Enzyme specificity: base recognition and hydrolysis of RNA by ribonuclease A

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Received 13 July 1983; revised version received 1 September 1983

The substrate specificity of pancreatic ribonuclease A is discussed in light of observations based on accurate X-ray structure analysis of several enzyme-nucleotide complexes. A hypothesis for protein-nucleic acid recognition is presented which proposes that: (a) pyrimidine bases in RNA are recognised by ribonuclease due to the charge complementarity of two groups (the amide nitrogen and the side chain oxygen (OG) of threonine 45) of the protein and relevant atoms in the heterocyclic base (O2 and N3 in pyrimidine nucleotides); (b) interaction of the protein with the ribose moiety of the nucleotides is non-specific; and (c) conformational flexibility in the region of the scissile P–O bond is provided by different locations of the phosphoryl oxygens, rather than by an overall translation of the phosphate moiety.

Substrate specificity *Base recognition* *Charge complementarity*
X-ray diffraction *Conformational flexibility* *Protein-nucleic acid interaction*

1. INTRODUCTION

Pancreatic ribonuclease A (RNase A) hydrolyzes [1] 3′,5′-phosphodiester linkages of RNA, where the 3′-linkage is attached to a pyrimidine base (fig. 1). The mechanism by which this hydrolysis occurs, whether 'in-line' [2–6] or 'adjacent' [7–9], has been widely investigated, but information on the specificity of the enzyme towards the monocyclic pyrimidine bases and the way this selectivity is brought about has been rather limited and imprecise. Although X-ray diffraction analysis [10,11] established separate binding sub-sites for purine and pyrimidine nucleotides (B2R2 and

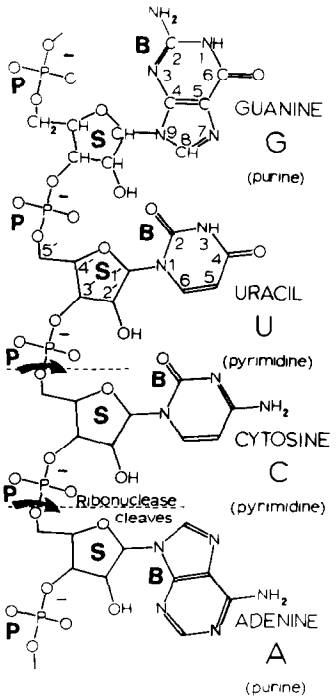
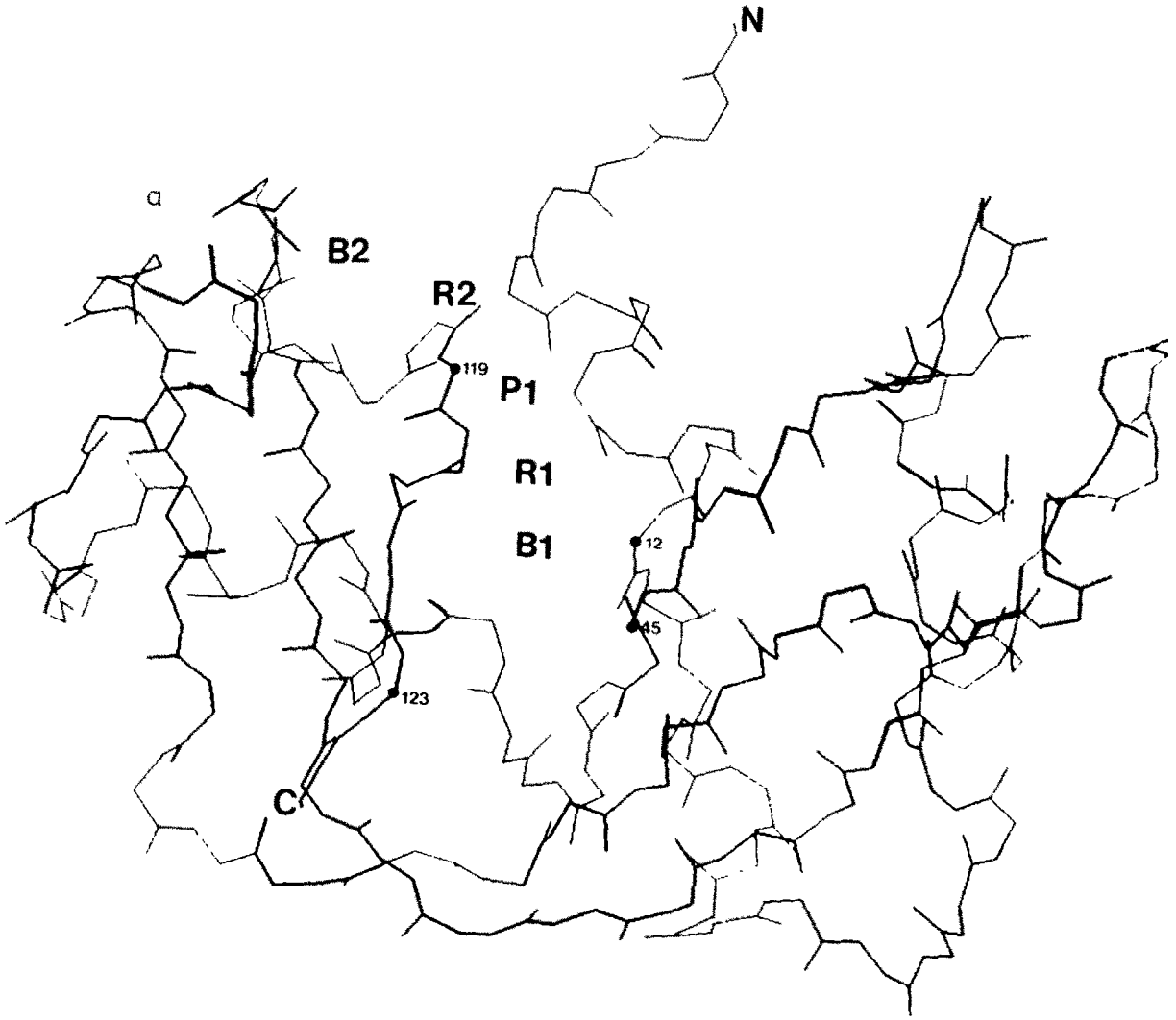


Fig.1. Ribonucleic acid showing nucleotides of the 4 commonly occurring bases. The base(B), ribose (S) and the phosphate (P) moieties of individual nucleotides are identified. The atomic numbering scheme for the bases and the region of ribonuclease attack (the P–O bond on the far side of nucleotides with pyrimidine bases) are also indicated.

Published by Elsevier Science Publishers B.V.
00145793/83/$3.00

B1R1, respectively, fig.2) details of enzyme–nucleotide interactions available from these studies did not provide adequate explanation for the preferential cleavage of modified purine nucleotides by RNase. These hypotheses on the specificity of RNase, as summarised in table 1, were based on kinetic [12,13] and spectroscopic [14–16] investigations, with support from observed positions of inhibitors located in low resolution crystallographic analyses of RNase-nucleotide complexes [10,11,17,18] of the bovine enzyme. The central premise was that pyrimidine nucleotides, which were seen to bind at the interior of the U-shaped active site cleft (B1R1p1, Fig.2a), interacted with the enzyme at 3 positions of the polypeptide chain, viz. a main chain nitrogen (N45) and two side chain oxygen atoms (OG45 and OG123) [10,17]. Due to steric hindrance and charge incompatibility of the larger purine derivatives at the B1R1 site, purine bases bind to the exterior of the active site (B2R2, fig.2a) and are thus not susceptible to RNase action. These postulates, while providing an overall rationalisation of the enzyme specificity, are inadequate to explain the RNase degradation [14] of purine polymers such as poly(formycin) (poly(F)) and poly(laurusin) (poly(L)). These binding specificity studies were, however largely dependent on the tentative results of the low resolution X-ray diffraction studies of enzyme-inhibitor complexes [17,18] lacking the advantages provided by present-day techniques.



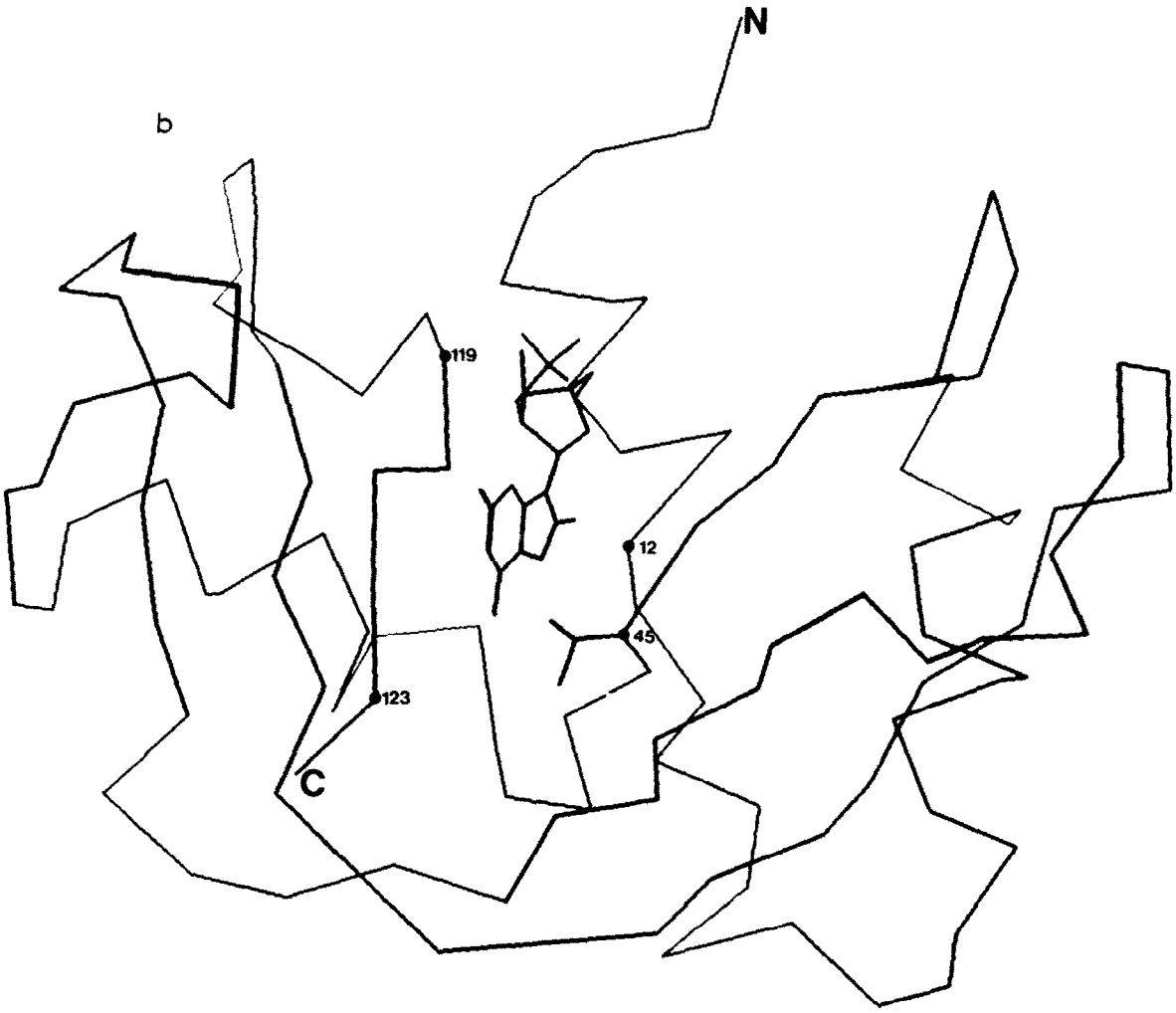


Fig.2.(a) A view of the main chain atoms of ribonuclease A deduced from the 1.45 Å analysis of the native enzyme. B1R1P1 indicate the relative positions of the base, sugar and phosphate for pyrimidine substrates. B2R2 are the locations for the base and ribose for most purine mononucleotides. C-alpha positions of the residues discussed in the text are marked. (b) Same view as fig.2a, showing the C-alpha backbone and the side chain of threonine 45 of the protein and the non-hydrogen atoms of the purine nucleotide bound in the active site of the ribonuclease A–O8-2′-GMP (ribonuclease A–8-oxo-guanosine-2′-phosphate) complex.

2. MATERIALS AND METHODS

Native crystals of bovine pancreatic RNase A (obtained from Sigma) were grown from 40% ethanol at pH 5.2–5.7 [19]. Enzyme–inhibitor complexes were prepared by soaking native crystals in different concentrations of individual nucleotides for varying lengths of time. Intensity data were collected from one crystal of each binary complex, such that about 5200 unique reflections (up to 2.3 Å resolution) were obtained for each inhibitor complex. Details of structure determination using restrained least-squares refinement and the interpretation of electron density maps are discussed in [20]. The least-squares fit between inhibitors was obtained by using the program FITZ [21] on Evans and Sutherland picture system 2.

Table 1

[Ref.]	Postulated protein–nucleotide interactions for base recognition	Remarks [Ref.]
[18,13]	Interactions non-specific with respect to base	Inconsistent with inhibitory effects of free bases [10]
[10,17]	Base identified at atoms O2, N3 and N4(O4) in C and U, H-bonded to protein atoms N45, OG45 and OG123, respectively	(a) Does not explain degradation of purine polymers [14,15]. (b) Inconsistent with activity of alanine 123 RNase derivative [24]
[12,18]	Base identified by 'amide fragment' at positions O2 and N3 in pyrimidines, H-bonded to protein atoms N45 and OG45, respectively	Inconsistent with observations that bases (e.g., F, L) lacking the 'amide fragment' are good substrates for the enzyme [14]
[14,15]	Base contact points N3 and O4(N4) in C and U, (and N7 and O6 in purines), H-bonded to protein atoms OG45 and OG123, respectively	(a) Inconsistent with H-bonding scheme observed in X-ray analyses [10,11,17]. (b) Inconsistent with observed properties of alanine 123 RNase derivative [24]

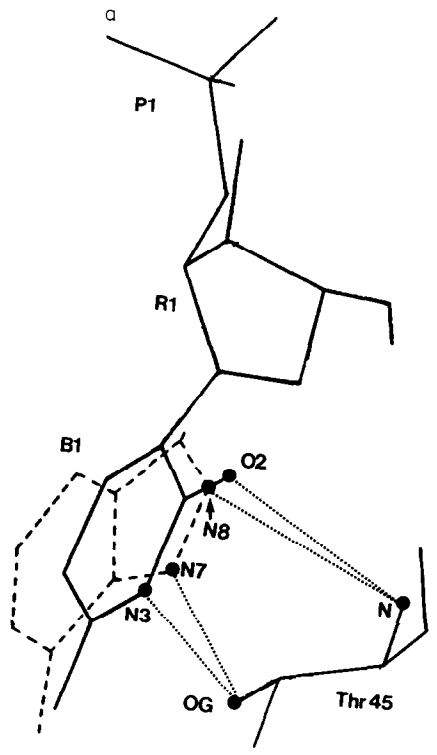
The base numbering scheme is given in fig. 1;
The base for formycin (F) is shown in fig. 3a;
The base for laurusin (L) is the same as for F with an oxygen atom substituted for the nitrogen atom in position 6 of F

3. RESULTS AND DISCUSSION

Accurate [19,20] X-ray diffraction studies of several enzyme mononucleotide complexes have revealed new details about the B1R1 site of ribonuclease A. Pyrimidine nucleotides bind (fig. 3(a)) at the active centre with the heterocyclic base interacting (via atoms O2 and N3) at only two locations of the protein; i.e., at an amide nitrogen (N45, an obligate donor), and a side chain oxygen of a threonine residue (OG45, donor or acceptor). In addition, a modified purine derivative, O8-2′GMP, (8-oxo-guanosine-2′-phosphate, fig. 3b), is found to bind at the pyrimidine binding site (fig.2b and 3b), with the same atoms (N45 and OG45) of the protein being involved in base binding. These observations clearly establish the requirements for base identification by ribonuclease A: an obligate proton acceptor within hydrogen bonding distance (<3.5 Å) of amide N45 and a polar group (either proton donor or acceptor) within H-bond proximity of OG45. Charge and spatial complementarity with these two 'contact points' on the enzyme seem to be a necessary and sufficient condition for bases to be recognized by RNase. Thus, in natural pyrimidines (C and U, fig.1) H-bonds at base positions O2 and N3 provide the correct electron configuration for base identification by the enzyme. In contrast, naturally occurring purines cannot fulfil this requirement, so that unless modified (for steric reasons at the 5-membered ring, fig.3b) purine nucleotides are not susceptible to RNase cleavage. Most modified purine bases found so far in RNA have changes (towards increased hydrophobicity) in the 6-membered ring [22], with the result that they cannot be 'identified' by RNase. Ribonuclease action on certain purine polymers such as poly(F) and poly(L) can be explained in terms of the realization of the charge requirements for base recognition via the substitution of the proton acceptor (N8) in the C8 position of the 5-membered ring of natural purine analogues (fig.1 and fig.3a). The presence of a threonine residue at position 45 in all known sequences of pancreatic ribonucleases [23] shows the constancy of the postulated protein 'contact points', thus supporting the base recognition hypothesis outlined above. The present hypothesis could account for the observation that the replacement of serine 123 with alanine 123 in RNase A

does not substantially affect the substrate binding properties of the alanine 123 derivative [24]. The replacement of the serine side chain in position 123 of the bovine enzyme by other amino acids (threonine, tyrosine) in the ribonucleases of other mammalian species (mouse, cuis, kangaroo) [23], corroborates the evidence that the nature of the residue at position 123 of the enzyme is not crucial for base recognition. Further, the versatility of threonine 45 by which it can either donate a proton (to N3 of cytidine) or act as an H-bond acceptor (from N3 of uridine) provides the enzyme with the flexibility of using the same 'contact points' in the identification of two different pyrimidine bases. By analogy, the more selective extracellular ribonucleases may have more rigid 'contact points' in order to express their specificity. The data on the complex of the guanyl-specific RNase, RNase T_1 [25], from the fungus *Aspergillus oryzae,* with 2′-GMP, indicates that the protein groups in RNase T_1 involved in base recognition (a carbonyl and an amide group) are indeed more rigid.

A further feature to emerge from the analyses on pancreatic RNase is that the sugar moiety of the nucelotide binds non-specifically. The mobility of the side chain of histidine 119 [20], which allows this imidazole to occupy preferentially one of two sites, assists in providing possible binding regions for the ribose. The base, identified as above, has a fixed orientation and the phosphate group is also found to occupy a similar position in all the binary complexes (fig. 3c). A superposition of the observed atomic locations of two inhibitors (O8-2′-GMP and UpcA [26], fig.3c) illustrates this clearly: when the bases are superimposed at the 'contact positions' the phosphorus atoms overlap, whereas the ribose moiety occupies distinct locations. The root-mean-square (r.m.s.) deviations between the 3 pairs of atoms (2 atoms at base identification points and the phosphorus atom), obtained after a least-squares fit is 0.4 Å (RMS deviation is 0.5 Å if the two 'contact points' of the protein, i.e. 5 atom pairs are included in the least-squares fit). The difference in the phosphoribosyl linkages (2′-phosphate in O8-2′-GMP and 3′-phosphate in UpcA) is accommodated by changes in the location of the phosphoryl oxygens (fig.3c), rather than by overall translation of the phosphate moiety. This indicates a flexibility around the scissile P–O bond on the far side of the ribose: a feature which may be essential in allowing the formation of the pentacoordinate intermediate (at the phosphorus atom) during catalysis. In the observed location, the phosphate group is close to the protein residues implicated in the catalytic mechanism (His 12 and His 119). This, together with the specific binding of the base indicates that the requisite for the initial step in hydrolysis by RNase (transphosphorylation) is the correct spatial orientation of the heterocyclic base and the phosphate moiety. The alignment of the base and the phosphate may also have a bearing on the seond hydrolysis) step of RNase cleavage. The procedures for transphosphorylation and hydrolysis may be different and, if so, the spatial relationship of the carbonyl group (O2) of the pyrimidine bases (fig.3c) and the phosphate group established at the end of the first step may have further mechanistic implications. All catalytic processes occur at the interior of the active site cleft; i.e., in the B1R1 ('catalytic') region, so that all nucleotides with 'identified' bases having the correct phosphate alignment will bind at the B1R1 site, regardless of whether the base is purine or a pyrimidine. The external B2R2 ('secondary') site appears to be essential in maintaining



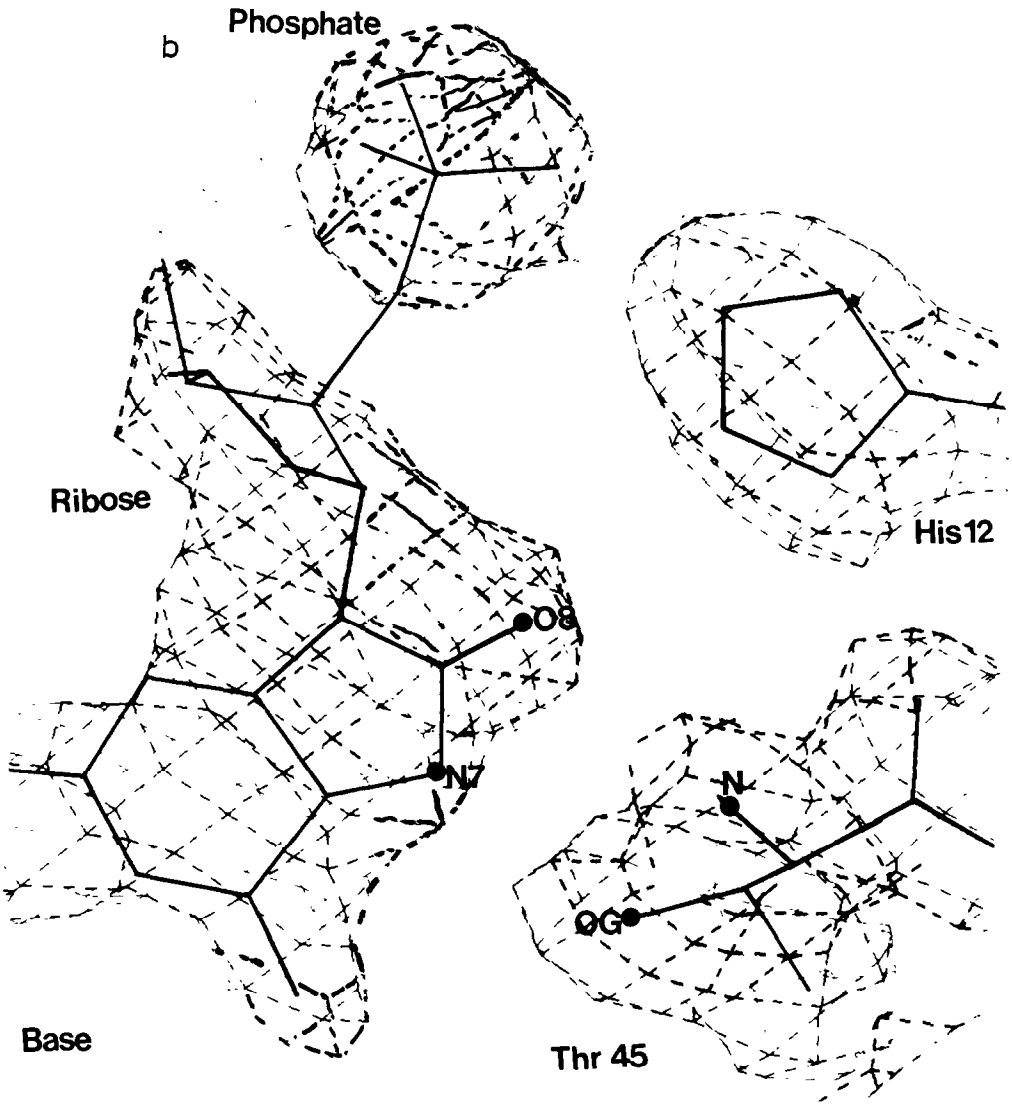
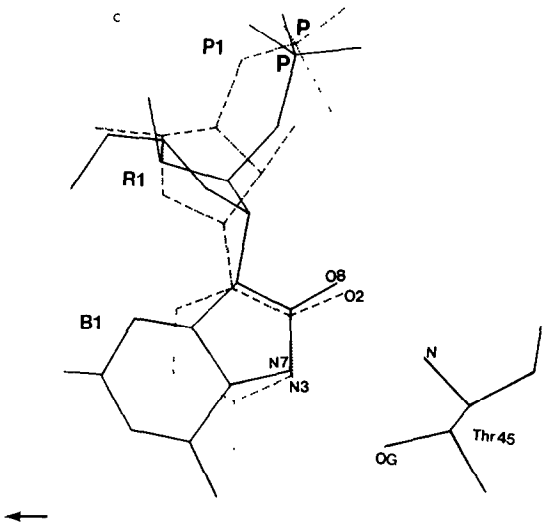


Fig.3.(a) The position of the nucleotide 2′-CMP (at the 'catalytic' site, B1R1P1) relative to the protein side chain threonine 45, as observed in the analyses of ribonuclease A–2′-CMP. The probable H-bonds between the cytosine (base) and threonine 45 are indicated. Dashed lines indicate the postulated base location for the purine analogue formycin. (b) Electron density map using $2|F_o|-|F_c|$ as coefficients in the 'catalytic' or B1R1P1 region for the purine mononucleotide (O8-2′-GMP) in the study of the ribonuclease A–8-oxo-guanosine-2′phosphate. The electron density for the protein side chains of threonine 45 and histidine 12 are shown for comparison. (c) The observed nucleotide conformations of O8-2′-GMP (full lines) and UpcA (dashed lines, only pyrimidine nucleotide shown) as seen in the X-ray structure analyses of ribonuclease A–O8-2′-GMP and ribonuclease S–UpcA complexes, respectively. The base recognition scheme is shown by the similarity in the H-bonding scheme between the purine (guanine in O8-2′-GMP) and pyrimidine (uracil, in UpcA) bases with the protein side chain threonine 45. In the two binary complexes, the position of the phosphorus atoms and the locations of the 'recognition' points of the heterocyclic bases are identical within experimental error. The location of the phosphoryl oxygens appear to be different in the two nucleotides. (UpcA is an analogue of UpA, where the 5′-oxygen is replaced by a carbon atom. Ribonuclease S is formed by the enzymatic hydrolysis of the peptide bond between residues 20 and 21 in RNase A).



the position of the nucleotide attached to the 5′-phosphoryl oxygen. As RNA, the natural substrate for RNase, is a polyribonucleotide, it is possible that there are other such subsidiary binding sites in the enzyme. Although there are indications [27] that the N-terminal region, close to the B2R2 region of the catalytic cleft (fig.2a), could provide further phosphate binding sites for RNA, the exact location of the new sub-sites must await further structural investigations of ribonuclease-oligonucleotide complexes.

ACKNOWLEDGEMENTS

I thank Dr D.S. Moss and Dr R.A. Palmer for discussions and encouragement during the course of this work. Financial support from the Science and Engineering Research Council is also greatly appreciated.

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