The Role of Non-catalytic Binding Subsites in the Endonuclease Activity of Bovine Pancreatic Ribonuclease A*

Mohamed Moussaoui†, Alícia Guasch‡§, Ester Boix‡, Claudi M. Cuchillo‡§, and M. Victòria Noguès†

From the ‡Departament de Bioquímica i Biologia Molecular, Facultat de Ciències and §Institut de Biologia Fonamental V. Villar-Palasi, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain

Bovine pancreatic ribonuclease A (RNase A)1 (EC 3.1.27.5) is a well known enzyme whose main physical, chemical, and enzymatic properties have been the subject of extensive reviews (Richards and Wyckoff, 1971; Blackburn and Moore, 1982; Eftink and Biltonen, 1987). RNase A is an endonuclease that cleaves 3',5'-phosphodiester linkages of single-stranded RNA when the base of the nucleotide in the 3' position is a pyrimidine. The use of well defined low molecular mass substrates such as pyrimidine 2',3'-cylic mononucleotides and dinucleoside monophosphates provided most of the kinetic data on which all mechanistic studies on RNase A have relied. Only a few kinetic studies have been carried out with longer oligonucleotides and homopolynucleotides such as poly(U) (Irie et al., 1984a, 1984b), but no detailed studies with RNA as substrate have been carried out. The reasons for this are (i) the difficulties in the kinetic analysis derived from the complex structural features of the RNA molecule, (ii) the difficulty of monitoring a very fast reaction in a reliable fashion, and (ii) the spectrophotometric methods used can only give an average measure of all the species produced during the reaction, without giving any idea about either the size distribution of the products or the characteristics of the bond broken. The use of HPLC techniques has circumvented some of the problems concerning the analysis of the products of the reaction. Using an anion exchange column it was possible to measure the products of the reaction at high concentrations of the low molecular mass substrate cytidine 2',3'-cylic phosphate (C>p). This method allowed the analysis of the partition of the reaction between the synthesis of the dinucleotide Cpc>p and the hydrolysis to 3'-CMP (Guasch et al., 1989; Cuchillo et al., 1991) and the separation between transphosphorylation and hydrolysis reactions using Cpc as substrate (Cuchillo et al., 1993). In addition to the catalytic center, several phosphate-binding subsites that recognize the negatively charged phosphates of RNA have been described (Parés et al., 1980; Parés et al., 1991; Fontecilla-Camps et al., 1994; Nogue’s et al., 1995) (Fig. 1). A non-catalytic phosphate-binding subsite (p2) adjacent to the catalytic center (p1) was postulated from the chemical modification of RNase A with the halogenated nucleotide 6-chloropurine 9-b-D-ribofuranosyl 5'-monophosphate (drRMP) (Parés et al., 1980). The reaction yielded a major derivative (derivative II) with the nucleotide label attached to the a-amino group. Different studies suggested that the phosphate group was bound in a specific phosphate-binding subsite, p2, and that Lys-7 and Arg-10 were involved in that subsite (Aris et al., 1981; Irie et al., 1986; deLlorens et al., 1989; Richardson et al., 1990). The structure of derivative II was recently solved by x-ray crystallography (Boqué et al., 1994), and the structure found is in accordance with the location of p2, B3, and R32 subsites at the N-terminal region of the protein. By means of

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2 The ribonuclease binding subsites are named as in deLlorens et al. (1989). B, R, and p stand for the base, ribose, and phosphate-binding subsites, respectively. B, R, and p1 are the main binding site where catalysis takes place. Subsites with subscripts 2 and 3 are on the 3'-side of the substrate's nucleotide chain whereas subsites with subscripts 0 and 1 are on the 5' side.
kinetic analysis of RNase A derivatives obtained by site-directed mutagenesis, an indirect role for the p2 subsite in the enzyme's catalytic mechanism was demonstrated (Boix et al., 1994). In the present work the pattern of oligonucleotide formation by RNase A using poly(C) as substrate was analyzed by means of reversed-phase HPLC, and the contribution of the binding subsite p2 to the product distribution was studied in both native RNase A and in p2-modified molecules. The results indicate that in the poly(C) cleavage RNase A does not act in a random fashion but rather prefers the binding and cleavage of the longer substrate molecules. In addition the phosphodiester bond broken should be 6–7 nucleotides apart from at least one of the ends to be preferentially cleaved by RNase A. Finally, deletion of p2 abolishes the endonuclease activity of RNase A, which is substituted by an exonuclease activity. All these facts can be explained in terms of the multisubsite structure of the enzyme.

EXPERIMENTAL PROCEDURES

Materials

Bovine pancreatic RNase (5× crystallized), d4RMP, poly(C), C–p and 3′-CMP were from Sigma. Acetonitrile HPLC grade was obtained from Farmitalia Carlo Erba (Milan, Italy) and ammonium acetate HPLC grade was from Scharlau (Ferosa, Barcelona, Spain). Distilled water treated with a Milli-Q water purification system (Millipore Corp., Milford, MA) was used throughout. All other reagents were of analytical grade. The reversed-phase HPLC column, Nova-Pak C18 (2×150 mm), was purchased from Waters Corp. (Milford, MA), and the anion exchange column Nucleosil 105B was obtained from Macherey, Nagel and Co. (Düren, Germany).

Apparatus

All HPLC experiments were carried out with a modular Waters system consisting of two pumps (model 510), a liquid chromatography injector (U6K), and an absorbance detector (model 490E). The system was controlled by the Maxima 820 program (Dynamic Solution, Division of Millipore Corp.). An Eppendorf centrifuge was used routinely to remove any particulate material before injecting the samples into the HPLC system.

Methods

Protein Purification

The RNase A fraction was obtained by a modification of the method of Taborsky (Alonso et al., 1986). Derivative II was obtained by reaction between d4RMP and RNase A according to Parés et al. (1980) and Alonso et al. (1986). K7Q and K7Q plus R10Q RNase A mutants were obtained and characterized according to Boix et al. (1994).

Analysis of the Digestion Products of Poly(C)

Digestion of Poly(C) and Separation of Oligo(CT) Ricic Acid—50 μl of a 5 mg/ml poly(C) solution in 10 mM Hepes-KOH pH 7.5 was digested with 10 μl of 30 nM RNase A at 25°C. At different time intervals, between 0 and 45 min, the products of the reaction were analyzed according to the method of McFarland and Borer (1979) by injecting 20 μl of the reaction mixture on a reversed-phase HPLC column (Nova-Pak C18 column) at time intervals from 0 to 45 min. See “Methods” for digestion and separation conditions. Note that in each chromatogram the best scale on the ordinate is used.
Separation of C\(_{p}\) and 3'-CMP—C\(_{p}\) and 3'-CMP produced in the poly(C) digestion by RNase A eluted from the Nova-Pak C18 column near the injection peak but so close to one another that no direct quantification was possible. The separation of these mononucleotides was achieved by concentrating the fractions eluted during the first 2 min in a Speed-Vac system. 200 \(\mu l\) of the concentrated sample was injected on an anion exchange column (Nucleosil 10SB) equilibrated with 0.2M ammonium acetate, pH 7.5. Sample separation was carried out with the starting eluent at a flow rate of 1 ml/min according to Alonso et al. (1985). Commercial C\(_{p}\) and 3'-CMP were used as standards.

RESULTS

Separation of the Poly(C) Digestion Products—The poly(C) used as substrate is a high molecular mass polymer. Fig. 2 (t = 0 min) shows that poly(C) is eluted as a single fraction by reversed-phase HPLC (Nova-Pak C\(_{18}\) column), indicating that although the sample is not electrophoretically homogeneous (information provided by Sigma) all high molecular mass components are eluted as a single peak and that oligonucleotides are not present in the sample at the initial conditions.

Fig. 2 also shows the elution profile by reversed-phase HPLC of the (Cp)\(_{n}\)C\(_{p}\) oligonucleotides obtained from the poly(C) digestion with a low concentration of RNase A at different times within the range 0–45 min. The elution position of the small oligonucleotides obtained from poly(C) digestion by RNase A was deduced from the pattern obtained after the reaction mixture was incubated for a long time (100 min) when no high molecular mass poly(C) was left (Fig. 3). The separation pattern found by McFarland and Borer (1979) for the chemical hydrolysis of poly(C) was also taken into account for the identification of the different peaks. Previous studies about the degradation of poly(C), poly(U), and poly(A) by RNase A had shown that the oligoribonucleotidic acids have a general structure of (Cp)\(_{n}\)C\(_{p}\) containing a 2',3'-cyclic phosphate terminus, except for the fragment arising from the 3' terminus of the initial molecules of substrate (Imura et al., 1965; Irie et al., 1984b). The two mononucleotide products 3'-CMP and C\(_{p}\) elute very near the injection peak and thus they cannot be directly measured. The smallest structure that can be clearly separated is the dinucleotide CpC\(_{p}\) with an elution time of around 4 min. Oligomers of increasing size elute sequentially as a function of the amount of organic phase in the eluent.

The analysis of the oligonucleotide size distribution (Figs. 2, 4, and 5) shows that under the conditions used only polynucleotide fragments are formed during the early stages of incubation. However, shortly thereafter (5 min) a clear trend toward the formation of oligonucleotides with a size of about 6–7 residues is observed. As expected, at the end of the process there is a clear increase in the number of small size oligonucleotides. These results suggest that the enzyme prefers binding and cleavage of long substrates and that to be preferentially cleaved by RNase A the phosphodiester bond has to be some six-seven nucleotides apart from at least one of the ends of the molecule.

The rate of appearance of mononucleotides C\(_{p}\) and 3'-CMP was followed by rechromatography on an anion exchange HPLC column (Nucleosil 10SB) of the fraction eluted during the first 2 min of chromatography of poly(C) digestion products on the Nova-Pak C\(_{18}\) column (Table I). The formation of mononucleotides is a very slow process. For example, after 45 min of poly(C) digestion when no poly(C) is left but oligonucleotides are present in the medium as RNase A substrates (Fig. 2), the absorbance area at 260 nm of mononucleotide fraction accounts for only 14% of the total, 92% of which is C\(_{p}\) and the remain-
ing 8% of which is 3'-CMP. In agreement with previous results on CpC digestion by RNase A (Guasch et al., 1989; Cuchillo et al., 1993) there is an accumulation of C->p in the reaction medium before its transformation to 3'-CMP.

Sequential Cleavage of Poly(C) Products—The action of RNase A on poly(C) gives rise to products which, in turn, are also substrates of the enzyme. This peculiarity results in a competition of the different substrate species for the enzyme leading to a partition of the catalyst between them. As shown by Fersht (1985) this partition is determined by the \( k_{cat}/K_m \) ratio of each competing substrate. Although in such a complex system as the present one it is very difficult to evaluate the \( k_{cat}/K_m \) ratios of the high molecular mass substrates, it is clear from Fig. 2 that there is a substrate size preference which, overall, can be viewed as a sequential process. The enzyme first acts on the longer substrates and then on the intermediate size substrates, and eventually all the C->p is converted to 3'-CMP.

This was checked by analyzing the evolution of some selected species. The original poly(C) substrate, the hexanucleotide (Cp)6Cp, and dinucleotide CpCp intermediates were chosen. Fig. 4 shows the experimental values for the evolution of the three species. The progress curves for the three species are very similar to what would be expected from two consecutive and irreversible reactions (Fersht, 1985) according to Scheme I.

\[ \text{Poly(C)} \xrightarrow{\text{RNase A}} \text{CpCp} \xrightarrow{\text{RNase A}} \text{3'-CMP} \]

The hexanucleotide species (Cp)6C->p behaves as a transient intermediate, whereas CpC->p accumulates during the observed reaction times. This product distribution suggested that RNase A shows a clear preference for polynucleotide or oligonucleotide substrates of high molecular mass rather than for low molecular mass substrates. These results can be explained according to the multisubsite structure of RNase A (Parés et al., 1991; Nogués et al., 1995). The formation of the RNA-RNase A complex is mainly driven by interactions between the phosphate groups of the substrate and the active site (p1) and the main phosphate-binding subsites (p0 and p2) of the enzyme (Fig. 1). However, other electrostatic interactions between phosphate groups of RNA and basic amino acid residues located at the surface of the protein are also involved in leading to optimal catalytic efficiency. The total occupancy of these binding subsites gives the best conformation for activity, and the additional binding energy clearly favors the action on the higher substrates.

Effect of the p2 Phosphate-binding Subsite on the RNase A Substrate Preference—The additional p2 phosphate-binding subsite adjacent to the 3'-side of the active site was postulated from the specific reaction of the halogenated nucleotide dRMP with RNase A (Parés et al., 1980). The major derivative, derivative II, is the result of an affinity-labeling reaction in which the phosphate group of the nucleotide binds to the specific p2 phosphate-binding subsite before the formation of the covalent

| Digestion time | Initial areasa | C->p (%) | 3'-CMP (%) |
|----------------|----------------|----------|------------|
| 0              | 1.2            | 90       | 10         |
| 15             | 8.6            | 92       | 8          |
| 45             | 14.6           | 91       | 9          |
| 100            | 16.0           | 91       | 9          |

\[ a \text{ Area percent of the fraction eluted during the two initial minutes of the Nova-Pak C18 column with respect to the total area.} \]
\[ b \text{ Distribution between C->p and 3'-CMP (as percent area) found in the initial fraction eluted from the Nova-Pak C18 column.} \]
Fig. 6. Model of the cleavage of an RNA chain by RNase A that explains the preference of the enzyme for long polynucleotide substrates. The model is based on the cooperative binding between the multiple protein subsites and the phosphates of the polynucleotide. Step 1, a long RNA chain binds to RNase A. Step 2, cleavage occurs in the active site resulting in the formation of two shorter oligonucleotide fragments, one of them ending with a 2',3'-cyclic phosphate (2',3'-c). Step 3, the cooperative binding is weakened in the oligonucleotide fragments and this favors their replacement by a longer chain. In subsequent reactions the shorter fragments will also be broken, and eventually the hydrolytic step (formation of a 3'-phosphate from the 2',3'-cyclic phosphodiester) occurs when most of the substrate has already been cleaved by transphosphorylation. Adapted from Parés et al. (1991).

**DISCUSSION**

The results shown in Fig. 2 indicate that the breakdown of poly(C) catalyzed by RNase A is not a random process even though all internucleotide bonds in the substrate are susceptible to attack by the enzyme. Instead, it can be considered as taking place roughly in consecutive steps; during the early part of the reaction longer fragments are expected for a random endolytic reaction, but as the reaction proceeds there is a significant accumulation of oligocytidylic acids of 6–7 residues, which in the final stages of the reaction are transformed into 3'-CMP (Fig. 4 and Table I). These results suggest that the enzyme prefers binding and cleavage of long substrates and that the phosphodiester bond broken should be some 6–7 residues apart from the end of the chain to be preferentially cleaved by RNase A. It should be noted that although this treatment is indicative of the behavior of RNase A on poly(C) it cannot be used to calculate accurately the kinetic parameters. This is because (i) although the hexanucleotide product has been taken as the main intermediate one should consider a broader population of products and (ii) the same enzyme concentration has been used for the two consecutive steps, i.e. the initial concentration, but...
the relative concentration of enzyme in each step is smaller as they are catalyzed by the same enzyme which, therefore, is partitioned between the two steps. An accurate knowledge of the $K_{cat}/K_m$ for each substrate would allow knowledge of the proportion of enzyme acting in each reaction.

These results are supported by kinetic studies that suggest the contribution of phosphate-binding subsites to the catalysis. Steady-state kinetic studies of RNase A with oligonucleotides as substrates indicate that the $K_{cat}$ values increase with the substrate size, the $K_{cat}$ value for UpApA and UpApG being 3–5 times higher than those of UpA (Irie et al., 1984a). The use of oligouridylic acids has shown similar results (Irie et al., 1984b). From these findings it is demonstrated that the $p_0$, $p_1$, and $p_2$ binding sites play an important role in catalysis. Moreover, the partitioned between the two steps. An accurate knowledge of the catalytic process has been analyzed in $p_2$ chemically modified takes place.

Occupancy of the RNase A subsites. Finally, the hydrolytic step be progressively cleaved in the order of maximum to minimum long chain of RNA occurs. Subsequently, shorter fragments will be produced with long substrates as a consequence of the occupation of all the phosphate-binding sites. The $p_2$ phosphate-binding subsite also plays an important role in the catalytic mechanism and contributes significantly to the endonuclease activity of RNase A.

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