Volume 333, number 3, 207–210 FEBS 13164 November 1993

Hypothesis

The role of 2′,3′-cyclic phosphodiesters in the bovine pancreatic ribonuclease A catalysed cleavage of RNA: intermediates or products?

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Received 23 July 1993; revised version received 10 September 1993

There is a considerable degree of ambiguity in the literature regarding the role of the 2′,3′-cyclic phosphodiesters formed during the reaction of RNA cleavage catalysed by ribonuclease. Usually the reaction is considered to take place in two steps: in the first step there is a transphosphorylation of the RNA 3′,5′-phosphodiester bond broken yielding a 2′,3′-cyclic phosphodiester which in the second step is hydrolysed to a 3′-nucleotide. Although in many occasions, either explicitly or implicitly, the reaction is treated as taking place sequentially, this is not the case as it has been shown that the 2′,3′-phosphodiesters are actually released to the medium as true products of the reaction and that no hydrolysis of these cyclic compounds takes place until all the susceptible 3′,5′-phosphodiester bonds have been cyclised. Comparison of the hydrolysis and alcoholysis of the 2′,3′-phosphodiesters catalysed by RNase A indicates that the hydrolysis reaction has to be considered formally as a special case of the transphosphorylation back reaction in which the R group of the R-OH substrate is just H. It is thus concluded that the 2′,3′-cyclic phosphodiesters formed in the ribonuclease A reaction are true products of the transphosphorylation reaction and not intermediates as usually considered.

Ribonuclease A; Transphosphorylation; Hydrolysis; 2′,3′-Cyclic phosphodiester; Reaction intermediate

1. INTRODUCTION

The sequence of events that take place in the depolymerization of RNA by RNase is usually represented as shown in Scheme 1 (for example see [1,2]). In the first step there is a transphosphorylation reaction from the 5′ position of one nucleotide to the 2′ position of the adjacent nucleotide with the formation of a 2′,3′-cyclic phosphodiester. In the second step the 2′,3′-cyclic phosphodiester is hydrolysed to a 3′-nucleotide. It is widely accepted [2] that the catalytic mechanism involves general acid–base catalysis: in the first step His-119 acts as an acid and His-12 as a base whereas in the second step the roles of both histidines are reversed.

The formation of 2′,3′-cyclic *intermediates* in the cleavage of RNA catalysed either by bases or by bovine pancreatic ribonuclease was first shown by Brown and Todd [3] and Markham and Smith [4]. Although these *chemical* intermediates are mandatory [5,6] their role in the overall process has not been properly assessed. Part of the confusion may arise from the fact that the RNase reaction as usually shown gives the impression that the whole process, from polynucleotide to 3′ product, takes place in a sequential manner without release of the cyclic intermediate, i.e. that the intermediate occurs as an *enzyme-bound* intermediate. Thus, in the mechanisms proposed by Hammes [7], Roberts et al. [8] and Rabin et al. [9] the 2′,3′-cyclic intermediate does not appear to dissociate from the enzyme. Further, Anslyn and Breslow [10] and Breslow et al. [11] discuss their mechanism of action of RNase on the basis of a complete cycle of the enzyme via a 2′,3′-cyclic intermediate. The same assumptions are also made by the group of Karplus [12] in their molecular dynamics study. This inaccurate sequential scheme is also incorporated in some textbooks such as Rawn [13] and Voet and Voet [14] just to mention some recent examples. Finally, a great deal of ambiguity is present in many articles and reviews where the cyclic intermediates are considered, implicitly or explicitly, sometimes as enzyme-bound intermediates and sometimes as free intermediates depending on the context.

Here we present experimental evidence that, taken together with other data from the literature, show that RNase does not catalyse the hydrolysis of polynucleotides via an *enzyme-bound* 2′,3′-cyclic intermediate but rather that this cyclic phosphodiester is a *true product* which is released into the solution. This released com-

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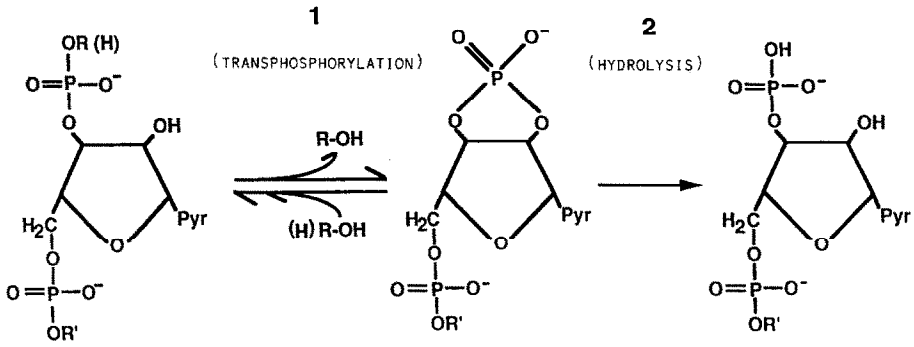
Abbreviations: RNase, ribonuclease; RNase A, bovine pancreatic ribonuclease A; C>p, cytidine 2′,3′-cyclic phosphate; CpC>p, cytidylyl-3′,5′-cytidine-2′,3′-cyclic phosphate; G>p, guanosine 2′,3′-cyclic phosphate.



Scheme 1

pound is only hydrolysed by the enzyme once practically all of the poly- or oligonucleotides have been used up. According to this idea, RNase catalyses two independent processes, a fact that has not always been realized and which may be the cause of certain difficulties concerning the RNase catalytic pathway. We propose that these two processes are reduced to one as represented by step 1 of Scheme 1 where R, in the forward direction, can be from a methyl group to a polynucleotide chain. In the latter case the OH group is attached to the 5′ position of a terminal ribose. In the reverse reaction R can also be a hydrogen atom and in this special case the reaction is a hydrolysis. The direction of the reaction is determined by a competition between substrates with different R groups, the overall thermodynamic equilibrium and the concentration of the different chemical species present. Here we consider the effect of different Rs upon the two reactions and we comment on the mechanism of cleavage of RNA. Although the following discussion concerns bovine pancreatic RNase A it is also applicable to other RNases which carry out their catalytic action via 2′,3′-cyclic phosphodiesters (e.g. RNase T_1 [15], barnase [16], human eosinophil-derived neurotoxin and human liver RNase [17], etc.).

2. EVIDENCE FOR A LAG IN 3′-MONONUCLEOTIDE FORMATION

A criterion for this reinterpretation of Scheme 1 is that there is a lag in the formation of the final product of the reaction, i.e. a 3′-pyrimidine mononucleotide or a polynucleotide ending in a 3′-pyrimidine nucleotide. Guasch et al., [18], analysed the reaction of RNase A on cytidylyl-3′,5′-cytidine-2′,3′-cyclic phosphate (CpC>p) by means of HPLC, and showed that no 3′-CMP appeared until all the dinucleotide had been converted to cytidine 2′,3′-cyclic phosphate (C>p). Here we show that with CpC too, little 3′-CMP is produced before all of the CpC has been converted to C>p, as illustrated in Fig. 1. The same pattern was obtained by Raines using UpA as substrate [19]. A similar result was found by Fersht and collaborators [16] who studied barnase, a *B. amyloliquefaciens* ribonuclease with specificity for purine bases. They showed that in the hydrolysis of GpA no release of proton due to the second step of the reaction, i.e. hydrolysis of G>p to 3′-GMP, was observed until most of the adenosine had been released.

Taken together, these results show that with simple substrates the hydrolytic reaction does not operate until practically all of the substrate has been transformed to free 2′,3′-cyclic nucleotide. This is also true for oligo and polynucleotides such as poly(C): no 3′-CMP appears until all oligonucleotides of intermediate size (which have a terminal 2′,3′-cyclic phosphate) have been transformed into C>p (M.V. Nogués, unpublished results). Raines has found the same result using a mutated RNase with specificity for poly(A) [19]. Polynucleotides and cyclic nucleotides can, therefore, be considered as competitive substrates for the enzyme with the longer nucleotides being transformed much more efficiently than the smaller cyclic nucleotides. This can be illustrated by reinterpreting the kinetic data of Witzel and Barnard [20] on the action of RNase A on CpA and C>p. From their kinetic parameters we calculate that the ratio of the rate of transphosphorylation (i.e. CpA to C>p) to the rate of hydrolysis (C>p to 3′-CMP), at pH 7.0 and 25°C, for the same substrate concentration is about 1,800. This ratio is even higher for longer oligonucleotides [21].

3. THE HYDROLYSIS REACTION IS FORMALLY EQUIVALENT TO THE BACK TRANSPHOSPHORYLATION REACTION: THE ROLE OF THE SUBSTRATE'S R GROUP

We now consider the backward direction of the transphosphorylation reaction and the hydrolysis reaction (Scheme 1). As pointed out above these pathways are essentially equivalent; the only difference is in the R group of the substrates which in the hydrolytic reaction is just H. Findlay et al. [22] showed that in the presence of alcohols both reactions occur (alcoholysis with R-OH, hydrolysis with H-OH). Hydrolysis only occurs because of the very high concentration (55 M) of H_2O with respect to ROH.

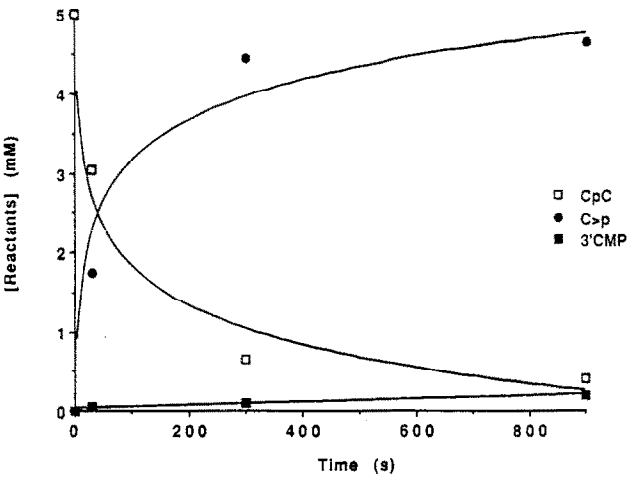


Fig. 1. Progress curves for the RNase A catalysed reaction on CpC. Initial conditions were: 4.5 mM CpC and 0.4 μM RNase A in 0.2 M sodium acetate/acetic acid buffer pH 5.5 at 25°C. Reaction was stopped by loading 20 μl aliquots onto an anionic exchange HPLC column. Chromatographic conditions were as in Guasch et al. [18].

The equivalence of the two pathways is such that, as pointed out by Richards and Wyckoff [1], 'it is not clear whether the RNase catalysed alcoholysis of 2',3'-cyclic nucleotides is similar to step 2 or to the reverse of step 1'. It is evident that the nature of the substrate's R group must be critical for the reaction through the establishment of interactions with specific regions in the enzyme, namely the B_2R_2 subsites (see [1,23] for the terminology of the binding subsites). It is logical to think that the more similar the substrate's R group to a nucleoside, adenosine in the case of RNase A, the more efficient the interaction will be. In this way the H of the water molecule would be clearly the less favoured structure. Jencks [24] has shown that 'an enzyme that utilizes the binding energy to the remainder of a specific substrate in order to fix a reacting hydroxyl group in the correct position can discriminate against an unpositioned water molecule by a factor of up to 3×10^3 from the entropy effect alone'. This assumption is further supported by the fact that the alcoholysis of cytidine 2',3'-cyclic phosphate catalysed by RNase A shows substantial rate increases with respect to the hydrolysis reaction depending on the type of alcohol [22]. Thus, methanol shows an 8-fold increase in the rate, and polyhydric alcohols have an even greater effect, a 10-fold increase in the case of ethylene glycol and a 22-fold increase for glycerol. The B_2R_2 subsites would assist in the correct positioning of the alcohol substrate molecule in such a way that proton abstraction by His-119 and nucleophilic attack by the resulting alkoxide ion on the phosphorus is greatly facilitated.

The dependence of the transphosphorylation rate on the nature of the leaving nucleoside seems to be a general feature of RNases. Thus, with RNase T_1, Steyaert et al. [25] showed that when the leaving nucleoside in guanosine dinucleotides (e.g. GpC) was replaced by the methyl group (GpMe), the transphosphorylation rate was reduced by three orders of magnitude. Of course, an induced-fit process caused by the binding of the substrate molecule could also be involved as it is known that the binding of substrate to RNase A produces a conformational change, leading to a more compact molecule [26].

4. THE ROLE OF RNASE IN DRIVING THE DIRECTION OF THE REACTION: THE PROBLEM OF SUBSITES

A corollary from the sequence of events that takes place according to the present interpretation is that, after every transphosphorylation and release of the cyclized product, there must be a reequilibration in the state of protonation of the active-site histidines in contraposition with the recovery of the original state of ionisation shown in the sequential schemes. It is possible that this re-equilibration is induced by the next substrate molecule if the mechanism of ligand substitution, as proposed by Jenkins [27], takes place as a means to dissociate the enzyme–product complex. In addition, the hydrogen bonding between Asp-121 and His-119 [28] could help in the reprotonation of the latter.

Here, the cyclic phosphodiester comes off much more rapidly than it is hydrolysed, even if the active site histidines are correctly protonated for hydrolysis. The time necessary for the release of the R-OH product, the entry of a water molecule in the active-site cleft and its correct positioning for a productive attack on the phosphorus is so long and the process so inefficient that it allows the displacement of the cyclic nucleotide from the enzyme by a longer molecule that can easily undergo forward transphosphorylation.

In the presence of very large substrates such as RNA itself the hydrolysis reaction is even less efficient. This can be explained, at least partially, by the subsite structure of the active site of the enzyme to which RNA is bound very tightly. Thus, when the 3',5'-phosphodiester bond of an RNA molecule has been broken, the product having the 2',3'-cyclised end is bound only to the 5' side of the catalytic site p_1, and no longer spans over to the 3' side (Fig. 2). In these circumstances the product of the forward transphosphorylation, which is also a substrate for hydrolysis, is easily displaced by another substrate molecule which can undergo forward transphosphorylation (i.e. a molecule that when bound to the enzyme will span subsites at both sides of p_1).

5. SUMMARY AND PROPOSAL

Although in the RNase A catalysed depolymerization process of RNA in an aqueous environment the hydrolysis of the 2',3'-cyclic phosphodiesters to 3'-nucleotides is an important reaction, we note that hydrolysis is formally equivalent to a reversal of the transphos-

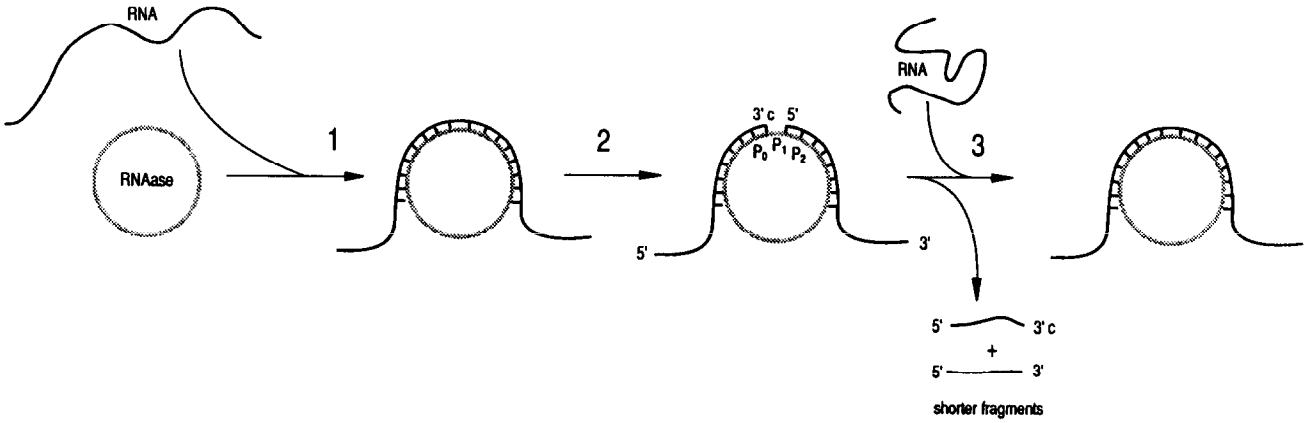


Fig. 2. Model of the cleavage of an RNA chain by RNase A (see Parés et al. [21]. The model is based on the co-operative binding between the multiple protein subsites and the phosphates of the polynucleotide. (1) A long RNA chain binds to RNase. (2) Cleavage occurs in the active site resulting in the formation of two shorter oligonucleotide fragments, one of them ending with a 2',3'-cyclic phosphate, indicated as 3'c. (3) The co-operative binding is weakened in the oligonucleotide fragments and this favours their replacement by a longer chain that fully occupies the RNase A binding sites. After the transphosphorylation step, both fragments of the cleaved polynucleotide leave the enzyme and are replaced by a new, long-chain, substrate molecule. In subsequent reactions the shorter fragments will also be broken, and eventually the hydrolytic step occurs when most of the RNA has already been cleaved in the transphosphorylation step.

phorylation of the 3',5'-phosphodiesters. The differences in rate between the transphosphorylation and hydrolysis can be explained in terms of the position of the chemical equilibrium, the concentration of the various chemical species present and the specific interactions of the different R-OH groups involved in the back reaction with several enzyme subsites. As a consequence of the formal equivalence between these two reactions we propose that the present classification of ribonuclease is changed from a hydrolase (EC 3.1.27.5) back to a transferase as in the 1978 classification (EC 2.7.7.16).

Acknowledgements: This work was supported by Grants PB88-0232 and PB91-0480 from the DGICYT of the Ministerio de Educación y Ciencia, Spain. We also thank the Fundació M.F. de Roviralta (Barcelona, Spain) for grants for the purchase of equipment. C.M.C. was awarded a Haut Niveau fellowship from the French Ministère de la Recherche et de la Technologie.

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