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The contribution of noncatalytic phosphate-binding subsites to the mechanism of bovine pancreatic ribonuclease A

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Abstract. The enzymatic catalysis of polymeric substrates such as proteins, polysaccharides or nucleic acids requires precise alignment between the enzyme and the substrate regions flanking the region occupying the active site. In the case of ribonucleases, enzyme-substrate binding may be directed by electrostatic interactions between the phosphate groups of the RNA molecule and basic amino acid residues on the enzyme. Specific interactions between the nitrogenated bases and particular amino acids in the active site or adjacent positions may also take place. The substrate-binding subsites of ribonuclease A have been characterized by structural and kinetic studies. In addition to the active site (p_1), the role of other noncatalytic phosphate-binding subsites in the correct alignment of the polymeric substrate has been proposed. p_2 and p_0 have been described as phosphate-binding subsites that bind the phosphate group adjacent to the 3′ side and 5′ side, respectively, of the phosphate in the active site. In both cases, basic amino acids (Lys-7 and Arg-10 in p_2, and Lys-66 in p_0) are involved in binding. However, these binding sites play different roles in the catalytic process of ribonuclease A. The electrostatic interactions in p_2 are important both in catalysis and in the endonuclease activity of the enzyme, whilst the p_0 electrostatic interaction contributes only to binding of the RNA.

Key words. Ribonuclease; binding sites; RNA; catalysis; enzyme kinetics.

Introduction

Formation of the enzyme-substrate complex is a basic step in enzymatic catalysis. In the case of enzymes that catalyse reactions with low molecular mass substrates, binding takes place only at the active site of the enzyme. However, in the case of enzymes acting on polymeric substrates, the interaction may be more complex due to the need for a correct alignment between the enzyme and the substrate regions flanking that which actually occupies the active site. This is a general feature seen in all enzymes that catalyse the breakdown of polymeric substrates, such as proteins, polysaccharides and nucleic acids.

In the case of proteinases the noncatalytic binding subsites were designated as S1, S2, S3 and so on, and S′1, S′2, S′3 and so on (amino-terminal and carboxy-terminal residues next to the scissile peptide bond, respectively). Lysozyme is an example of a glycosidase that catalyses the hydrolysis of a complex polysaccharide. The binding of its substrate takes place in a well-defined deep cleft in which six binding subsites have been defined (A, B, C, D, E and F). Cleavage takes place in the glycosidic bond between the residues that bind to subsites D and E [1].

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Enzyme-substrate binding for nucleic acids may be very different because of the substrate structure (single- or double-stranded) and the specificity of the enzyme. The binding of DNase I, a nuclease that acts on double-stranded DNA but with a low specificity for base sequence has been analysed by X-ray crystallography. From the DNase I-d(GGTATACC)$_2$ complex it has been shown that DNase I binds in the minor groove of a right-handed DNA duplex, and to the phosphate backbones on either side over 5 bp resulting in a widening of the minor groove [2]. On the other hand, more specific enzymes such as type II restriction enzymes need the specific recognition of the substrate in the regions of the DNA near to the scissile bonds. Only in a few of these enzymes, notably Eco RI and Eco RV, has the substrate binding been analysed: each of these enzymes follows a quite different mechanism for DNA recognition and/or cleavage [3].

In the case of RNases and RNA, enzyme-substrate binding may be directed by electrostatic interactions between the phosphate groups of the substrate and basic amino acid residues (Lys and Arg) of the protein structure as well as by specific interactions between nitrogenated bases and amino acids in the active site or adjacent positions. Bovine pancreatic RNase A-binding subsites are the best characterised from the point of view of both structure and kinetics, but binding subsites in other RNases of the same superfamily such as onconase and eosinophil-associated RNases or microbial RNases such as barnase have also been analysed. According to the nomenclature for the RNase A-binding subsites [4], B_1R_1 corresponds to the enzyme region which binds the nucleoside moiety that is linked through the 3′ position to the phosphate group located at the phosphate-binding site p_1 in the active centre. B_2R_2 is the enzyme region which binds the nucleoside moiety that is linked through the 5′ position to the phosphate at p_1. p_0 and p_2 are additional noncatalytic phosphate-binding subsites (fig. 1).

Barnase

Barnase is an extracellular enzyme secreted by *Bacillus amyloliquefaciens* that shows RNase activity. From the catalytic point of view, the enzyme reaction is very similar to that of RNase A: a transphosphorylation from the 5′ position of a nucleotide to the 2′ position of the adjacent nucleotide takes place, and a 2′,3′-cyclic phosphate product is formed. In an aqueous environment a hydrolysis reaction that cleaves the 2′-phosphate bond forming a nucleoside 3′-phosphate eventually occurs. The proposed mechanism is similar to the acid-base mechanism described for RNase A, although barnase uses a histidine (His-102) and a glutamate (Glu-73) residues instead of two histidines (His-12 and -119) as in the case of RNase A [5]. Barnase is specific for phosphodiester bonds that have a guanine in the 3′ position (GpN) although the catalytic efficiency increases with the substrate length, and the presence of a phosphate group on the 3′ position of the second nucleotide increases the rate of the transphosphorylation reaction. This effect is more pronounced when a third cytidine nucleotide is present (GpUpC or GpApC). It has been suggested that the third base modifies the position of the second phosphate, lowering the tran-sition-state energy [6]. X-ray crystallography of the complex of barnase and the oligodeoxynucleotide d(CGAC) [7] indicates that the guanosine plays a major role in the binding of the tetranucleotide and that the adenine base also contributes to the binding. However, most of the interaction energy is provided by the negative charge on the 3′-phosphate of the guanosine nucleotide and, to a lesser extent, by that of the adenosine nucleotide interacting with basic groups of the enzyme.

Onconase

Onconase has a lower specific activity towards common substrates in relation to pancreatic RNase A. Onconase activity is higher for RNA and polyuridylic acid [poly(U)] than for polycytidylic acid [poly(C)], while pancreatic RNases prefer poly(C). Substrate preference for uridine in the B_1 site and guanosine in the B_2 site [8], as deduced from activity assays with dinucleotides, is shared with other frog RNases studied [9, 10]. A computer model of onconase with d(UpG) involves Lys-33 in uridine B_1 specificity (position of Val-43 in RNase A) and Ser-54, Asn-56 and Glu-91 (positions 65, 72 and 111 in RNase A) in the B_2 binding [11].

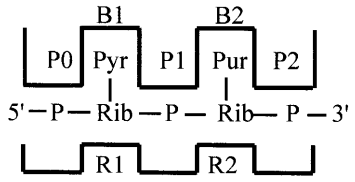

Figure 1. Schematic representation of the binding subsites of RNase A according to the nomenclature proposed in ref. 4. B corresponds to the nitrogenated base-binding sites. B_1 is specific for pyrimidines and B_2 prefers purines. R corresponds to ribose sites and p to phosphate sites; p_1 is the active centre, and p_0 and p2 are noncatalytic binding sites.

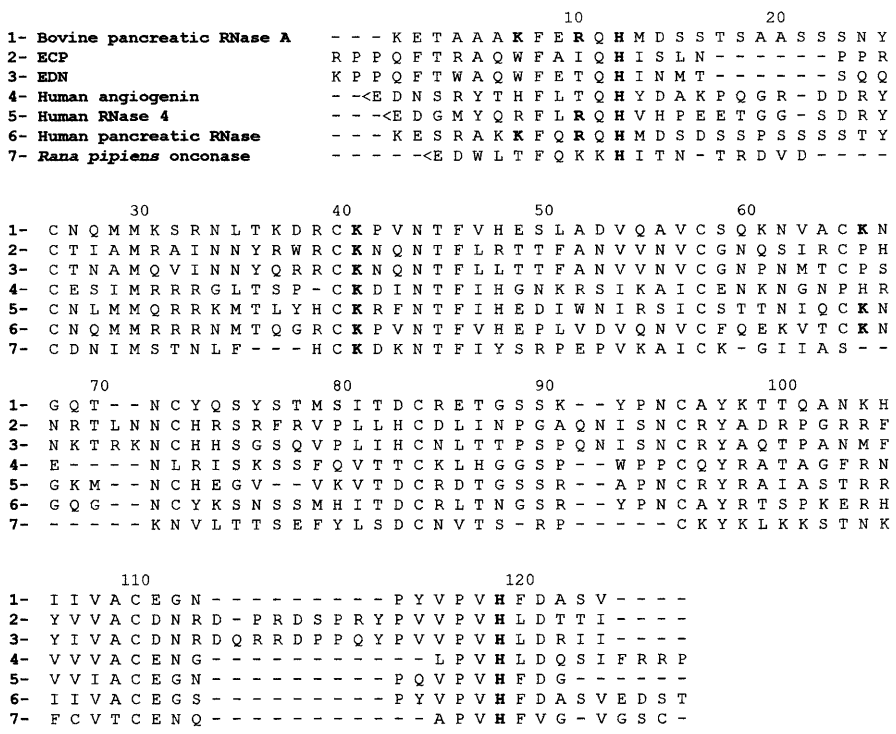

Figure 2. Alignment of the amino acid sequences of members of the RNase superfamily [45]. Numbering is allocated according to the RNase A sequence. Deletions in the amino acid sequences are indicated by dashes. Amino acid residues belonging to the p_0, p_1 and p_2 phosphate-binding sites are shown in bold characters.

Eosinophil-associated RNases

Eosinophil derived neurotoxin (EDN) has a preference for poly(U) in relation to poly(C), as described for other nonpancreatic RNases, but retains B_1 and B_2 specificity of pancreatic RNase A when using dinucleotide substrates, with an important reduction in catalytic efficiency [12]. In order to identify residues involved in putative secondary binding subsites, the recombinant EDN structure has been superimposed to the RNase A-thymidylic acid tetramer [p(T)$_4$] complex [13]. In this model p_1, B_1, R_1, B_2 and R_2 of the oligonucleotide can make specific interactions with the enzyme. Asn-70 (Asn-71 in RNase A) and Asp-112 (Glu-111 in RNase A) can be identified in the B_2 subsite and Thr-42 (Thr-45 in RNase A) in B_1. The role of Ser-123 in the B_1 site of RNase A may be performed by Gln-40 (Val-43 in RNase A) in EDN. Like Ser-123, Gln-40 can either accept or donate hydrogen bonds, suggesting that both cytosine and uracil can accommodate in the EDN B_1 site, thus explaining its observed substrate specificity. R_1 and R_2 are involved in van der Waals contacts with the Trp-7 ring and with Leu-130, respectively. In this study neither p_0 nor p_2 could be located. In the rEDN X-ray crystallographic structure there are two sulphate anion-binding sites. The position of the first sulphate anion mimics the p_1 site, interacting with Gln-14, His-15, His-129 and Leu-130 (positions 11, 12, 119 and 120, respectively, in RNase A). The second sulphate anion, which makes contacts with the side-chain atoms of Arg-36 and Asn-39 and the main-chain atoms of Asn-39 and Gln-40 (positions 39, 42 and 43, respectively, of RNase A), will likely represent p_{-1} [13].

Eosinophil cationic protein (ECP) substrate specificity and catalytic efficiency are not very well known. Preliminary studies show a much lower catalytic activity with oligonucleotides than EDN [14, 15]. Residues identified in EDN for B_1 specificity such as Thr-42 and Gln-40, and residue Asn-70 for the B_2 site, are conserved in all known sequences of EDN and ECP from primates [16].

Beintema [17] has suggested that the low RNase activity of ECP, in relation to RNase A, could be due to the lack of a basic residue at positions 66 or 122 (RNase A numbering). In the EDN sequence Arg-131 (position 122 in RNase A) could supply an equivalent positive charge, but this is not the case in ECP, which has a Thr residue at this position (fig. 2).

The binding sites of RNase A

The characterisation of several binding subsites in R-Nase A that contribute to the binding of the polymeric substrate has been carried out by means of structural and kinetic approaches. Several works have extensively reviewed these aspects of RNase A [18–20]. RNase A is the most studied pyrimidine-specific RNase. Depolymerization of RNA shows specificity for the phosphodiester bonds that have a pyrimidine nucleotide at the 3′ position. The pyrimidine-binding site has been designated as B_1R_1 and the phosphate active site as p_1. In addition, this enzyme shows preference for purine nucleotides at the 5′ position of the cleaved bond, and B_2R_2 defined this purine-binding site. Other phosphate-binding subsites in addition to the active site have been proposed. Of these the best characterized are p_2 and p_0, which are adjacent to the active site in the 3′ direction and the 5′ direction, respectively (fig. 1).

Structural studies

X-ray diffraction studies of complexes of RNase A with several substrate analogues of the mononucleotide, dinucleotide and oligonucleotide types have shown the amino acid residues involved in the B_1, R_1, p_1, B_2 and R_2-binding subsites. The contribution of these studies has been the subject of a recent review by Gilliland [21]. The interactions of nucleotides at the B_1, R_1 and p_1 subsites are well characterized: Ser-123, Asp-83 and Thr-45 are amino acid residues that contribute to the binding of the base at B_1, the sugar in R_1 has few direct interactions with the enzyme, and the pucker conformation depends on the type of sugar and the phosphate linkage. In the p_1 phosphate active site, His-12, His-119 and Lys-41 are the amino acids that participate in the catalytic process. In the B_2 binding-site, interactions with Glu-111, Gln-69 and Asn-71 have been described in some complexes, and although this site shows different conformations, stacking interaction with His-119 is always observed.

The RNA-RNase A complex has been mimetized by the binding of four oligomers of tetradeoxyphosphoadenosine [$d(pA)_4$] to a single molecule of RNase A [22]. From this study a virtual continuous RNA strand of 12 nucleotides has been described. The 12 phosphates of the RNA strand, beginning from the 5′ terminus,

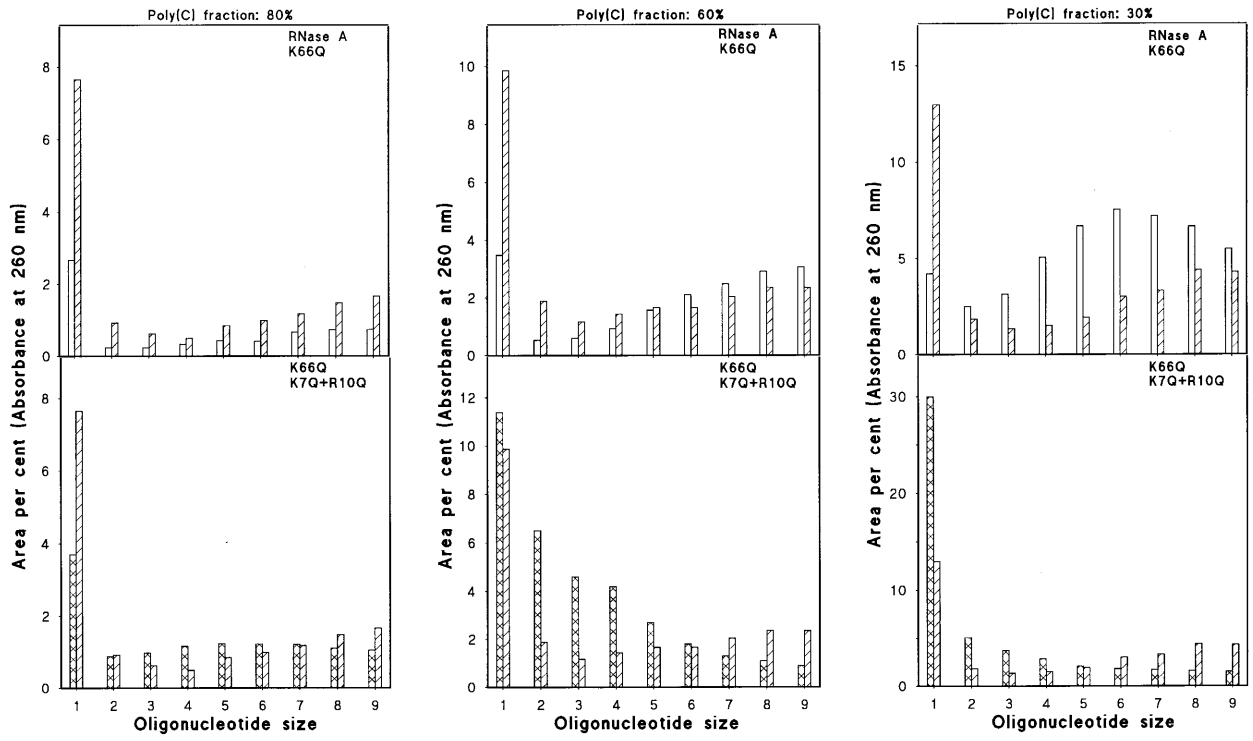

Figure 3. Comparison of the $(Cp)_nC > p$ ($n = 0–8$) formation from poly(C) cleavage by RNase A (□), and RNase A modified forms (K66Q-RNase A (▨) and K7Q + R10Q-RNase A (⊠)). Area percentage in each case has been determined from the area of the corresponding peak separated by reversed-phase HPLC of the products from poly(C) cleavage [26]. Due to the different enzyme activities, comparisons have been established using as reference the same undigested poly(C) fraction. Note that in each graphic, the best scale on the ordinate is used.

would bind by electrostatic interactions with nine basic amino acid residues (Lys-7, Lys-41, Lys-66, Arg-85, Arg-39, Lys-91, Lys-98, Arg-33 and Lys-31). Fontecilla-Camps et al. [23] have solved the crystal structure of the d(ApTpApApG)-RNase A complex. This structure has contributed to the characterization of different binding subsites, especially p_0, $B_1R_1p_1$ and $B_2R_2p_2$.

Kinetic studies

Steady-state kinetic studies with oligonucleotides [24, 25] suggested that p_0, p_1 and p_2 are the phosphate-binding subsites important for catalysis. However, steady-state kinetics with poly(U) [25] or poly(C) (M. Moussaoui et al. unpublished observations) show that the catalytic efficiency for these high molecular mass RNA analogues is higher, and in the case of poly(C) is in the range expected for a diffusion-limited reaction. These results indicate that additional substrate-enzyme interactions must contribute to the binding of these polymeric substrates.

As the assays for kinetic studies are, in general, based on spectrophotometric methods, in the case of the use of RNA or analogues as substrates the observed absorbance change due to the depolymerization process is the result of the sum of different reactions that take place at the same time. This is due to the fact that the product of an initial reaction is also a substrate of the same enzyme and, most likely, with a different catalytic efficiency. For this reason, Moussaoui et al. [26] analysed the pattern of oligonucleotide formation by RNase A using poly(C) as substrate. Product separation by high-pressure liquid chromatography (HPLC) shows that the enzyme does not act randomly but rather prefers the binding and cleavage of the longer substrate molecules and that the phosphodiester bond broken should be 6–7 residues apart from the end of the chain to be preferentially cleaved by RNase A (fig. 3). From the pattern of product formation and substrate preference it has been demonstrated that the cleavage of an RNA chain is based on the cooperative binding structure of RNase A and the phosphates of the polynucleotide [18].

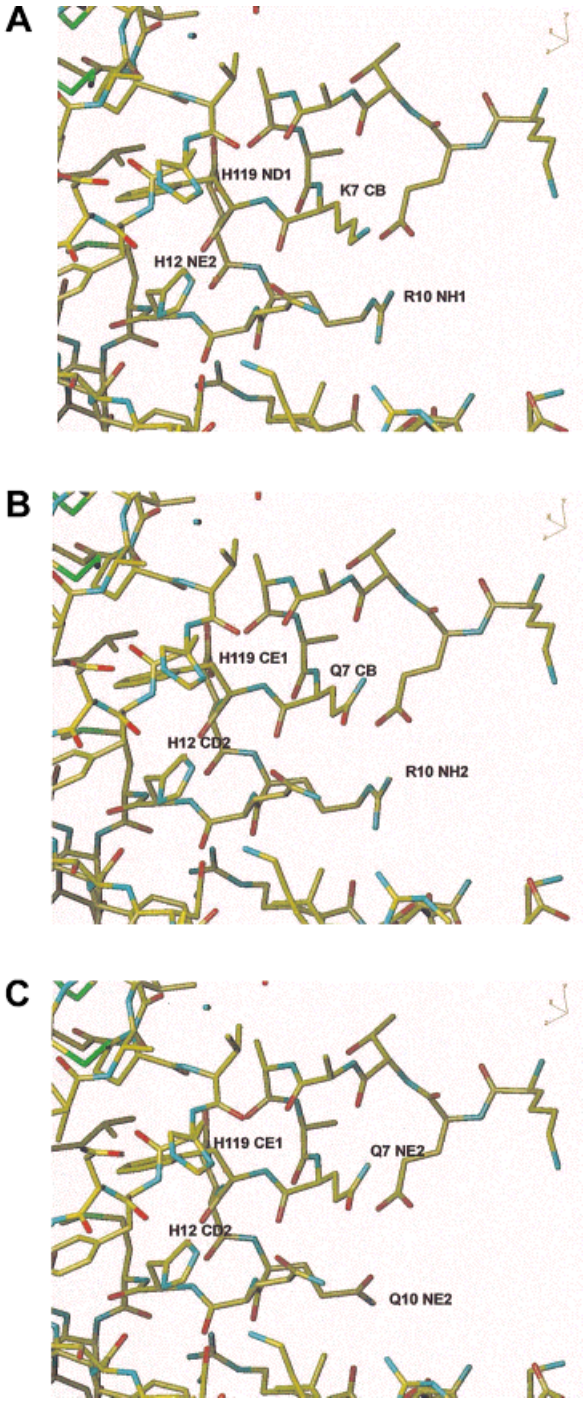

Figure 4. (*A*) Structure of the region around Lys-7, Arg-10 and the active site of RNase A (7RSA.pdb [46]). (*B*) Possible structure of the same region in the mutant K7Q-RNase A, and (*C*) in the double mutant K7Q + R10Q-RNase A. Pictures were generated using TURBO-FRODO [47].

The role of p_0 and p_2 in the catalytic process of RNase A

The p_2 phosphate-binding subsite

The p_2 phosphate-binding subsite binds the phosphate group adjacent to the 3′ side of the phosphate in the active site. This site was hypothesized from the affinity-labelling reaction of RNase A with 6-chloropurine riboside 5′-monophosphate, a 5′-adenosine monophosphate (AMP) analogue [27]. The reaction yields a major derivative (derivative II) with the nucleotide label bound through position 6 of the purine base to the

α-amino group of the enzyme. The specificity of the reaction and the protection efficiency of natural nucleotides indicate that the phosphate group of the labelling nucleotide binds to a specific phosphate-binding subsite (p_2). ^{1}H-nuclear magnetic resonance (NMR) studies of derivative II, RNase A and their complexes with natural nucleotides supported the existence of this subsite [28–31]. Kinetic studies on the effect of the nucleotide inhibitors 5′-AMP and adenosine 3′,5′-biphosphate (pAp) on the hydrolysis of cytidine 2′,3′-cyclic phosphate (C > p) and the kinetic parameters obtained for dinucleoside monophosphates and trinucleosides diphosphates also suggest the existence of the p_2 site [24]. As a result of chemical modification studies with pyridoxal 5′-phosphate, it was postulated that Lys-7 and Arg-10 are constitutive of this subsite [32]. These amino acid residues are conserved in mammalian pancreatic RNases but are not present in RNases with low activity such as ECP or angiogenin (fig. 2). Basic amino acid residues are also present at these positions in human RNase 4 [33], but this enzyme is poorly characterized from the kinetic point of view with respect to this subsite [34]. For a discussion of this enzyme see the article by Hofsteenge et al. in this issue.

The structure of derivative II obtained by X-ray crystallography [35] described the location of the labelling nucleotide at the N-terminal region of the enzyme. The phosphate group of the nucleotide interacts with Lys-7 and it is close to His-119 in the 'up' conformation, but no interactions with Arg-10 were observed. The crystal structure of the complex between RNase A and the oligonucleotide d(ApTpApApG) indicates a clear salt bridge interaction between Lys-7 and the phosphate group joining adenosines A^3 and A^4 [23]. Recently, the crystal structure of RNase A complexes with the potent low molecular mass inhibitors 5′-diphosphoadenosine 3′-phosphate (ppA-3′-p) and 5′-diphosphoadenosine 2′-phosphate (ppA-2′-p) have been solved [36]. In both cases the residues in the active site are oriented as in free RNase A, with the exception of His-119 and Lys-7. Lys-7 occupies different positions in all three structures, and it appears that its position is determined by the interactions with the amino group of its side chain. The 5′β-phosphates of both ppA-3′-p and ppA-2′-p occupy the active site (p_1), and Lys-7 forms hydrogen bonds with the 5′α-phosphates of both nucleotides. Only in the case of ppA-3′-p, does Lys-7 interact with the 3′-phosphate group that according to the binding subsites model should bind to p_2.

The role of the amino acid residues in the p_2-binding subsite with respect to RNase A catalysis has been analysed by site-directed mutagenesis (fig. 4). Steady-state kinetic parameters of the K7Q, R10Q and K7Q + R10Q RNase A mutants using C > p and RNA as substrates (table 1) confirmed that the p_2 subsite plays

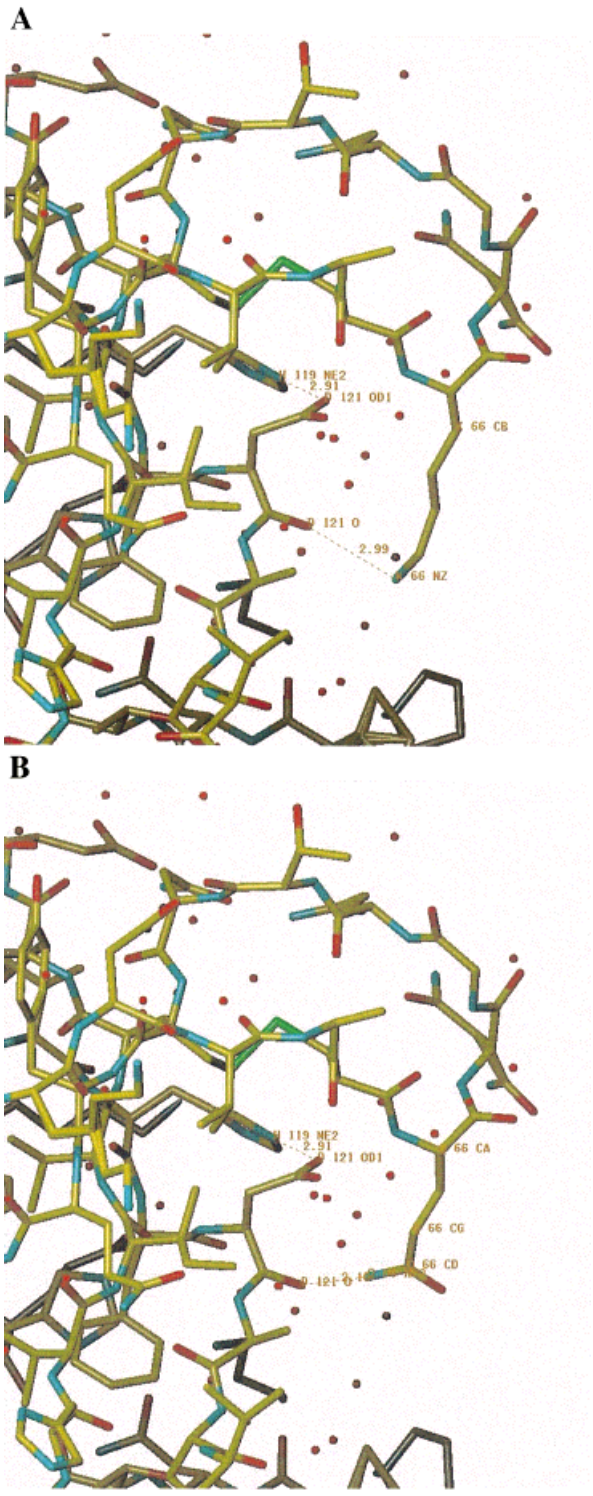

Figure 5. (*A*) Structure of the region around Lys-66 and the active-site of RNase A (7RSA.pdb [46]) in which the hydrogen bond between the side chain of Lys-66 and the main chain carbonyl of Asp-121 is shown. (*B*) Possible structure of the same region in the mutant K66Q-RNase A. The hydrogen bond between residue 66 and Asp-121 is conserved in this mutant. Pictures were generated using TURBO-FRODO [47].

Table 1. Kinetic parameters of RNase A and K66Q and K7Q + R10Q mutants of RNase A.

Enzyme	C > p		poly(C)	
	K_m (mM)	k_{cat} (min^{-1})	K_m (mg/ml)	Relative $V_{max}/[E_0]$†
RNase A	1.06 ± 0.10	137 ± 11	0.28 ± 0.03	100
K66Q-RNase A	1.07 ± 0.16	118 ± 10	0.71 ± 0.09	112
K7Q + R10Q-RNase A†	0.88 ± 0.06	11 ± 1	0.97 ± 0.05	6

Enzyme activities were determined spectrophotometrically in 0.2 M sodium acetate, pH 5.5 at 25 °C.
$[E_0]$ is the final enzyme concentration. The values for native RNase A are taken as 100. †Taken from ref. 37.

a central role in the binding of the polymeric substrate poly(C). Moreover, the results suggest the participation of these residues in the catalytic mechanism of RNase A, since the catalytic efficiency of these recombinant enzymes is diminished not only in the case of the RNA analogue poly(C) but also in the case of the low molecular mass substrate C > p, which only binds to the catalytic site ($B_1R_1p_1$). K_m values are not modified for the C > p substrate, but an increase in the values for poly(C) is observed. The synthetic activity of RNase A, the reversal of the transphosphorylation reaction, that takes place at C > p concentrations higher than 5 mM, gives rise to the formation of CpC > p. This reaction is also diminished in this recombinant enzyme, thus indicating that, in addition to the binding of a C > p molecule in $B_1R_1p_1$, binding of the second C > p substrate molecule takes place through the phosphate group that binds to p_2 [37]. The contribution of this subsite to the enzymatic process has been analysed both with an RNase A chemically modified in p_2 (derivative II) and by means of site-directed mutagenesis (K7Q + R10Q-RNase A). Poly(C) was used as substrate, and the products were separated by HPLC. In both cases deletion, or blocking, of p_2 modifies the pattern of poly(C) digestion described for native RNase A and abolishes its endonuclease activity (fig. 3), which is substituted by an exonuclease activity [26].

The p_0 phosphate-binding site

The existence of a p_0 phosphate-binding subsite was proposed by Mitsui et al. [38] from X-ray diffraction studies of RNase A-adenosyl 3′,5′-cytidine (ApC) complexes and model building. The authors also suggested that the binding takes place through Lys-66. Previous studies of the association constants of uridine 2′(3′),5′-bisphosphate and 2′(3′)-monophosphate (mixed isomers) (2′(3′)-UMP) with RNase A and with the corresponding carboxymethylhistidine-119 indicated that the 5′-phosphate group of the nucleoside bisphosphate contributes to substrate binding [39]. Steady-state kinetic studies of uridine 5′-phosphate-2′,3′-cyclic phosphate (pU > p) hydrolysis showed a fivefold increase in k_{cat}/K_m as compared with U > p [40].

Molecular dynamic simulations [41] show that Lys-66 and other positively charged groups located near the active site may play a role in stabilizing the transition state through an intervening water network. Model building and computer graphics studies show that Lys-66 is a good candidate as constituent of p_0 [42]. No other contributions to the study of the role of Lys-66 as phosphate-binding subsite appeared until the crystallographic studies of the RNase A-d(ApTpApApG) complex were published [23]. In this complex, Lys-66 does not bind to the substrate analogue; but because this region can adopt different orientations, a conformation in which Lys-66 would interact, in solution, with the first phosphate group of the oligonucleotide was not excluded.

The interest in this residue is correlated with the proposed role of this amino acid residue in the catalytic activity of RNase. This residue is conserved in all mammalian pancreatic RNases [17], but is substituted in other proteins of the RNase superfamily that show a low ribonucleolytic activity such as angiogenin and human eosinophil cationic protein (fig. 2). Beintema [17] hypothesized that the absence of Lys-66 can be substituted by a positive group at position 122, as is the case for turtle pancreatic RNase and human nonsecretory RNase (EDN or RNase 2). The absence of a positive group in both positions is observed only in proteins with low RNase activity.

The role of electrostatic interactions in substrate binding and the catalytic process was checked by analysing the behaviour of the K66Q-RNase A mutant (fig. 5). The kinetic parameters for C > p and poly(C) as substrates are shown in table 1. K_m and k_{cat} values for C > p are not modified in K66Q-RNase A with respect to the native enzyme. However, using poly(C) as substrate the k_{cat} value is unmodified, whereas an increase in the K_m value is observed. Again, these results can be interpreted according to the model for the interaction to the binding subsites. In the range of substrate concentrations (1–5 mM, pH 5.5), where RNase A shows

Michaelis-Menten kinetics (M. Moussaoui et al., unpublished observations), the C > p substrate binds only to $B_1R_1p_1$, and thus no change in K_m is seen with this mutant. However, the increase of K_m for the polymeric substrate poly(C) can only be due to the abolition of the electrostatic interaction in the p_0 site adjacent to the active site. On the other hand, the unmodified k_{cat} values for both substrates mean that the basic group of Lys-66 is not essential to the catalytic mechanism. These results do not, however, exclude a possible role for this amino acid residue in the network of water molecules proposed by Brünger et al. [41]. This network can be maintained in the K66Q-RNase A mutant because of the hydrogen-bonding capacity of glutamine (fig. 5). To reconcile these facts with the proposed role for this amino acid in RNases with high catalytic activity [17], it is important to point out that the proteins with low RNase activity have different amino acid residues in positions 66 or 122. ECP has a proline residue that can modify the water network, whereas in angiogenin this position is occupied by a histidine residue. In the latter case, low RNase activity has been explained by the occupation of the pyrimidine-binding site by a Glu or Gln-117 side chain (position 122 in RNase A) [43, 44].

Depolymerization of poly(C) by K66Q-RNase A follows a pattern similar to that of native RNase A (fig. 3). However, the accumulation of oligonucleotides containing six to seven residues observed with the native enzyme is not so clear with the mutant. It is very likely that deletion of the p_0 subsite decreases the endonucleolytic efficiency of the enzyme, giving rise to this mixed endo- and exonucleolytic behaviour.

In conclusion, the p_0 and p_2 binding subsites adjacent to the active site in the 5′ and the 3′ direction, respectively, play a different role in the catalytic process of RNase A. From kinetic studies with C > p and poly(C) as substrates, and the poly(C) depolymerization process, we can conclude that electrostatic interactions in p_2 play an indirect role in the catalytic mechanism and in the endonuclease activity of the enzyme. The p_0 electrostatic interaction appears to contribute only to the binding of the polymers, as an increase in the K_m value for poly(C) is observed with no apparent effect on either the catalytic mechanism or in the product formation.

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