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Interaction of Cytidylyl(3′→5′)cytidylyl(3′→5′)cytidylyl(3′—5′)guanylic Acid with Ribonuclease T_1 diazotized with Diazonium 1-(H)-tetrazole

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Ribonuclease T_1 modified with diazonium-1-(H)-tetrazole (DHT-RNase T_1) reported by Kasai has been known to be inactive towards RNA, but active towards guanylyl (3′→5′)-cytidine (GpC). In order to explain this peculiar phenomenon, a couple of experiments were performed. DHT-RNase T_1 is almost inactive towards poly G and poly C as well as RNA, but active against GpCpC. Michaelis constants of DHT-RNase T_1 for GpC was about twice as large as that of native RNase T_1. Although *Ki* value for 2′,(3′)-GMP of DHT-RNase T_1 was about 4 times as large as that of native RNase T_1, that of $(Cp)_3Gp$ for DHT-RNase T_1 was more than 60 times larger than that for the native enzyme. From the results described above, it was suggested that a peculiar nature of DHT-RNase T_1 towards RNA was probably due to the unfavorable effect of the modification on the binding of guanylic acid residue having polynucleotide at 5′-OH side.

Many attempts have been done on the clarification of structure-function relationship of ribonuclease T_1 from *Aspergillus oryzae* (E.C. 2.7.7.26) by means of kinetics, chemical modification and biophysical studies.[2] Among the many chemical modification studies, the reaction of RNase T_1 with diazonium 1-(H)-tetrazole (DHT) reported by Kasai was quite interesting, since the modified RNase T_1 (DHT-RNase T_1) had no activity towards RNA, but was active towards guanylyl(3′—5′)cytidine(GpC). According to his report, the chemical modification by DHT occurs on NH_2-terminal alanine, ε-amino group of lysine and $tyrosine_{45}$ and/or $tyrosine_4$.[3] In this report, an attempt was done to clarify why DHT-RNase T_1 is inactive towards RNA in spite of its activity towards GpC, and proposed a hypothesis that chemical modification by DHT made the binding of guanylate covalently bound with oligonucleotide on its 5′-OH side unfavorable.

Experimental

Substrate——Guanylyl (3′→5′) cytidine was prepared from 2′,3′-cyclic GMP and cytidine by the method of Ukita, *et al.*[4] Poly I and poly G were purchased from Boehringer Manheim. Cytidylyl (3′→5′) cytidylyl-(3′→5′)cytidylyl(3′→5′) guanylic acid was obtained from the hydrolyzate of poly CG(C/G=3) by RNase T_1 and purified by paper chromatography on Toyo Roshi No. 51 filter paper using the following system, 2-propanol: conc. NH_4OH: H_2O (7 : 1 : 2 v/v). Guanylyl (3′→5′)cytidylyl(3′→5′) cytidine was obtained from Miles Co. Ltd.

Enzyme——RNase T_1 was prepared according to the method of Takahashi.[5] DHT-RNase T_1 was prepared according to Kasai's procedure.[2]

Enzyme Assay——(a) Enzyme assay using RNA as substrate. The assay of enzymatic activity was carried out by modified Kunitz procedure.[6] To 2 ml of Tris–HCl (0.1 M, pH 7.5) containing 1 mg RNA, 10—20 μl of enzyme solution was added and the decrease in optical density at 300 nm was followed by a

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2) K. Takahashi, T. Uchida, and F. Egami, "Advances in Biophysics," Vol. 1, ed. by K. Kotani, University of Tokyo Press, Tokyo, 1970, p. 503.
3) H. Kasai, "The 21th Symposium on Protein Structure," Chiba, Japan, 1970, p. 90.
4) T. Ukita, M. Irie, M. Imazawa, Y. Furuichi, H. Nishimura, and T. Sekiya, *Seikagaku*, **40**, 363, (1967) (in Japanese).
5) K. Takahashi, *J. Biochem.*, **49**, 1 (1960).
6) M. Kunitz, *J. Biol. Chem.*, **164**, 563, (1946).

Shimadzu UV 200 spectrophotometer at 23°. (b) Enzyme assay using poly G and poly I as substrate. The activity was measured by the increase of optical density at 250 nm for poly G and 260 nm for poly I by mixing 2 ml of homopolynucleotide solution in Tris-HCl (0.1 M, pH 7.5) with 20 μl of RNase T_1 or DHT-RNase T_1 (1 mg/ml). The final polynucleotide concentration was 10^{-4} M in nucleotide units. (c) Enzyme assay using GpCpC as substrate. GpCpC (0.9 optical density unit in 1.5 ml of 0.1 M Tris-HCl buffer, pH 7.5) was incubated with 5 μg of RNase T_1 or DHT-RNase T_1 and the increase in optical density at 280 nm was followed by recording spectrophotometer at 23°. (d) Enzyme assay using GpC as substrate. The RNase T_1 and DHT-RNase T_1 activities were measured at pH 5.0 (0.01 M, acetate buffer) at 25° in the presence or absence of inhibitor. To the 2 ml of reaction mixture containing $0.25—1.0\times10^{-4}$ M of GpC, 20 μl of enzyme (1 mg/ml) was added and the reaction was followed by the increase in optical density at 280 nm.[7] To measure the inhibition constant, 1.12×10^{-4} M of 2′,(3′)-GMP or 0.6×10^{-4} M $(Cp)_2Gp$ was added to the reaction mixture. *Ki* values were obtained by Lineweaver and Burk's plot.[8]

Amino Acid Analyses——Amino acid analyses were performed according to the method of Spackman, *et al.*[9] by Nihondenshi 6AH amino acid analyzer.

Results and Discussion

DHT-RNase T_1 was prepared according to Kasai's procedure.[3] The amino acid composition of DHT-RNase T_1 used for the experiments reported here is shown in Table I. DHT-RNase T_1 lost about each one residue of alanine, lysine and tyrosine and the results agreed well with those of Kasai.[3]

RNase T_1 hydrolyzes RNA preferentially at the 3′ side of guanylic acid or inosinic acid residue. Thus the activity of DHT-RNase T_1 towards homopolynucleotides, poly G and poly I were measured as model compounds (Table II). The results indicated that DHT-RNase T_1 is inactive towards homopolymers as well as RNA. Since DHT-RNase T_1 could hydrolyze GpC to Gp and cytidine, it is not unlikely that the hydrolysis mechanism of

Table I. Amino Acid Composition of Modified RNase T_1[a]

Amino acid	DHT-RNase T_1[b]	RNase T_1
Trp[c]	—	— (1)
Lys	0.15	0.99(1)
His	2.84	3.02(3)
Arg	0.83	0.98(1)
Asp	15.3	15.5 (15)
Thr	4.95	5.40(6)
Ser	13.0	13.2 (15)
Glu	9.50	9.01(9)
Pro	3.70	3.72(4)
Gly	12.0	12.0 (12)
Ala	5.90	7.01(7)
Cys/2	3.58	3.50(4)
Val	6.00	5.40(8)
Met	0	0 (0)
Ile	0.81	1.15(2)
Leu	2.32	2.78(3)
Tyr	7.58	8.50(8)
Phe	3.30	3.40(4)

a) The data were obtained from the amino acid analyses of 24 hours hydrolyzate of the enzyme in 6 N HCl at 110°. The data were calculated assuming that RNase T_1 contains 12.0 residues of alanine. The figures in parenthesis were the theoretical values.[1]
b) The DHT-RNase T_1 analyzed has less than 5% activity of the native RNase T_1 as far as measured using RNA as substrate.
c) not determined

7) M. Irie, *J. Biochem.*, **63**, 649 (1968).
8) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
9) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1955).

Table II. Hydrolyses of RNA, Poly G, Poly I and GpCpC by RNase T_1 and DHT-RNase T_1

Substrate	Activity[a]	
	RNase T_1	DHT-RNase T_1[b]
RNA	100	9.1
Poly G[c]	100	14
Poly I[c]	100	14
GpCpC[d]	100	60

a) Activity was expressed as the percentage of the activity of RNase T_1.
b) DHT-RNase T_1 used for this experiment was 9.1% active as compared to the activity of the native RNase T_1 using RNA as substrate.
c) Activity was measured using assay method (b) described in the text.
d) Activity was measured using assay method (c) described in the text.

DHT-RNase T_1 is altered from RNase T_1 by chemical modification in essence. Therefore, the loss of the activity of DHT-RNase T_1 towards RNA, poly G and poly I may be related to the polymeric nature of substrates, not to the presence of a particular bases in RNA other than guanine.

As RNase T_1 is a kind of endonuclease having specificy towards guanine base, when RNase T_1 is incubated with oligonucleotide such as XpYpZp....XpZpXpGpXpYpZp....XpYp (X, Y and Z are the bases of nucleic acid components other than guanine), RNase T_1 might bind with Gp portion of the oligonucleotide preferentially. Therefore, the influence of oligonucleotide portion at 3'-OH side or 5'-OH side of guanine on DHT-RNase T_1 was studied. First of all, the hydrolysis of GpCpC (obtained from Miles Co. Ltd.,) as a example of oligonucleotide having more nucleotides than GpC at the 3'-OH side of guanylic acid, by RNase T_1 and DHT-RNase T_1 was studied. DHT-RNase T_1 hydrolyzed GpCpC at the rate of 60% of RNase T_1 under the conditions used. Since as shown in Table III, *Km* value of DHT-RNase T_1 towards GpC was about twice higher than that of RNase T_1, it could be possible that *Km* value of DHT-RNase T_1 for GpCpC was higher than that of RNase T_1. If this is the case, V_{max} value of DHT-RNase T_1 for GpCpC should be higher than 60% of that of RNase T_1. The results indicated that diazotization of RNase T_1 did not cause serious influence on the hydrolysis of GpXpYp....(X, Y are the bases of nucleic acid other than guanine).

Then, the binding of $(Cp)_3Gp$ with RNase T_1 was studied. RNase T_1 was inhibited by $(Cp)_3Gp$ competitively like by guanylate[10] (not shown here). The *Ki* values of GMP and $(Cp)_3Gp$ for RNase T_1 and DHT-RNase T_1 were measured using GpC as substrate at pH 5.0. The results are shown in Table III. As already reported by Kasai, *Km* value of DHT-RNase

Table III. *Km* and *Ki* values of Modified RNase T_1 at pH 5.0

	Substrate or inhibitor	RNase T_1	DHT-RNase T_1[a]
Km(M)	GpC	0.72×10^{-4}	1.38×10^{-4}
		(2.3×10^{-4})	(5.9×10^{-4})[b]
Ki(M)	2',(3')-GMP	0.1×10^{-4}	0.41×10^{-4}
	$(Cp)_3Gp$	0.21×10^{-4}	1.25×10^{-3}

a) DHT-RNase T_1 used has less than 5% of activity of the native RNase T_1 using RNA as substrate. The enzymatic assay was performed using GpC as substrate as described in the text.
b) These are taken from the data of Kasai[2] measured at pH 7.0.
c) Activity was measured using the assay method (b) described in the text.
d) Activity was measured using the assay method (c) described in the text.

10) M. Irie, *J. Biochem.*, **56**, 495 (1964).

T_1 for GpC was twice as large as that of RNase T_1,[3] and similarly, *Ki* value of DHT-RNase T_1 for 2′,(3′)-GMP was about four times as large as that of the native RNase T_1.

In the case of RNase T_1, *Ki* value for $(Cp)_3Gp$ was slightly higher than that for 2′,(3′)-GMP. Although we were unable to measure the exact *Ki* value for $(Cp)_3Gp$ of DHT-RNase T_1, the value was more than 60 times higher than the *Ki* value for $(Cp)_3Gp$ of the native RNase T_1. The experiments shown above seem to indicate that modification of RNase T_1 by DHT-group is quite unfavorable for the binding of guanylic acid residue having large nucleotides chain at 5′-OH side. Whether this phenomenon is due to the steric interference of DHT-group or the change in conformation of RNase T_1 is not clear at present. According to Kasai, optical rotatory dispersion or circular dichroism curves for RNase T_1 and DHT-RNase T_1 around 220 nm were very similar, but some differences were observed around at 270 nm. Of the three groups modified by DHT, the modification of tyrosine residue might be responsible for this phenomenon, because the introduction of trinitrophenyl group at lysine and NH_2-terminal alanine residues of RNase T_1 did not give such character to RNase T_1.[11] From the evidence described above, it was concluded that a peculiar nature of DHT-RNase T_1 towards RNA was probably due to the unfavorable effect of the modification on the binding of guanylic acid residue having polynucleotides at 5′-OH side.

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Synthesis of Pyrrolo[2,1,5-*cd*]indolizine, Cycl[3,2,2]azine[1]

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Cycl[3,2,2]azine (III) was synthesized from II by Pd-C dehydrogenation.

Previous approaches to the synthesis of cyclazines have been largely based on annelation procedures employing quinolizines,[3] indolizines[4] or pyrrolizines.[5]

The dehydrogenation by Pd-C of readily accessible octahydropyrrolo[2,1,5-*cd*]indolizine(II) now reported is a new approach to the synthesis of pyrrolo[2,1,5-*cd*]indolizine(III), otherwise known as cycl[3,2,2]azine (Fig. 1).

II was readily obtained in yield of 48% as the free base by dry-distilling ethyl 3-oxoindolizidine-5-propionate(I) with the same, or half the amount of soda lime as described in the previous paper.[1a]

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