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Ribonuclease 4, an evolutionarily highly conserved member of the superfamily

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Abstract. The structural and enzymatic properties of RNase 4 are reviewed. This RNase shows a much higher interspecies similarity (approximately 90%) than the other members of the RNase A superfamily. The enzyme is ubiquitous, with the highest amounts present in liver and lung. Its unique uridine specificity results from alterations in and around the pyrimidine-binding site. In particular, the shortened C-terminus and the side chains of Phe-42, Asp-80 and Arg-101 appear to be involved. RNase 4 binds tightly to the intracellular RNase inhibitor, with a K_d of 4×10^{-15} M.

Key words. RNase substrate specificity; RNase evolution; RNase inhibitor.

Introduction

RNase 4 has been isolated from different animals, and appeared in the literature under various names. Shapiro et al. isolated a 'tumour RNase' from human colon carcinoma cells (HT-29) with enzymatic properties that differed from all then known RNases [1]. Independently, Morita et al. obtained chromatographic and immunological evidence for the existence of at least seven different RNases in bovine tissues and body fluids, amongst them RNase BL4 [2]. In a search for RNases that interact with porcine RNase inhibitor (RI; [3]), Hofsteenge et al. identified RNase PL3 and determined its primary structure [4]. The soon thereafter reported amino acid sequences of RNase BL4 [5] and tumour RNase from human plasma [6] demonstrated the three enzymes to be orthologous. Following the proposal of Zhou and Strydom, we will use the name RNase 4 [6].

Structural properties

The primary structure of RNase 4 from human [6], cow [5] and pig [4] has been determined by protein sequencing, whereas that of rat was deduced from the complementary DNA (cDNA) sequence [7]. The enzyme consists of a polypeptide chain of 119 amino acids (fig. 1), which contains a pyroglutamic acid residue at position 1. None of the enzymes contains a recognition site for N-glycosylation. The sequence of RNase 4 is most similar to that of RNase 1 (table 1), with conservation of the four disulphide bridges, as well as catalytically important residues (His-12, His-119, Lys-41)**. In addition, several residues that are important for substrate binding, Phe-120, Thr-45, Asn-44 and Lys-66**, have also been conserved.

No experimentally determined three-dimensional structure of RNase 4 is available yet. A model obtained by comparative modelling, using the computer program Swiss-Model [8], displays an overall fold which is very similar to that of RNase A (fig. 2). The major difference that is predicted is the conformation of the loop 58–71.

A salient feature of the RNase 4 family, which distinguishes it from other RNase families, is its high interspecies similarity (~90%). The four sequences in figure 1 differ only in 26 positions, and 11 of these contain conservative substitutions. This degree of conservation (81–94% identity) is much higher than for other RNase families in the same animals (table 2).

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Apparently, there exists strong evolutionary pressure to maintain the structure and function of RNase 4. This strongly suggests that RNase 4 performs a more specialized function than simply digestion or 'cleaning-up' of RNA [6]. It probably involves specific RNA-protein interactions, since RNase 4 displays a high degree of preference for cleavage after uridine (see below). Whether, in addition, specific interactions with proteins or other biomolecules have contributed to the strong structural conservation is presently unclear.

Molecular biology

The nucleotide sequence of the cDNA encoding RNase 4 from pig [9], humans [10] and rat [7], as well as that of the human gene [11], has been determined. The human genome contains a single copy of the gene encoding RNase 4, which is located on chromosome 14 [11]. The intronless gene is transcribed into a messenger RNA (mRNA) of ~2 kb [11, 12] that is found in most tissues. It is present at the highest level in liver, but is absent from brain (table 3). In porcine tissues RNase 4 mRNA is most abundant in liver and lung. In contrast to humans, it can also clearly be detected in brain but not in kidney, thymus or testis [9]. At present, it is not known which cells in a particular tissue express RNase 4, nor whether the levels of mRNA correlate with that of the RNase 4 protein. The mRNA encoding RNase 4 differs in two respects from that of other RNases. Both in porcine and human tissues, its size is significantly larger than that of the mRNA encoding other human RNases [11]. In addition, in human liver, kidney and pancreas a second, larger mRNA (~2.4 kb) exists [11, 12].

RNase 4 is also detectable in human white blood cells. Using Northern analysis, Futami et al. demonstrated its mRNA in human peripheral blood leukocytes [12]. More specifically, Egesten et al. obtained immunological evidence for the presence of RNase 4 in the cytoplasmic granules of peripheral blood monocytes, but not in granulocytes or lymphocytes. The mRNA could, how-

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          1                   10                  20                  30
porcine   <Q D R M Y Q R F L R Q H V D P D . A T G G N D A Y C N L M M Q
bovine    <Q D R M Y Q R F L R Q H V D P D . E T G G N D S Y C N L M M Q
human     <Q D G M Y Q R F L R Q H V H P E . E T G G S D R Y C N L M M Q
rat       <Q D R M Y Q R F L R Q H V D P E . G T G G S D N Y C N V M M Q
RNase A    K E T A A A K F E R Q H M D S S T S A A S S S N Y C N Q M M K
          1                   10                  20                  30
                              # *

                             40                  50                  60
porcine    R R K M T S H Y C K R F N T F I H E D I W N I R S I C S T S
bovine     R R K M T S H Q C K R F N T F I H E D L W N I R S I C S T T
human      R R K M T L Y H C K R F N T F I H E D I W N I R S I C S T T
rat        R R R M T S T Q C K R F N T F I H E D I W N I R S I C D T A
RNase A    S R N L T K D R C K P V N T F V H E S L A D V Q A V C S Q K
                       40                  50                  60
                         *     # #

                             70                  80                  90
porcine    N I Q C K N G Q M N C H E . . G V V K V T D C R E T G S S R A P
bovine     N I Q C K N G Q M N C H E . . G V V R V T D C R E T G S S R A P
human      N I Q C K N G K M N C H E . . G V V K V T D C R D T G S S R A P
rat        N I P C K N G N M N C H E . . G I V R V T D C R E T G S S V P H
RNase A    N V A C K N G Q T N C Y Q S Y S T M S I T D C R E T G S S K Y P
                       70                  80                  90
                #

                            100                 110                 119
porcine    N C R Y R A M A S T R R V V I A C E G N P E V P V H F D K
bovine     N C R Y R A K A S T R R V V I A C E G N P E V P V H F D K
human      N C R Y R A I A S T R R V V I A C E G N P Q V P V H F D G
rat        N C R Y R A R A S T R R V V I A C E G T P E V P V H F D R
RNase A    N C A Y K T T Q A N K H I I V A C E G N P Y V P V H F D A S V
                  100                 110                 120     124
                                      #                   * # *   #
```

Figure 1. Comparison of the primary structures of RNase 4 from pig, humans, cow and rat. The sequence of bovine pancreatic RNase A is shown for comparison. Residues that have not been conserved between RNase 4s are shown in bold. Catalytic residues have been indicated with '*', whereas residues that are involved in substrate binding have been indicated with '#'. <Q denotes pyroglutamic acid. The sequences of pig, cow and humans were taken from the SWISSPROT database and that of rat from ref. 7. The RNase 4 numbering is shown above the structure, whereas that of RNase A is shown underneath.

Table 1. Percentage sequence identity between RNase 4 and various RNases, calculated for several animals*.

Species	RNase 1	RNase 2	RNase 3	Angiogenin	RNase 6†
Human	43	31	30	39	32
Cow	47	−‡		41	36
Pig	47	−‡		37	41
Rat	46	36‡		nd	nd

*Amino acid sequences were obtained from the SWISSPROT and GENEMBL databases and compared using the program 'Gap' in the Wisconsin Package Version 9.1, GCG, Madison, WI. †This enzyme is called RNase k6 in ref. 34. ‡Distinct genes for RNases 2 and 3 occur only in primates. Therefore, in nonprimates no distinction can be made between these two RNases. Rat eosinophil cationic protein [35] is equally similar to human RNase 2 and 3 (~50%).

Table 2. Percentage sequence identity of a particular RNase in various animalsa*.

	RNase 4	RNase 1	RNase 6	Angiogenin
Human/cow	88	76	72	64
Human/pig	87	74	72	68
Human/rat/mouse†	81	62	nd	77
Cow/pig	94	79	75	68
Cow/rat	84	65	nd	nd
Pig/rat	83	65	nd	nd

*For details see legend to Table 1. †Angiogenin only.

Table 3. Distribution of the mRNA encoding RNase 4 (from refs 9, 11 and 12).

Tissue	Humans	Pig
Brain	−	+
Heart	+	+/−
Kidney	+	−
Liver	+++	++
Lower gut	+	+
Lung	+	++
Muscle	+	+
Ovary	+	+
Spleen	+	+
Stomach	nd	+
Testis	+	−
Thymus	+	−
Upper gut	+	+
Uterus	nd	+
Pancreas	+	nd
Peripheral blood leukocytes	+	nd
Placenta	+/−	nd
Prostate	+	nd

Table 4. Substrate specificity of RNase 4 from different species.

	k_{cat}/K_m (M^{-1} s^{-1})	
	human*	porcine†
UpA	2.5×10^5	2.5×10^5
CpA	6.3×10^2	6.7×10^2

	Relative activity					
	human*	porcine†		bovine		rat‡
Poly(C)	1	1	1	1	1	1
Poly(U)	> 650	66	17‡	0.18§	6‡	20

* From Shapiro et al. [1]. † From Vicentini et al. [9]. ‡ From Zhao et al. [7]. § From Hosoya et al. [5].

ever, only be detected after culturing monocytes for 16 h. It has been suggested that the appearance of the mRNA correlates with the differentiation into macrophages [13]. It is not entirely clear how these results fit in with those obtained with human promyelocytic leukaemia cells (HL-60) in culture. In that case, RNase 4 mRNA synthesis is induced when HL-60 cells are differentiating towards the neutrophil lineage, but not when converting to the monocyte/macrophage lineage [11].

Enzymatic properties

Like all RNases of this superfamily, RNase 4 only cleaves RNA after pyrimidine bases [1]. Whereas the prototypical RNase A from bovine pancreas has a preference for cytidine over uridine, human RNase 2 and 3 [14], iguana RNase 1 [15], frog RNases [16] and the four RNase 4s (table 4) show a preference for uridine. For RNase 4 this specificity is very pronounced and has been observed with cyclic nucleotides, dinucleotides and homopolymers [1, 6]. Although the results with the latter substrates seem to differ between species (table 4), this almost certainly reflects differences in the assays used (e.g. pH, temperature and methods of detection of cleavage), rather than intrinsic differences in the molecules. This is corroborated by the close agreement between the values of k_{cat}/K_m for the cleavage of uridylyl-3′,5′-adenosine (UpA) and cytidylyl-3′,5′-adenosine (CpA) by the human and porcine enzyme, which were obtained under identical conditions [1, 6]. The initial observation that bovine RNase 4 is cytidine-preferring [5] has been proposed to

result from a possible contamination with another RNase [6]. Indeed, a reexamination of another preparation of this enzyme by Zhao et al. showed its specificity for polyuridylic acid (poly(U)) over polycytidylic acid (poly(C)) [7].

Comparison of the values of k_{cat}/K_m for the cleavage of UpA and CpA by RNase A (3.5×10^6 and 4.5×10^6 M^{-1} s^{-1}) and RNase 4 (table 4) reveals that the uridine specificity of the latter results from a suppression of the activity with cytidine-containing substrates. This difference is most likely due to dissimilarities in the structural details of the pyrimidine-binding pockets of RNase 4 and RNase A. A brief examination of the binding of pyrimidines to RNase A is, therefore, useful. The pyrimidine-binding pocket (B1 site; [17]) is formed by His-12, Val-43, Asn-44, Thr-45 and Phe-120, with the main interactions formed by hydrogen bonds with Thr-45 and van der Waals contacts with Phe-120. The main chain NH of Thr-45 donates a hydrogen bond to O2 of either pyrimidine base, whereas its $^{\gamma}$OH accepts a hydrogen bond from NH-3 of uridine and donates a hydrogen bond to N-3 of cytidine [17]. In addition to these residues, the $^{\gamma}$OH of Ser-123 has been implicated in the binding of uridine, either by hydrogen bonding directly [18], or via water molecules [19] to O4. This amino acid does not, however, play a role in the binding of cytidine [18, 20]. Another auxiliary residue is Asp-83. Although it does not directly contact the pyrimidine base, it influences binding of uridine through accepting a hydrogen bond from $^{\gamma}$OH of Thr-45 [21].

The structural features that suppress CpA cleavage have been examined by site-directed mutagenesis of porcine RNase 4 [6, 22]. Although Thr-45 has been conserved in all RNases sequenced to date, its function does not appear to be conserved. Mutation of Thr-45 into Ala in RNase A decreases the cleavage of uridine- and cytidine-containing substrates to approximately the same extent [23]. In angiogenin, however, it reduces $(k_{cat}/K_m)_{CpA}$ 10-fold stronger than $(k_{cat}/K_m)_{UpA}$. Surprisingly, the T44A mutation in RNase 4 increases $(k_{cat}/K_m)_{CpA}$ 5-fold but at the same time reduces $(k_{cat}/K_m)_{UpA}$ 300-fold [22]! Thus, the side chain of Thr-44 appears to be part of the structure that suppresses the cleavage of CpA. Apparently, the exact role of Thr-45 depends on structural features unique to each RNase.

In RNase 4, one of the residues that could influence Thr-44 is Phe-42 (Val-43 in RNase A). Model building suggests that this large aromatic residue can reach up to

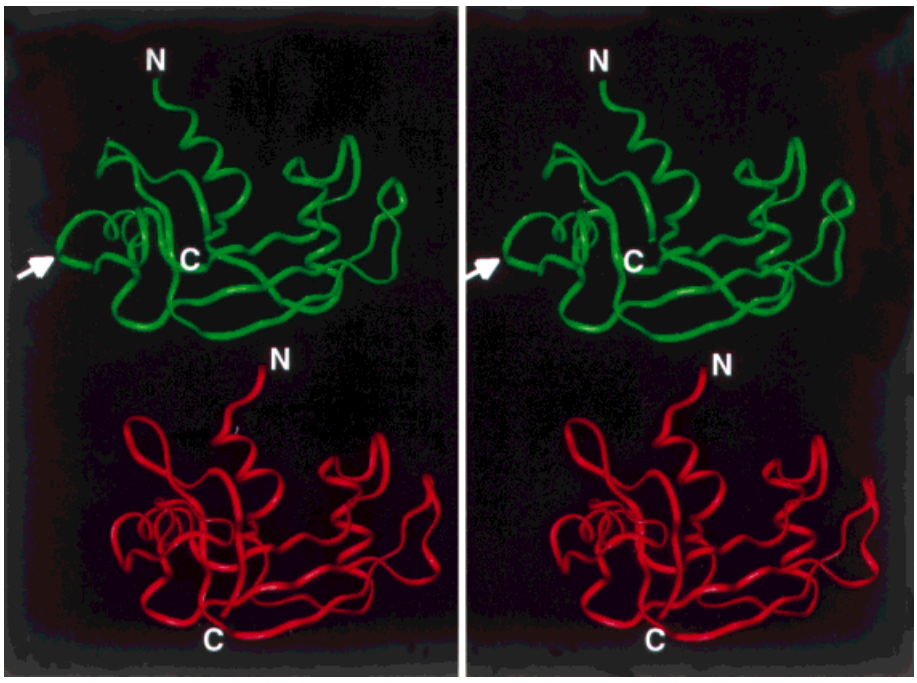

Figure 2. Three-dimensional model of RNase 4. Stereoview of the model of RNase 4, obtained by comparative modelling using the program Swiss-Model [8]. The backbone of RNase 4 is depicted in green, whereas that of bovine pancreatic RNase A is shown in red. The loop 58–71, which is predicted to have a different conformation, is indicated with an arrow. A complete set of coordinates is available upon request.

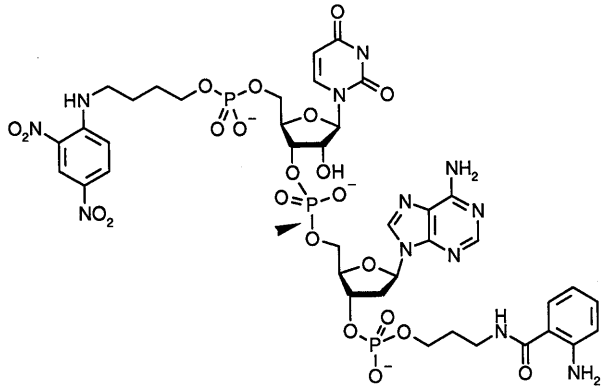

Figure 3. Structure of the fluorogenic RNase substrate DNPp **UpdA**paa [28]. The scissile bond has been indicated with an arrowhead.

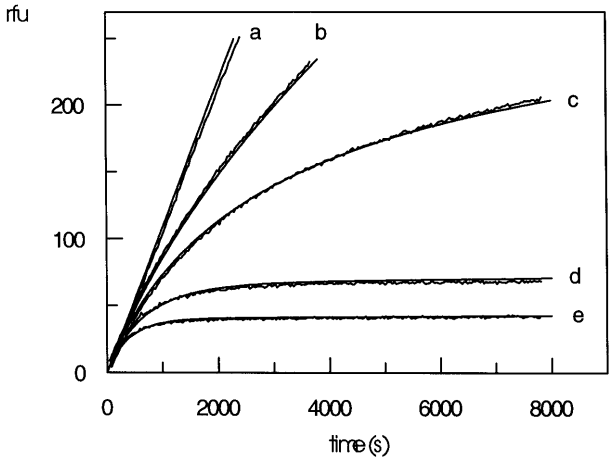

Figure 4. Slow tight-binding inhibition of porcine RNase 4 by porcine RI. Different concentrations of RI were incubated with 37.2 μM substrate (DNPpdApdApdAp**UpdA**paa), and the reaction was started by adding RNase 4 (5.8 pM) under the conditions described in ref. 24. Product formation was monitored by the increase in fluorescence at 415 nm. (a) 0 pM RI, (b) 4.1 pM RI, (c) 6.84 pM 19 RI, (d) 13.67 pM RI, (e) 20.5 pM RI. The lines represent the best fit of the data to the slow, tight-binding equation [30], with the parameters mentioned in the text. rfu, relative fluorescence units.

and possibly interact with Thr-44. Indeed the mutation F42V increases $(k_{cat}/K_m)_{CpA}$ 19-fold, but $(k_{cat}/K_m)_{UpA}$ only 3-fold. More interestingly, the improving effect of the T44A mutation on the cleavage of CpA was not observed in the F42V mutant [22]. This shows that Phe-42 also suppresses CpA cleavage and strongly suggests that part of its effect is, directly or indirectly, exerted by influencing Thr-44. An indirect effect could involve Asp-80 (Asp-83 in RNase A). As pointed out above, in RNase A this residue makes a hydrogen bond to $^{\gamma}$OH of Thr-45, improving cleavage of poly(U). It has, on the other hand, hardly any influence on the cleavage of poly(C) [21]. In line with the observation in RNase A, the mutation D80A in RNase 4 reduces $(k_{cat}/K_m)_{UpA}$. Unexpectedly, however, the mutation improved the cleavage of CpA (A. Vicentini and J. Hofsteenge, unpublished results), such that RNase 4 D80A is strongly cytidine-preferring. So far three residues, Phe-42, Thr-44 and Asp-80, have been identified that suppress the cleavage of CpA. Two of these, Thr-44 and Asp-80, have, on the other hand, a positive influence on the cleavage of UpA. This kind of differential behaviour has not been observed in RNase A and angiogenin, and it points once more to the fact that the character of the pyrimidine-binding pocket of each of these RNases is unique. Therefore, a complete interpretation of the role of the residues in RNase 4 that suppress CpA cleavage will crucially depend on the determination of the three-dimensional structure of this RNase.

Another region which is different in RNase 4 compared with, for example, RNase A and could affect the substrate specificity is the C-terminus which in RNase 4 is two residues shorter [9]. This could influence the properties of the B1 site through the absence of a residue equivalent to Ser-123. Furthermore, the negatively charged α-carboxylate would be close to the B1 pocket of RNase 4. Alternatively, the shortened C-terminus in RNase 4 may create the space for another residue to point into, and form part of, the B1 site [6].

Interaction of RNase 4 with the RNase inhibitor

All investigated mammalian RNases of this superfamily interact with a 50-kDa RNase inhibitor (RI) that occurs in the cytoplasm of virtually all mammalian cells. The inhibitor forms a very tight, reversible, one-to-one complex with RNase [3]. The dissociation constant of the enzyme inhibitor complex, as well as the association and dissociation rate constants have been determined for RNase A ($K_i = 67$ fM [24, 25]), angiogenin ($K_i = 0.7$ fM [25, 26]) and human RNase 2 ($K_i = 0.9$ fM [27]), showing them to be amongst the strongest protein-protein interactions known. A two-step mechanism of inhibition was demonstrated [24, 25]. The first step involves the rapid formation of an enzyme-inhibitor complex, EI, followed by a slower isomerization of EI to a tight complex EI*:

$$E + I \rightleftharpoons EI \rightleftharpoons EI^*$$

Preliminary data have been reported for the interaction of porcine RNase 4 with human RI, showing an upper limit for the dissociation constant of 40 pM. Competition experiments revealed, however, that the binding is at least nine-fold tighter than that of RNase A [1]. In order to facilitate the study of the RNase-RI interaction, Zelenko et al. have developed a fluorogenic RNase substrate consisting of the dinucleotide UpdA to which a fluorophore, *o*-aminobenzoic acid (aa), and a quencher, 2,4-dinitroaniline (DNP) have been attached via phosphodiester linkages (DNPp**UpdA**paa; fig. 3) [28]. Cleavage of the phosphodiester bond on the 3′ side of uridine causes a large increase in fluorescence. The value of k_{cat}/K_m of RNase 4 with this substrate is 18-fold higher than with UpA [29]. A further 13-fold increase could be obtained by introducing three deoxyadenosine-phosphate moieties (pdA) on the 5′ side of the uridine (DNPpdApdApdAp**UpdA**paa). Most important, the K_m of this substrate for porcine RNase 4 is 4 μM [29]. The high turnover number of this substrate allows the detection of low pM concentrations of RNase, a condition necessary to measure the tight interaction between RI and RNase 4. Furthermore, by using substrate concentrations several times the value of K_m, the tightness of the interaction can be attenuated such that the apparent inhibition constant becomes measurable (see e.g., ref. 30). Using this substrate, progress curves of the inhibition of porcine RNase 4 by porcine RI have been measured at five inhibitor concentrations (fig. 4). Analysis of the data according to the equation for slow, tight-binding inhibition [30] yields a dissociation constant of $4.0 +/- 0.4$ fM and association and dissociation rate constants of 1.53×10^8 M^{-1} s^{-1} and 6.1×10^{-7} s^{-1}, respectively [29]. RNase 4 binds RI 17-fold tighter than RNase A (due to a decrease in the dissociation rate constant), which corresponds to an increase in binding energy of nearly 7 kJ/mol. The three-dimensional structures of the pRI-RNase A [31] and hRI-angiogenin complex [32] have been reported. A comparison of these, together with mutagenesis studies [33], reveals that a major portion of the binding energy is contributed by the C-terminus of RI (residues 430–456; pRI numbering) and active site residues of the enzymes (Gln-11, Lys-41 and His-119). Although these residues are also present in RNase 4, it will be more difficult to predict why RNase 4 binds stronger than RNase A. The structure of the complexes of RI with RNase A and angiogenin show that RI binds each of its ligands in a largely unique way. Even residues from the same region of the enzyme molecule interact in a different way with the inhibitor [32]. A detailed understanding of the RI-RNase 4 interaction will require the elucidation of the crystal structure.

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