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Sequence-specific backbone ^1H , ^{13}C , and ^{15}N resonance assignments of human ribonuclease 4

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Abstract

Human ribonuclease 4 (RNase 4) is the most evolutionarily conserved member of the 8 canonical human pancreatic-like RNases, showing more than 90% identity with bovine and porcine homologues. The enzyme displays ribonucleolytic activity with a strong preference for uracil-containing RNA substrates, a feature only shared with human Eosinophil Derived-Neurotoxin (EDN, or RNase 2) and Eosinophil Cationic Protein (ECP, or RNase 3). It is also the shortest member of the human family, with a significantly truncated C-terminal tail. Its unique active-site pocket and high degree of conservation among vertebrates suggest that the enzyme plays a crucial biological function. Here, we report on the ^1H , ^{13}C and ^{15}N backbone resonance assignments of RNase 4, providing means to characterize its molecular function at the atomic level by NMR.

Keywords

Orthologues; Ribonucleases; RNase A; Evolutionary conservation; RNA

Introduction

Bovine pancreatic ribonuclease A (RNase A) is one of the best-studied enzymes of the 20th century. The enzyme gives its name to an extensive superfamily of pancreatic-like structural homologues found in mammals and vertebrates. In human, eight canonical members were identified to date, *i.e.* RNases 1–8 (Sorrentino, 2010), which have been found in the pancreas (Weickmann et al., 1981), in urine and kidney (Cranston et al., 1980, Iwama et al., 1981, Mizuta et al., 1990), in blood serum (Rabin and Tattrie, 1982), in the placenta (Zhang et al., 2002), in the liver (Zhang et al., 2003) and in seminal plasma (De Prisco et al., 1984), to name a few. While all pancreatic-like human members conserve varying degrees of ribonucleolytic activities and a high structural similarity, each member appears to have also

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acquired additional biological functions (Sorrentino, 2010, Boix et al., 2013). These alternative roles include antibacterial, antipathogenic, cytotoxic and neurotoxic activities (Durack et al., 1981), angiogenesis (Strydom et al., 1985), and immunosuppressive activity (Bystrom et al., 2011). It is believed that all members are secretory proteins, although it was recently suggested that RNase 8 might act through a different mechanism in light of the hydrophilic nature of its amino terminal extension (Chan et al., 2012).

RNase 4 is the shortest member among human RNase A representatives, with a primary structure of 119 residues. It is also the most evolutionarily conserved orthologue among mammalian species, showing ~90% identity with bovine, porcine, and rat RNases (Fig. 1) (Zhou and Strydom, 1993). Because of the high degree of conservation among mammalian species, it is hypothesized that RNase 4 plays a crucial biological function. Although this role remains elusive, RNase 4 has been linked to *in vitro* cytotoxicity in carcinoma cell lines, together with the induction of cellular migration and the proliferation of human umbilical vein endothelial cells (Di Liddo et al., 2010).

RNase 4 retains all the important catalytic residues found in members of the RNase A superfamily (His12, His116 and Lys40, Fig. 2), in addition to 8 strictly conserved cysteine residues involved in the formation of 4 disulfide bridges. It contains a unique deletion site of two residues (residues 77–78, RNase 1 numbering), and no motif for *N*-linked carbohydrates (Zhou and Strydom, 1993). Like RNase 5 (angiogenin), its first amino acid residue is a pyroglutamic acid (Hofsteenge et al., 1998). It has a sequence identity of 43%, 31%, 30% and 39% with human pancreatic RNase 1, eosinophil-derived neurotoxin (EDN, RNase 2), eosinophil cationic protein (ECP, RNase 3) and angiogenin (RNase 5), respectively (Zhou and Strydom, 1993).

The enzyme has a marked preference for uracil-containing RNA substrates, a unique feature shared with liver-type members of the family (Shapiro et al., 1986, Zhao et al., 1998). It was proposed that Phe42 (Val43 in RNase 1) and Arg41 (Pro42 in RNase 1), two residues located in the vicinity of the ligand, could potentially be responsible for this substrate selectivity (Zhou and Strydom, 1993).

The crystal structure of the free and d(Up)-bound RNase 4 was determined at atomic resolution (PDB entries 1RNF and 2RNF) (Terzyan et al., 1999). Here, we report on the ¹H, ¹³C and ¹⁵N backbone resonance assignments of RNase 4 (BMRB entry 19844), which will further be used to monitor backbone conformational exchange at different stages of the reaction coordinates. By comparing the dynamic profile of RNase 4 with that of previously reported pancreatic RNases (Gagné et al., 2012), we hope to further our understanding of the role played by protein motions during enzyme catalysis. We also ran a ConSurf analysis, a bioinformatics tool used to estimate the evolutionary conservation of amino acid positions based on the phylogenetic relations between homologous sequences (Ashkenazy et al., 2010). Results confirm the important conservation of key residues involved in ligand binding among vertebrates.

Methods and experiments

DNA constructs, expression and purification of RNase 4

The cDNA sequence of human RNase 4 was codon-optimized for *Escherichia coli* expression and subcloned into a *NdeI/HindIII*-digested pJexpress414 vector under the control of the T7 promoter (DNA2.0, Menlo Park, CA USA). The construct was transformed into *E. coli* BL21(DE3) and protein expression was induced with 1 mM IPTG for 4 hours in $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled M9 minimal medium supplemented with non-essential amino acids (Invitrogen, Grand Island, NY US), metals and ammonium acetate. Cells were lysed by sonication and inclusion bodies were recovered by centrifugation. Proteins were refolded using a combination of oxidized/reduced glutathione for 72 hours, as described (Doucet et al., 2009). RNase 4 was purified on HiTrapQ HP and MonoS ion exchange columns using an ÄKTA Purifier (GE Healthcare, Piscataway, NJ US). The final purity was estimated by SDS-PAGE and a total of 12.8 mg of >98% pure RNase 4 was obtained from 2 L of cell culture.

Solution NMR spectroscopy

NMR samples were prepared in 15 mM sodium acetate and pH 5.0. The protein was quantified by UV-Vis spectrophotometry using a theoretical extinction coefficient of 7082 $\text{M}^{-1}\text{cm}^{-1}$. All NMR experiments were recorded at 298 K on samples containing 0.5–0.7 mM $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ -labeled RNase 4 in 15 mM sodium acetate at pH 5.0 with 10% $^2\text{H}_2\text{O}$ in 5-mm Shigemi NMR tubes filled with 350 μL of protein solution. NMR experiments were carried out on Agilent 500 MHz and 800 MHz NMR spectrometers equipped with triple-resonance cold probes and pulsed-field gradients. For backbone resonance assignments of wild-type human RNase 4, 2D HSQC, 3D TOCSY-HSQC and 3D NOESY-HSQC were collected and sequence-specific assignments of the backbone atoms were achieved by running a series of independent CBCA(CO)NH, HNCACB and HNCO 3D experiments. All heteronuclear NMR experiments were processed by NMRPipe and analyzed using Sparky and NMRView.

Evolutionary conservation of amino acid positions in RNase 4

The evolutionary conservation of amino acid positions based on the phylogenetic relations between homologous sequences was performed using the ConSurf Server (<http://consurf.tau.ac.il/>) (Ashkenazy et al., 2010). The sequence alignment was built using MAFFT and the orthologues were collected from UNIREF90 using the CS-BLAST algorithm with an E-value of 0.0001. The minimum and maximum %ID for homologues were respectively 35 and 95, with a maximum number of 150 sequences. A Bayesian method was used for the conservation score calculation with a JTT model of substitution.

Results

Assignments and data deposition

The ^1H - ^{15}N two-dimensional HSQC spectrum of RNase 4 shows well-dispersed resonances, typical of other pancreatic-type RNases (Fig. 3). The enzyme contains 4 proline residues, so the expected number of backbone ^1H - ^{15}N peaks was 114 (excluding N-terminal). From this

number, 110 backbone ^1H - ^{15}N peaks could be assigned, corresponding to 96.5% of the total number of non-proline residues. Similarly, resonance assignments were achieved for 93% of non-proline $\text{C}\alpha$ and 96.3% of non-proline and non-glycine $\text{C}\beta$. The chemical shift assignments have been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 19844. Secondary structure assignments were corroborated by assignment of sequential and medium range NOEs in ^{15}N -edited three-dimensional NOESY-HSQC spectra and from measurements of ^1H side chains in ^1H - ^{15}N TOCSY-HSQC experiments. Most unassigned residues are located on the C-terminal region of the protein, illustrating possible exchange broadening caused by increased millisecond dynamics. The chemical shift assignment of resonances for human RNase 4 is the first step in the analysis of protein structure and dynamics, free in solution and in complex with ligands.

Evolutionary conservation of amino acid positions in RNase 4

The ConSurf Server was used to estimate evolutionary conservation of amino acid positions based on the phylogenetic relations between RNase 4 orthologues. Using free human RNase 4 (PDB entry 1RNF), a conservation analysis among family members provided a total of 194 hits in CSI-BLAST, 188 of them being unique sequences. The final calculation was performed on the 150 sequences with the lowest E-value.

The conservation scores calculated by ConSurf are shown on the 3D structure of RNase 4 (Fig. 4). $\beta 1$, $\beta 2$ (residues 78–81), $\beta 3$ (residues 103–107) and $\beta 4$ demonstrate the most conserved regions between RNase 4 homologues, while $\alpha 1$, $\beta 3$ (residues 95–102), L2 and L4 show moderately conserved regions. As expected, high scores were observed for all cysteines (Cys25, Cys39, Cys57, Cys64, Cys71, Cys81, Cys92 and Cys107), an inherent structural characteristic shared by the vast majority of RNase A homologues. Many residues involved in ligand binding also showed high scores. It was previously shown that conserved residues Gln11, His12, Lys40, Asn43, Thr44, Cys64, Asn70, Ala106, Val115, His116 and Phe117 are involved in d(CpA), 5'-ADP, 2',5'-ADP, dUp, 5'-UDP or 3'-CMP binding to RNase A (Zegers et al., 1994, Leonidas et al., 2003, Jenkins et al., 2005). Although RNase A family members demonstrate important variations in substrate affinities, this binding core remains highly conserved throughout evolution.

Conclusion

Backbone ^1H , ^{13}C , and ^{15}N resonance assignments for RNase 4 were achieved using a series of two- and three-dimensional resonance experiments. These assignments are extremely valuable in providing a framework by which to compare functional and dynamic properties of RNase 4 with its structural homologues, as well as studying key residues involved in the binding of various ligands with the enzyme.

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References

- Ashkenazy H, Erez E, Martz E, Pupko T, Ben-Tal N. ConSurf 2010: calculating evolutionary conservation in sequence and structure of proteins and nucleic acids. *Nucleic Acids Res.* 2010; 38:W529–33. [PubMed: 20478830]
- Boix E, Blanco JA, Nogues MV, Moussaoui M. Nucleotide binding architecture for secreted cytotoxic endoribonucleases. *Biochimie.* 2013; 95:1087–97. [PubMed: 23274129]
- Bystrom J, Amin K, Bishop-Bailey D. Analysing the eosinophil cationic protein--a clue to the function of the eosinophil granulocyte. *Respir Res.* 2011; 12:10. [PubMed: 21235798]
- Chan CC, Moser JM, Dyer KD, Percopo CM, Rosenberg HF. Genetic diversity of human RNase 8. *BMC Genomics.* 2012; 13:40. [PubMed: 22272736]
- Cranston JW, Perini F, Crisp ER, Hixson CV. Purification and properties of ribonucleases from human urine. *Biochim Biophys Acta.* 1980; 616:239–58. [PubMed: 7213638]
- De Prisco R, Sorrentino S, Leone E, Libonati M. A ribonuclease from human seminal plasma active on double-stranded RNA. *Biochim Biophys Acta.* 1984; 788:356–63. [PubMed: 6466685]
- Di Liddo R, Dalzoppo D, Baiguera S, Conconi MT, Dettin M, Parnigotto PP, et al. In vitro biological activity of bovine milk ribonuclease-4. *Mol Med Report.* 2010; 3:127–32.
- Doucet N, Watt ED, Loria JP. The flexibility of a distant loop modulates active site motion and product release in ribonuclease A. *Biochemistry.* 2009; 48:7160–8. [PubMed: 19588901]
- Durack DT, Ackerman SJ, Loegering DA, Gleich GJ. Purification of human eosinophil-derived neurotoxin. *Proc Natl Acad Sci U S A.* 1981; 78:5165–9. [PubMed: 6946462]
- Gagné D, Charest LA, Morin S, Kovrigin EL, Doucet N. Conservation of flexible residue clusters among structural and functional enzyme homologues. *J Biol Chem.* 2012; 287:44289–300. [PubMed: 23135272]
- Hofsteenge J, Vicentini A, Zelenko O. Ribonuclease 4, an evolutionarily highly conserved member of the superfamily. *Cell Mol Life Sci.* 1998; 54:804–10. [PubMed: 9760989]
- Iwama M, Kunihiro M, Ohgi K, Irie M. Purification and properties of human urine ribonucleases. *J Biochem.* 1981; 89:1005–16. [PubMed: 6788751]
- Jenkins CL, Thiagarajan N, Sweeney RY, Guy MP, Kelemen BR, Acharya KR, et al. Binding of non-natural 3'-nucleotides to ribonuclease A. *FEBS J.* 2005; 272:744–55. [PubMed: 15670155]
- Leonidas DD, Chavali GB, Oikonomakos NG, Chrysina ED, Kosmopoulou MN, Vlassi M, et al. High-resolution crystal structures of ribonuclease A complexed with adenylic and uridylic nucleotide inhibitors. Implications for structure-based design of ribonucleolytic inhibitors. *Protein Sci.* 2003; 12:2559–74. [PubMed: 14573867]
- Mizuta K, Awazu S, Yasuda T, Kishi K. Purification and characterization of three ribonucleases from human kidney: comparison with urine ribonucleases. *Arch Biochem Biophys.* 1990; 281:144–51. [PubMed: 2383019]
- Notredame C, Higgins DG, Heringa J. T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol.* 2000; 302:205–17. [PubMed: 10964570]
- Rabin EZ, Tattire B. The isolation, purification, and properties of a ribonuclease (Mr 18 000) from human uremic serum, and its relation to the human urinary ribonuclease (Mr 33 000). II. Properties of the enzymes. *Biochim Biophys Acta.* 1982; 701:132–7. [PubMed: 7055583]
- Sayle RA, Milner-White EJ. RASMOL: biomolecular graphics for all. *Trends Biochem Sci.* 1995; 20:374. [PubMed: 7482707]
- Shapiro R, Fett JW, Strydom DJ, Vallee BL. Isolation and characterization of a human colon carcinoma-secreted enzyme with pancreatic ribonuclease-like activity. *Biochemistry.* 1986; 25:7255–64. [PubMed: 3467790]
- Sorrentino S. The eight human “canonical” ribonucleases: molecular diversity, catalytic properties, and special biological actions of the enzyme proteins. *FEBS Lett.* 2010; 584:2194–200. [PubMed: 20388512]
- Strydom DJ, Fett JW, Lobb RR, Alderman EM, Bethune JL, Riordan JF, et al. Amino acid sequence of human tumor derived angiogenin. *Biochemistry.* 1985; 24:5486–94. [PubMed: 2866794]

- Terzyan SS, Peracaula R, de Llorens R, Tsushima Y, Yamada H, Seno M, et al. The three-dimensional structure of human RNase 4, unliganded and complexed with d(Up), reveals the basis for its uridine selectivity. *J Mol Biol.* 1999; 285:205–14. [PubMed: 9878400]
- Weickmann JL, Elson M, Glitz DG. Purification and characterization of human pancreatic ribonuclease. *Biochemistry.* 1981; 20:1272–8. [PubMed: 6784751]
- Zegers I, Maes D, Dao-Thi MH, Poortmans F, Palmer R, Wyns L. The structures of RNase A complexed with 3'-CMP and d(CpA): active site conformation and conserved water molecules. *Protein Sci.* 1994; 3:2322–39. [PubMed: 7756988]
- Zhang J, Dyer KD, Rosenberg HF. Human RNase 7: a new cationic ribonuclease of the RNase A superfamily. *Nucleic Acids Res.* 2003; 31:602–7. [PubMed: 12527768]
- Zhang J, Dyer KD, Rosenberg HF. RNase 8, a novel RNase A superfamily ribonuclease expressed uniquely in placenta. *Nucleic Acids Res.* 2002; 30:1169–75. [PubMed: 11861908]
- Zhao W, Kote-Jarai Z, van Santen Y, Hofsteenge J, Beintema JJ. Ribonucleases from rat and bovine liver: purification, specificity and structural characterization. *Biochim Biophys Acta.* 1998; 1384:55–65. [PubMed: 9602056]
- Zhou HM, Strydom DJ. The amino acid sequence of human ribonuclease 4, a highly conserved ribonuclease that cleaves specifically on the 3' side of uridine. *Eur J Biochem.* 1993; 217:401–10. [PubMed: 8223579]

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Homo sapiens      1 QDGM YQRLRQHVP EETGGSDRYCNLMMORRKMTLYHCKRFNTFIHEDIWNIRSICSTT
Pan troglodytes   1 QDGM YQRLRQHVP EETGGSDRYCNLMMORRKMTLYHCKRFNTFIHEDIWNIRSICSTT
Bos taurus        1 QDGM YQRLRQHVP EETGGSDRYCNLMMORRKMTSHQCKRFNTFIHEDIWNIRSICSTT
Sus scrofa        1 QDGM YQRLRQHVP EETGGSDRYCNLMMORRKMTSHYCKRFNTFIHEDIWNIRSICSTT
Rattus norvegicus 1 QDGM YQRLRQHVP EETGGSDRYCNLMMORRKMTSTQCKRFNTFIHEDIWNIRSICSTT
Mus musculus      1 QDGM YQRLRQHVP EATGGSDRYCNLMMORRKMTSVQCKRFNTFIHEDIWNIRSICSTT

Homo sapiens      61 NITCKNGRMNCHEGVVKVTDCRDTGSSRAPNCRYRAASTRRVVIACEGNFQVPVHFDDG
Pan troglodytes   61 NITCKNGRMNCHEGVVKVTDCRDTGSSRAPNCRYRAASTRRVVIACEGNFQVPVHFDDG
Bos taurus        61 NITCKNGRMNCHEGVVKVTDCRDTGSSRAPNCRYRAASTRRVVIACEGNFQVPVHFDDK
Sus scrofa        61 NITCKNGRMNCHEGVVKVTDCRDTGSSRAPNCRYRAASTRRVVIACEGNFQVPVHFDDK
Rattus norvegicus 61 NITCKNGRMNCHEGVVKVTDCRDTGSSVPHNCRYRAASTRRVVIACEGNFQVPVHFDDR
Mus musculus      61 NITCKNGRMNCHEGVVKVTDCRDTGNSKAPNCRYRAASTRRVVIACEGNFQVPVHFDDR

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Fig. 1.

Sequence alignment of RNase 4 members found among vertebrates. Alignment was performed with T-Coffee (Notredame et al., 2000) using the following orthologues: *Homo sapiens* (human), *Pan troglodytes* (chimpanzee), *Bos taurus* (cattle), *Sus scrofa* (wild boar), *Rattus norvegicus* (brown rat) and *Mus musculus* (mouse). Residues showing >90% identity (similarity) are highlighted in black (grey). Alignment was processed with BoxShade 3.21.

Fig. 2. Schematic representation of human RNase 4. Three-dimensional view of RNase 4 (PDB entry 1RNF) highlighting the approximate active-site position of 3'-UMP (red), resulting from a structural overlay with RNase A-3'-UMP (PDB entry 1O0N). Catalytic residues His12, Lys40, His116 and Lys65 are shown as blue sticks. PyMOL was used to build all structures (Sayle and Milner-White, 1995).

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Fig. 4. Evolutionary conservation of amino acid positions among vertebrate RNase 4 members. Residue positions are colored by their conservation grades using the color-coding bar, with turquoise-through-maroon indicating variable-through-conserved residues. Color ramping is identified on (A) the structure and (B) the amino acid sequence of RNase 4 (PDB entry 1RNF). The figure was generated using a PyMOL script generated by the ConSurf Server.