THE MECHANISM OF PURINE POLYNUCLEOTIDE HYDROLYSIS BY RIBONUCLEASE A

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1. Introduction

It is generally held that bovine pancreatic ribonuclease (RNAase) is an enzyme specific for phosphodiester bonds in which the 3′-linked nucleotide is a pyrimidine [1]. Slow hydrolysis of purine nucleotides has nevertheless been observed in ribonuclease preparations and is sometimes attributed to other contaminating nucleases [2].

In this communication, we wish to report observations made by ^{31}P NMR which indicate that the hydrolysis of single stranded poly(A) in the presence of ribonuclease proceeds via a 2′,3′-cyclic intermediate. The kinetics of the reaction suggest that the cyclization step is enzymatic, and the hydrolysis is completed non-enzymatically by the basic solvent (pH 7.90, held constant), yielding a mixture of 2′ and 3′-AMP.

2. Experimental

Phosphorus NMR spectra were obtained at 40.5 MHz on a Varian Associates XL-100-15 spectrometer equipped with a Nicolet Technology Corporation (Mountain View, California) Fourier Transform accessory and a NIC-1080-16K Computer. Heteronuclear proton noise decoupling was achieved with a Varian gyrocode spin decoupler model V 4421. Sensitivity was increased by using quadrature detection and base line stability was improved by usual shifting of the RF-pulse phase. Purified poly(A) (60 mM in phosphodiester groups) from Sigma and RNAase A (60 μM) from Worthington were dissolved in 0.1 M Tris–HCl buffer pH 7.90. All spectra were recorded at 42°C in 12 mm cells. Chemical shifts are expressed from 85% H_3PO_4 as an external standard.

Abbreviations: RNAase = Bovine Pancreatic Ribonuclease A, poly(A) = polyadenylic acid, poly(U) = polyuridylic acid, AMP = adenosine monophosphate, His = histidine, NMR = nuclear magnetic resonance

3. Results

Figure 1 shows the ^{31}P spectra of poly(A) in the presence of RNAase over a period of 7 days. Initially, only the single peak of the poly(A) (δ = +0.76 ppm) is seen (fig.1). The small separate peak of the 5′-terminal residue often seen in the spectra of shorter polynucleotide chains is not seen in this spectrum because of the relatively high degree of polymerization (mol. wt ∼ 100 000). After approximately 1 h, an additional peak with a δ of −20.39 ppm a position characteristic of the 2′,3′-cyclic AMP [3] begins appearing (fig.1). This peak does not appear in control samples of poly(A) held under identical conditions (pH, concentration, ionic strength and temperature) except for the presence of RNAase. Its appearance can therefore be attributed to enzymatic catalysis. The reaction proceeds, as might be expected, only at tem-

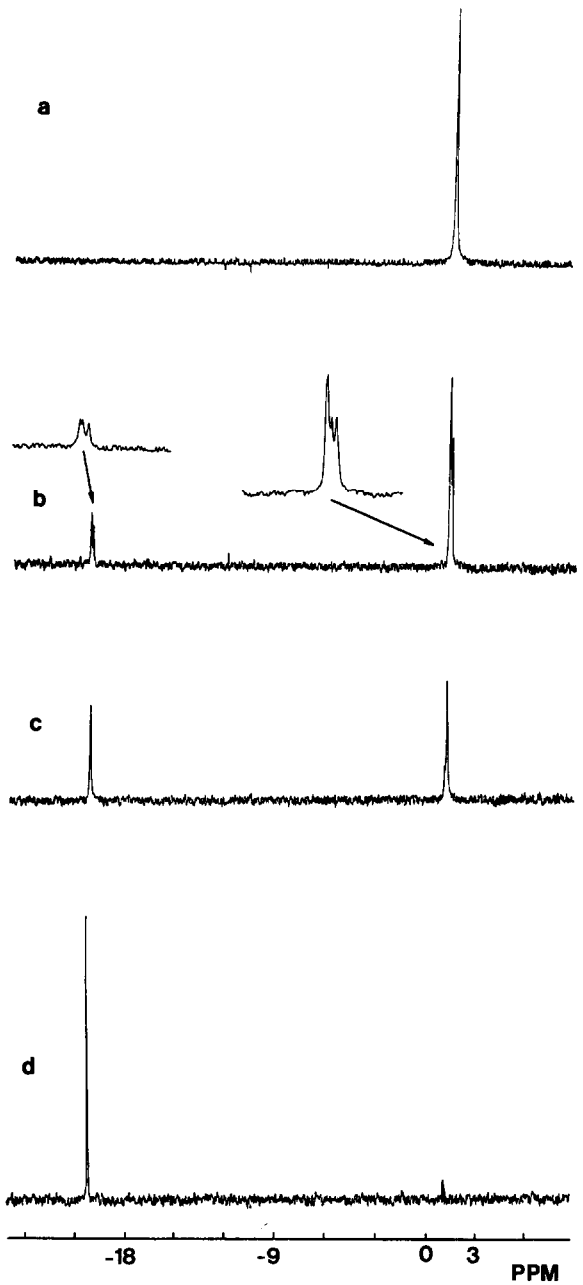

Fig.1. Proton decoupled ^{31}P spectra illustrating the time course of poly(A) hydrolysis in presence of RNAase A at pH 7.90. (a) Initial, (b) 32 h (c) 82 h (d) 142 h. Spectra are fully proton-noise decoupled. Acquisition time 5.45 s. Digital resolution 0.18 Hz. Sensitivity enhancement corresponding to 0.3 Hz artificial line broadening. 52 Pulses. Inserts on spectrum (b) represent the −21.5 to −19 ppm (left) and −1 to + 1.5 ppm (right) regions of the spectrum.

peratures above 35°C, i.e., only on partially unstacked poly(A) [4].

As the concentration of the cyclic intermediate increases, heterogeneity appears in the poly(A) peak, as well as in the cyclic AMP peak (fig.1), indicating the gradual depolymerisation of poly(A) yielding a mixture of oligonucleotides of different length (from 0.75–0.59 ppm) and of short oligonucleotides containing a cyclized residue (from −20.25 to −20.39 ppm). The signal attributable to the final product is initially broad and complex then becomes narrower as the cyclization reaction nears completion. This indicates that initially the product is a mixture of mono-, di- and perhaps other short oligonucleotides which gradually become digested by repeated interaction with the enzyme.

The cyclization reaction is essentially completed in 160 h. The final hydrolysis product, a mixture of 2′- and 3′-nucleotides does not begin appearing until 150 h (fig.2). The resonances corresponding to 2′-AMP and 3′-AMP (fig.2) are readily identified as the basis of their chemical shifts and coupling constants patterns [3]. This second step of the reaction was not followed to completion and is believed to be non-enzymatic.

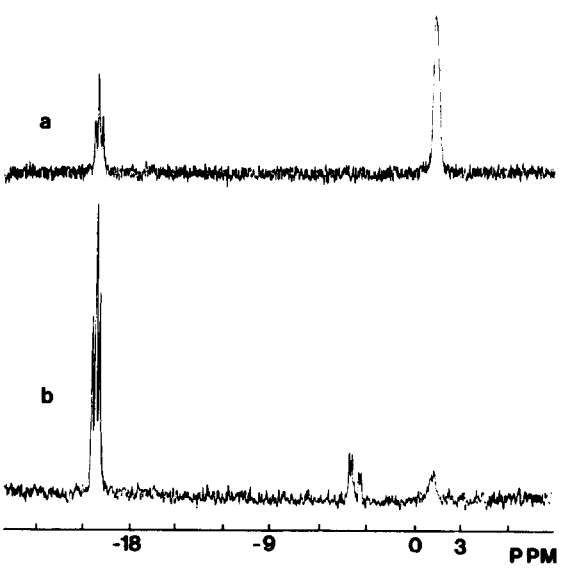

Fig.2. Undecoupled ^{31}P spectra illustrating the time course of poly(A) hydrolysis in presence of RNAase A at pH 7.90. (a) 50 h of reaction. 520 pulses. See legend of fig.1 for conditions. (b) 155 h of reaction. 520 pulses. Same conditions as above except for 0.5 Hz line broadening for sensitivity enhancement. Low field doublet 2′-AMP. High field doublet 3′-AMP.

4. Discussion

These results indicate that RNAase is capable of carrying out the cyclization but not the hydrolytic step of its two-step mechanism on purine polynucleotides. The structural interpretation of this finding is that poly(A), like poly(U), forms a complex at the active site of ribonuclease, but in a manner which permits alignment of the phosphate with one of the active site histidines but not with the other. This allows the proton transfer from the 2′-hydroxyl of the ribose to histidine 12 required for the formation of the pentacovalent intermediate with subsequent cyclization [5], but does not permit the second proton transfer from a water molecule to His 119 required for hydrolysis of the cyclic nucleotide. The necessary degree of distortion could result from the fact that the ribose is attached to N_9 of the imidazole ring in purine nucleotides, but to N_1 of the pyrimidine ring in pyrimidine nucleotides. Thus, even if the fit of the pyrimidine moiety of adenine were perfect at the pyrimidine-specific (B_1) site of RNAase [2], the position of the ribose with respect to His 12 and His 119 would be different in the two cases. Examination of molecular models indicates that the resulting change in the position of the phosphate is greater relative to His 119 than relative to His 12. The less favorable mutual orientation of the 2′-hydroxyl and His 12 probably accounts for the extreme slowness of the cyclization reaction in the purine case. The larger displacement of the phosphate relative to His 119 accounts for the failure of the in-line mechanism to go to completion.

Acknowledgements

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