands wrap around each other to form the central cleft of RNase P, with α-1 also being a part of the central cleft (Gopalan et al., 1997). The central cleft interacts with the 5′ leader of pre-tRNA and governs substrate specificity. Between the parallel β-strands of the central cleft, there is an α-helix forming an unusual left-handed βαβ crossover connection, termed as the RNR motif based on the three highly conserved residues Arg-Asn-Arg (Kazantsev et al., 2003; Reiter et al., 2010; Stams et al., 1998). The RNR motif is composed of around 18 amino acids. The sequence of the RNR motif in *B. subtilis* is Lys-Xaa$_{4-5}$-Ala-Xaa$_2$-Arg-Asn-Xaa$_2$-(Lys or Arg)-Arg-Xaa$_2$-(Arg or Lys), where Xaa refers to any amino acid (Stams et al., 1998). The RNR motif is considered to stabilize binding interactions of the protein component with RNase P RNA owing to its highly basic nature. Furthermore, reorientation of the RNase P RNA takes place when in complex with protein component that places the conserved RNR motif close to the catalytic site (Niranjanakumari et al., 1998), and brings loop L12 of the RNase P RNA near the catalytic site (Sharkady and Nolan, 2001). Kazantsev et al. (2003) report that many residues in the RNase P protein component form an intramolecular conserved bridge that stabilizes the folded structure of the protein.

As RNase P is the only enzyme responsible for 5′ end processing of all different pre-tRNAs, it could be a potential target for novel drugs against pathogenic bacteria, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The bacterial RNase P is inhibited by retinoids and aminogylcosides; aminogylcosides can discriminate between bacterial and archaeal RNase P enzymes (Mikkelsen et al., 1999; Papadimou et al., 1998; Kawamoto et al., 2008). A real time assay to screen for RNase P inhibitors has been described (Liu et al., 2014). Apart from small molecule inhibitors, specific inhibition of gene expression has been demonstrated through external guide sequence technology that mimics RNase P-mediated cleavage (Davies-Sala et al., 2015). This platform has the potential of developing novel therapeutic interventions.

We have earlier shown that *M. tuberculosis* RNase P generally functions like the other bacterial RNase Ps, and a histidine present in the RNR motif in mycobacterial RNase P protein, in place of asparagine, is functionally well tolerated (Singh et al., 2016). A multiple sequence alignment of *M. tuberculosis* RNase P protein component with those of *E. coli* and *B. subtilis*, shows several differences within the RNR motif and central cleft of the mycobacterial protein. The current study was undertaken to investigate the roles of five unique residues, Val27, Ala70, Arg71, Ala77 and Asp124 at locations where conserved residues are present in other bacterial RNase Ps, and two putative critical residues, Phe23 and Arg93 in the protein component of *M. tuberculosis* RNase P in the functional activity of the enzyme. The unique residues Ala70, Arg72, and Ala77 are in RNR motif, whereas Val27 and Arg93 fall in the central cleft. The selected residues were substituted with those present in other bacterial proteins or alanine, and functionally characterized as components of holenzyme. The current study demonstrates that the residues Phe23, Val27 and Ala70 of *M. tuberculosis* RNase P protein component are important for its interaction with the pre-tRNA substrate. The residues, Arg72 and Arg93 are involved in interaction with other residues within the protein for proper folding.

2. Materials and methods

2.1. Cloning of DNA encoding pre-tRNA alanine and RNase P RNA and protein components

The DNA encoding the RNA and protein components of RNase P, and pre-tRNA$_{alanine}$ of *M. tuberculosis* were amplified from *M. tuberculosis* genomic DNA by PCR and cloned into pGEM-4, pVex11 and pGEM-T vectors, respectively (Singh et al., 2016).

2.2. Site-directed mutagenesis in the protein component

Site-directed mutagenensis was used to obtain various mutants of RNase P protein of *M. tuberculosis* (Li and Wilkinson, 1997). Two complementary 33-mer DNA primers containing the desired nucleotide change corresponding to mutation, in the middle of the primers were designed. The plasmid DNA containing the DNA of wild type RNase P protein was amplified by PCR using the two primers with NEB Phusion polymerse. Competent DH5α cells were transformed and plated on LB-ampicillin plates to obtain discrete colonies. The colonies were screened by restriction digestion analysis to identify the desired clones which were further confirmed by DNA sequencing.

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