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Original Paper

Kinetic Studies on Ribonuclease F1 Using Diribonucleoside Phosphates as Substrates

(ribonuclease F1 / enzyme kinetics / diribonucleoside phosphate)

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Transphosphorylation of diribonucleoside phosphates (GpA, GpC, GpU, ApA, CpC and UpU) to corresponding nucleoside 2',3'-cyclic phosphate + nucleoside by ribonuclease F1 was followed by high-performance liquid chromatography and the kinetic constants, K_m and k_{cat}, were determined. The K_m values were similar for all the standard substrates, GpN's but the k_{cat} values followed the order GpC > GpA > GpU. This suggests that binding affinity of a substrate to the enzyme is determined primarily by the guanine binding recognition subsite and that the enzyme contains a second subsite preferring C > A > U which contributes to catalysis by interacting with the leaving nucleoside. All the non-standard substrates, NpN's, showed similar k_{cat} / K_m values which were approximately six orders of magnitude smaller than those for GpN's, although individual K_m and k_{cat} values varied considerably: small k_{cat} for CpC and UpU, and large K_m for ApA. This means that ribonucleas F1 degrades GpN's 10^6 times more effectively than NpN's. The kinetic constants for hydrolysis of guanosine 2',3'-cyclic phosphate by ribonuclease F1 were also determined. It showed almost the same K_m but three orders of magnitude smaller k_{cat} compared to GpN's.

INTRODUCTION

We have long been studying the structure and function of ribonuclease (RNase) F1 isolated from the culture filtrate of a phytopathogenic fungus *Fusarium moniliforme*. Our studies have shown that RNase F1 belongs to the RNase T1 family, whose representative, RNase T1 from *Aspergillus oryzae*, is a guanine-specific endoribonuclease and is one of the best-characterized enzymes from both structural and functional viewpoint. In the RNase T1 family, RNase F1 takes up a unique position. It is rather distant evolutionarily from RNase T1[1]. Structurally, however, RNases F1 and T1 are very similar in the core including the active site, but they differ considerably in the periphery[2]. With such a background, detailed comparison of their functions will give further insight into the action mechanism of this family of enzymes.

RNase T1 is a guanine-specific endoribonuclease: it splits internucleotde bonds in RNA between 3'-guanylic acid and 5'-hydroxyl group of an adjacent nucleotide with intermediary formation of guanosine 2',3'-cyclic phosphate (G > p), which is ultimately hydrolyzed to guanosine 3'-phosphate (G3'p). Therefore, if a minimal substrate, dinucleoside monophosphate GpN, is used, the reaction proceeds as follows.

$$GpN \rightarrow G > p + N \quad (1)$$

$$G > p + H_2O \rightarrow G3'p \quad (2)$$

Step 1 (transphosphorylation) proceeds much faster than step 2 (hydrolysis). Earlier kinetic studies with GpN substrates have shown that nucleoside N has a considerable effect on the rate of step 1[3,4]. The k_{cat} values for transphosphorylation follow the order GpC > GpA > GpU, whereas the K_m values for these substrates are very similar. These results suggest that RNase T1 has a second subsite in addition to the guanine recognition subsite and that the second subsite preferring C > A > U contributes to catalysis by interacting with the leaving nucleoside. Although the nature of this subsite remained unclear by structural studies alone, Steyaert *et al*. have shown through site directed mutagenesis that the subsite includes Asn 36 and Asn 98 which are far apart in the tertiary structure[5]. In view of the state of conservation of these residues in the primary structure of RNase F1, kinetic studies on the effect of the second nucleoside of GpN's would be a good test for their conclusion. Furthermore, we demonstrated earlier that RNase F1 is capable of

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degrading nonstandard type of substrates NpN, though very slowly[6]. In this paper, we also carried out kinetic studies on this type of substrates.

MATERIALS AND METHODS

Materials — RNase F1 was prepared as described previously[7]. The enzyme concentration was determined on the basis of absorbance at 280 nm using a molecular absorbance of 13,900 $M^{-1}cm^{-1}$. Barium salt of G > p was synthesized according to Smith *et al*[8]. The barium salt was converted to sodium salt by passage of its solution through a column of Dowex 50, Na^+ form. Diribonucleoside phosphates, GpA, GpC, GpU, ApA, CpC and UpU were the products of Sigma. Their concentrations were determined spectrophotometrically after alkaline hydrolysis.

Kinetics — The initial velocity v was measured at various substrate concentrations. The kinetic constants, K_m and k_{cat}, were determined by a computer-aided nonlinear least square regression method according to the Michaelis-Menten equation

$$v = k_{cat}es/(K_m + s)$$

where e = enzyme concentration and s = substrate concentration. The substrate concentration range was chosen so that it contained the K_m when possible.

Transphosphorylation of GpN substrates and hydrolysis of G>p — These were followed by high performance liquid chromatography (HPLC) using an anion exchange column, IEX-540DEAE (Toso, 7.5 i.d. x 150 mm). Isocratic elution at a flow rate of 1.0 ml/min was performed with 0.5 M KH_2PO_4. The detection was carried out spectrophotometrically at a wavelength of 260 nm. Retention times in minute of the relevant compounds were as follow; adenosine (6.3), cytidine (4.8), uridine (5.1), G3'p (8.7), G > p (10.4), GpA (8.5), GpC (6.4), and GpU (7.2). Therefore, the substrate and the products thereof were well resolved in any case. Reaction mixtures contained in a total volume of 80 μl: various amounts of the substrate, 4 μmol of Tris-HCl buffer (pH 7.5), and RNase F1 whose amount was 30 fmol for GpC, 73 fmol for GpA and GpU, and 8.2 pmol for G > p. The mixture was incubated at 37 ℃ and 10 μl aliquots were withdrawn at 15 min intervals for analysis by the HPLC. The remaining substrate was quantitated on the basis of peak height, and the initial velocity was calculated from the initial slope of the decrease curve.

Transphosphorylation of NpN substrates — This was followed by the HPLC method described previously[6] except that 0.15 M, instead of 0.25 M, KH_2PO_4 was used as the eluent in order to attain better resolution. The method was slightly different from that described above simply due to renewal of the HPLC apparatus, but the basic principle of the method remained unchanged. Reaction mixtures contained in a total volume of 80 μl: various amounts of the substrate, 4 μmol of Tris-HCl buffer (pH. 7.5), and 1.6 nmol of RNase F1. The mixture was incubated at 37 ℃ and 2 μl aliquots were withdrawn at 1 h intervals for analysis by the HPLC. The amount of nucleoside N was determined from the peak area, and the initial velocity was calculated from the initial slope of the increase curve.

RESULTS AND DISCUSSION

Kinetic studies on RNases using diribonucleoside phosphates are abundant in literature. In these studies, reaction rates were usually determined on the basis of hyperchromicity (increase of absorbance) caused by cleavage of the substrates. The method is simpler than the HPLC method described here. However, our HPLC method permits to study a wide range of substrate concentration allowing determination of kinetic constants with adequate precision. Furthermore, it reveals clearly what reaction is going on. For example, with all the GpN substrates, the reaction products were always G > p and nucleoside N: no G3'p was observed even at the time when the substrate was completely consumed. On the other hand, the spectrophotometric method can only tell that the substrate is being cleaved.

The obtained kinetic data fitted well to the Michaelis-Menten equation (data not shown), giving the kinetic constants k_{cat} and K_m with adequate precision. In the case

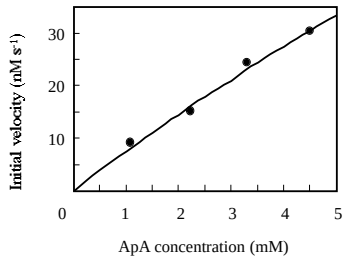

Fig. 1. Dependence of the initial velocity of ApA transphosphorylation by RNase F1 on its concentration.

of ApA, however, the data shown in Fig. 1 demonstrate that the K_m value is far beyond the concentration range examined. We could not go to a higher substrate concentration from a practical reason, thus we could only get approximate estimates of the kinetic constants. However, the parameter k_{cat} / K_m calculated from the initial slope of the curve in Fig. 1 is reliable. The kinetic constants thus obtained are listed in Table I.

Table I. Kinetic constants for the hydrolysis of G > p and the transphosphorylation of diribonucleoside phosphates by RNase F1

Substrate	K_m(mM)	k_{cat}(s^{-1})	k_{cat} / K_m (M^{-1} s^{-1})
G > p	0.42 ± 0.10	(5.3 ± 0.5) x 10^{-1}	1.3 x 10^3
GpA	0.93 ± 0.17	(2.8 ± 0.2) x 10^2	3.0 x 10^5
GpC	1.01 ± 0.32	(8.2 ± 1.2) x 10^2	8.1 x 10^5
GpU	1.09 ± 0.14	(1.1 ± 0.1) x 10^2	1.0 x 10^5
ApA[a]	*ca.*30	*ca.* 1.2 x 10^{-2}	3.8 x 10^{-1}
CpC	0.64 ± 0.10	(3.6 ± 0.2) x 10^{-4}	5.6 x 10^{-1}
UpU	0.96 ± 0.06	(3.0 ± 0.1) x 10^{-4}	3.2 x 10^{-1}

[a] Each kinetic constant, K_m or k_{cat}, could not be determined accurately because of high K_m, but the k_{cat} / K_m is reliable.

The following points can be made from these results.

1) The K_m values are similar for all the GpN's and G>p, suggesting that the binding affinity of a substrate to the enzyme is dependent primarily on the guanine nucleotide moiety entering the recognition subsite.

2) The k_{cat} values for transphosphorylation of GpN's follow the order GpC > GpA > GpU. This implies that RNase F1 contains a second subsite preferring C > A > U which contributes to catalysis by interacting with the leaving nucleoside.

3) The k_{cat} value for the hydrolysis of G > p is three orders of magnitude smaller than those for the transphosphorylation of GpN's.

4) When N = C or U (pyrimidine nucleoside), the K_m values for NpN's are almost the same as those for GpN's, but the k_{cat} values are approximately six orders of magnitude smaller.

5) When N = A, the K_m value is much larger, whereas the k_{cat} value is approximately four orders of magnitde smaller than those for GpN's.

6) The k_{cat} / K_m values are similar for all the NpN's and are approximately six orders of magnitude smaller than those for GpN's. This means that RNase F1 degrades the GpN's 10^6 times effectively than the NpN's.

Points 1) and 2) are exactly what has been observed with RNase T1[3,4]. This indicates that the second subsite, which only affects the catalysis, is well conserved in RNases T1 and F1. Steyaert *et al.* have shown through site-directed mutagenesis that Asn 36 and Asn 98 of RNase T1 are parts of this subsite[5]. However, as shown in Fig. 2, Asn 98 is conserved in RNase F1 but Asn 36

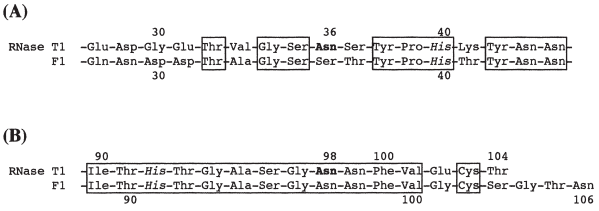

Fig. 2. Alignment of the amino acid sequences of RNases T1 and F1 around Asn 36 (A) and Asn 98 (B) of RNase T1. Conserved residues are boxed. Asn 36 and Asn 98 of RNase T1 are shown in boldface letters. The active site residues, His 40 and His 92 in RNase T1 numbering, are in italics.

is not. Moreover, inspection of the tertiary structures of RNase T1 (Protein Data Bank ID: 9RNT) and RNase F1 (1FUT) has revealed that the regions around Asn 36 are very different whereas those around Asn 98 are well conserved, as anticipated from the degrees of sequence homology. Therefore, the role played by Asn 36 of RNase T1 should be reconsidered in the light of our study. Point 3) has also been observed with RNase T1. Yoshida and Ohtsuka reported the kinetic constants K_m = 2.99 mM and k_{cat} = 2.88 s^{-1} at pH 7.5 and 37 ℃ (the same condition as ours)[9]. Therefore, the k_{cat} value for RNase F1 is approximately five times smaller than that for RNase T1. No explanation is possible at present for this substantial difference.

Point 4) implies that CpC or UpU binds to the enzyme equally well as GpN's even without G that is recognized by the primary subsite, but that the transphosphorylation to follow proceeds extremely slowly. This suggests that the main role of guanine base binding by the primary recognition site is to orient the substrate in a position that enables the reaction to take place rapidly. In contrast, ApA can bind to the enzyme only weakly as demonstrated by its large K_m value. This means that the primary recognition subsite discriminates adenine from guanine strictly. Once ApA binds to the enzyme, however, the transphosphorylation proceeds two orders of magnitude more rapidly than for CpC or UpU. In terms of k_{cat} / K_m, as a consequence, the GpN's are degraded 10^6 times

effectively than the NpN's. Our studies on RNase F1 have given for the first time a reliable estimate of the degree of specificity which is approximately 10^6 : 1 in favor of guanine against other bases. It can, therefore, be said that the guanine specificity of enzymes of the RNase T1 family is not absolute but relative, although this high level of discrimination power can be considered practically guanine-specific. There is difference, however, in the behavior of the enzyme toward bases other than guanine. It discriminates A from G strictly at the binding level and expels the former. On the other hand, it binds C or U equally well as G, but the transphosphorylation does not proceed rapidly.

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