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Original Paper

Interaction of Ribonuclease F1 With Various Guanine Nucleotides as Studied by Difference Absorption Spectra

(ribonuclease F1 / guanine nucleotide / difference absorption spectrum)

Mari IIZUKA* and Hiroshi YOSHIDA*,**

Interaction of various guanine and related nucleotides with ribonuclease F1 was studied by means of difference absorption spectra. Spectrophotometric titrations were carried out on a total of 16 nucleotides which were classified into three groups; (A) guanine and hypoxanthine nucleotides (6), (B) 8-substituted guanine nucleotides (6) and (C) dinucleotides (4) (the numbers of nucleotides are shown in parentheses). Computer-aided data processing enabled determination of the difference molar absorbance coefficient $\Delta\varepsilon_{max}$ and the dissociation constant of the ribonuclease F1-nucleotide complex K_d for each nucleotide. Among group A nucleotides, guanosine 2'-phosphate showed the lowest K_d (the highest affinity) and inosine 2'-phosphate the highest K_d (the lowest affinity). The difference between their binding free energy changes was estimated to reach 2.3 kcal/mol. Among group B nucleotides, 8-aminoguanosine 2'-phosphate exhibited the lowest K_d, which was even lower than that of guanosine 2'-phosphate. The difference in their binding free energy changes was 1.0 kcal/mol. Group C nucleotides had higher K_d than their common component, guanosine 3'-phosphate, suggesting destabilizing effect on complex formation by an adjacent nucleotide. All these results were similar but not completely the same for those reported on ribonuclease T1.

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 it belongs to the well-known RNase T1 family. This family of RNases are known as guanine-specific: they split internucleotide bonds in RNA between 3'-guanylic acid and 5'-OH group of an adjacent nucleotide with intermediary formation of guanosine 2',3'-cyclic phosphate, which is ultimately hydrolyzed to guanosine 3'-phosphate. A crucial characteristic of these enzymes is, therefore, to specifically recognize and bind a guanine nucleotide. Back in 1965, Sato and Egami reported that the absorption spectrum of RNase T1-guanosine 2'-phosphate complex differ from the sum of the spectrum of each component[1]. The difference between them, which is called difference absorption spectrum, has since become a method of choice to study interactions between the enzyme and various guanine nucleotides, leading to accumulation of abundant data[2-5].

Here, we have investigated interactions of RNase F1 with guanine and related nucleotides and have compared the results with those for RNase T1. It has been shown that the two RNases have similar but not completely the same guanine nucleotide binding properties.

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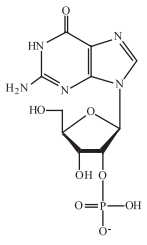
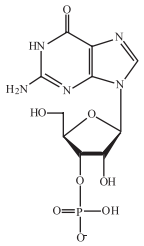
MATERIALS AND METHODS

Materials — RNase F1 was prepared as described previously[6]. The enzyme concentration was determined on the basis of absorbance at 280 nm using molecular absorbance of 13,900 M^{-1} cm^{-1}[7]. The nucleotides used in this study are shown in Fig. 1. They are classified into three groups; (A) guanine and hypoxanthine nucleotides, (B) 8-substituted guanine nucleotides and (C) dinucleotides. Group A nucleotides were purchased from Sigma. Group B nucleotides were synthesized as described previously[5]. Group C nucleotides were isolated from a complete digest of yeast RNA by RNase T1 using anion-exchange chromatograpy except for GfpC which was a gift from Dr. M. Danno of Protein Research Institute, Osaka, Japan.

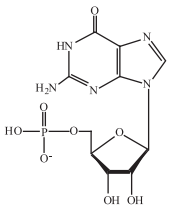
(A) Guanine and Hypoxanthine Nucleotides



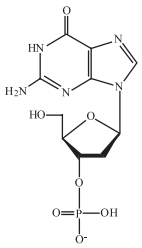
Guanosine 2'-phosphate
G-2'-*P*



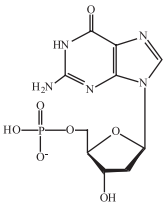
Guanosine 3'-phosphate
G-3'-*P*



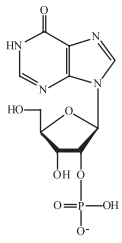
Guanosine 5'-phosphate
G-5'-*P*



Deoxyguanosine 3'-phosphate
dG-3'-*P*

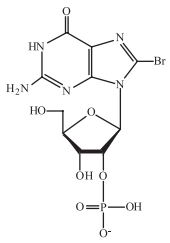


Deoxyguanosine 5'-phosphate
dG-5'-*P*

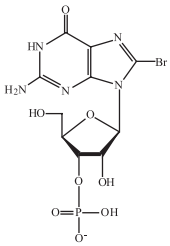


Inosine 2'-phosphate
I-2'-*P*

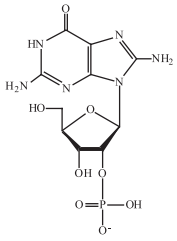
(B) 8-Substituted Guanine Nucleotides



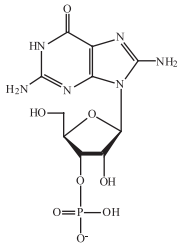
8-Bromoguanosine 2'-phosphate
8-BrG-2'-*P*



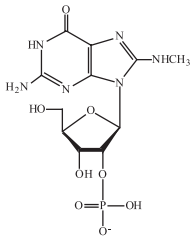
8-Bromoguanosine 3'-phosphate
8-BrG-3'-*P*



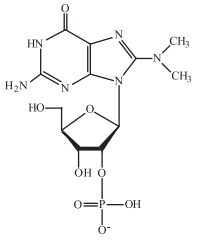
8-Aminoguanosine 2'-phosphate
8-NH_2G-2'-*P*



8-Aminoguanosine 3'-phosphate
8-NH_2G-3'-*P*

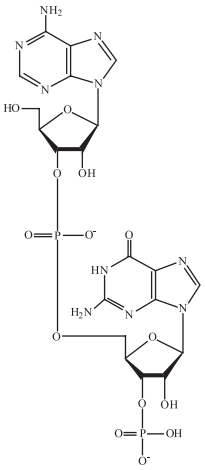


8-Methylaminoguanosine 2'-phosphate
8-MeNHG-2'-*P*

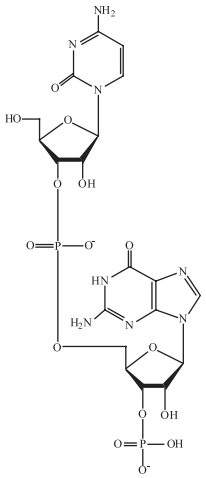


8-Dimethylaminominoguanosine 2'-phosphate
8-Me_2NG-2'-*P*

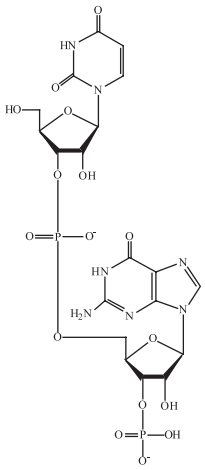
(C) Dinucleotides



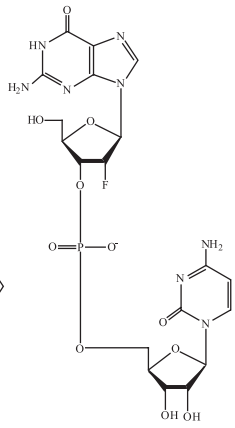
Adenylyl-3',5'-guanosine
3'-phosphate
ApGp



Cytidylyl-3',5'-guanosine
3'-phosphate
CpGp



Uridylyl-3',5'-guanosine
3'-phosphate
UpGp



2'-Deoxy-2'-fluoroguanylyl-
3',5'-cytidine
GfpC

Fig. 1. The structure, nomenclature and abbreviation of the nucleotides used in this study. Strictly, GfpC is a dinucleoside phosphate but is included in dinucleotides here.

Determination of Dissociation Constant of RNase F1-Nucleotide Complex — This was carried out on the basis of difference absorption spectra caused by complex formation as described previously in detail[8]. All measurements were made in 10 mM sodium acetate buffer (pH 5.5) containing 0.1 M NaCl at 25 ℃. In short, an enzyme E and its ligand L are assumed to form a 1 : 1 complex, EL.

$$E + L \rightleftharpoons EL$$

Note that "ligand" is a general term for a substance interacting with a protein and is equivalent to "nucleotide" here. Then, the dissociation constant K_d is defined as follows.

$$K_d = \frac{[E]\ [L]}{[EL]}$$

Starting from this equation, the following relationship can be derived:

$$\Delta A_{max} = (\Delta\varepsilon_{max}/2)\{[E]_T + [L]_T + K_d - \sqrt{([E]_T + [L]_T + K_d)^2 - 4[E]_T[L]_T}\} \quad (1)$$

ΔA_{max} is the difference absorbance at the maximum wavelength. Suffix T shows the total concentration of each component and $\Delta\varepsilon_{max}$ is the difference molar absorbance coefficient. Measurements of absorbances at the maximum wavelength (λ_{max}) and the crossover point (λ_0) of the difference spectrum at incremental $[L]_T$ enabled calculation of ΔA_{max} after each addition. A data set thus obtained, ΔA_{max} *vs.* $[L]_T$, was processed using Kaleidagraph, giving best fit values of $\Delta\varepsilon_{max}$ and K_d.

RESULTS AND DISCUSSION

Representative data for 8-NH_2G-2'-*P* are shown in Figs. 2 and 3. Although we do not present other data, essentially similar results were obtained for all the nucleotides with different degrees of deviation. The parameters thus determined are shown in Table 1. Among group A nucleotides, I-2'-*P* is outstanding in its high K_d, hence low affinity to the enzyme. The free energy change of the binding can be calculated from the equation, $\Delta G^o = RT\ln K_d$. Then, the free energy change difference ($\Delta\Delta G^o$) between the binding of G-2'-*P* and I-2'-*P* reaches 2.3 kcal/mol. X-Ray crystallographic studies have shown that 2-NH_2 group of the guanine ring forms two hydrogen bonds with Glu 46 and Asn 98 of RNase F1[9]. Disruption of these hydrogen bonds caused by elimination of the NH_2 group, which leads to a change

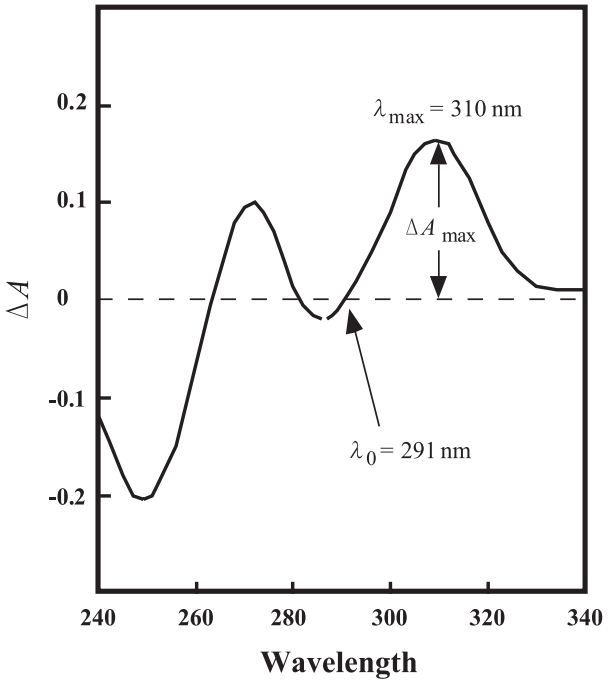


Fig. 2. The difference absorption spectrum for the RNase F1-8-NH_2G-2'-*P* complex. The initial concentrations of RNase F1 and the nucleotide were 50.7 μM and 52.3 μM, respectively. The experimental procedure to obtain the spectrum was as described previously[8].

y = m1/2*(50.9+M0+m2-SQRT((50.9+M0+m2)*(50.9+M0+m2)-203.6*M0))

	Value	Error
m1	0.003342	0.000019
m2	0.463	0.108

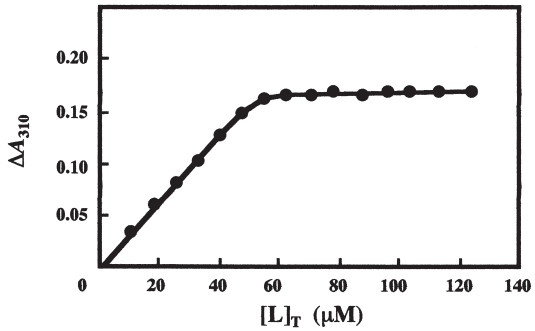


Fig. 3. The titration of RNase F1 with 8-NH_2G-2'-*P*. To 1.5-ml of 50.9 μM RNase F1 solution were added successively 1-μl aliquots of 8.76 mM nucleotide solution up to a total of 15 μl. After each addition, absorbances at 310 nm and 290 nm were measured, yielding a data set shown here: the difference absorbance ΔA_{310} as a function of total nucleotide concentration $[L]_T$. The data was processed using Kaleidagraph to give the two parameters m1 ($\Delta\varepsilon_{max}$) and m2 (K_d). The best-fit curve is shown by a continuous line. The calculation result by the software is shown at the upper part in a summarized form, the equation at the first line being the expression of equation (1) in conformity to the software, where y and M0 are ΔA_{max} and $[L]_T$, respectively. Note that m1 is expressed in μM^{-1} cm^{-1} here.

Table 1. Parameters for interaction of RNase F1 with various nucleotides.

Nucleotide	λ_{max} (nm)	λ_0 (nm)	$\Delta\varepsilon_{max}$ (M^{-1} cm^{-1})	K_d (μM)
(A) Guanine and Hypoxanthine Nucleotides				
G-2'-*P*	290	271	3,890 ± 75	2.5 ± 0.7
G-3'-*P*	289	275	4,150 ± 240	7.5 ± 2.1
G-5'-*P*	289	273	3,930 ± 90	71.7 ± 3.8
dG-3'-*P*	289	273	3,960 ± 60	26.2 ± 1.2
dG-5'-*P*	289	273	4,010 ± 70	39.0 ± 2.0
I-2'-*P*	285	259	1,700 ± 70	123 ± 12
(B) 8-Substituted Guanine Nucleotides				
8-BrG-2'-*P*	294	268	4,240 ± 60	2.3 ± 0.4
8-BrG-3'-*P*	295	271	4,240 ± 160	9.4 ± 2.0
8-NH_2G-2'-*P*	310	291	3,340 ± 20	0.46 ± 0.11
8-NH_2G-3'-*P*	309	289	2,780 ± 160	5.7 ± 2.2
8-MeNHG-2'-*P*	317	296	3,730 ± 55	9.1 ± 0.7
8-Me_2NG-2'-*P*	302	277	3,390 ± 100	49.8 ± 3.8
(C) Dinucleotides				
ApGp	291	262	7,280 ± 1,100	23.2 ± 4.3
CpGp	292	276	5,940 ± 240	43.9 ± 4.8
UpGp	290	276	5,590 ± 95	17.9 ± 1.3
GfpC	290	273	6,480 ± 100	58.1 ± 2.0

from G-2'-*P* to I-2'-*P*, will explain quantitatively the destabilization of the enzyme-nucleotide complex described here. For all the guanine nucleotides, the qualitative nature of difference spectra was similar and $\Delta\varepsilon_{max}$ values were more or less the same. The order of affinities for the enzyme was: G-2'-*P* > G-3'-*P* > dG-3'-*P* > dG-5'-*P* > G-5'-*P*. The role played by 2'-OH group in the binding is less clear. Comparison of G-3'-*P* *vs.* dG-3'-*P* showed a binding energy difference of 0.7 kcal/mol in favor of G-3'-*P*, but comparison of G-5'-*P* *vs.* dG-5'-*P* gave a contrary result, a slight difference (0.3 kcal/mol) in favor of dG-3'-*P*. The widely accepted enzyme mechanism of RNase T1 assumes interaction between 2'-OH group and Glu 58 resulting in enhanced nucleophilicity of the OH group[10]. This active site Glu residue is conserved in RNase F1. Our results suggest that this interaction may not contribute much to the binding.

Group B nucleotides were shown to bind RNase F1 with similar but definitely different affinities as the parent guanine nucleotides. The crystallographic study has revealed that the position 8 of the guanine ring is oriented outward from the guanine nucleotide binding site[9]. Therefore, it is quite understandable that a substituent as bulky as dimethylamino group can be introduced to this position without too much influence on the binding. However, subtle differences in the binding affinities were observed for these nucleotides. The order of affinities was: 8-NH_2G-2'-*P* > 8-BrG-2'-*P* (≈ G-2'-*P*) > 8-NH_2G-3'-*P* > (G-3'-*P*) > 8-MeNHG-2'-*P* ≈ 8-BrG-3'-*P* > 8-Me_2NG-2'-*P*. Especially noteworthy is the fact that 8-NH_2G-2'-*P* binds to the enzyme even more tightly than G-2'-*P* ($\Delta\Delta G^o$ = -1.0 kcal/mol). Although this free energy difference is comparable to formation of a hydrogen bond, we consider that direct interaction between the 8-NH_2 group and the enzyme is unlikely because of the above mentioned structure of the active site. Rather, its effect on electronic state and/or conformation of the guanine nucleotide may affect the binding affinity. Further studies will be necessary to understand the mechanism for the enhanced affinity.

Our results on group C nucleotides, cannot be interpreted straightforward. The qualitative nature of the difference spectra remained the same as with G-3'-*P* alone, indicating that the principal cause of the difference spectra was the binding of the guanine nucleotide moiety. Yet, values of $\Delta\varepsilon_{max}$ were considerably larger than that for G-3'-*P*, suggesting participation of the adjacent nucleotide or nucleoside in the binding. However, values of

K_d were larger than that for G-3'-*P* indicating destabilization in a range 0.5 - 1.2 kcal/mol. Therefore, the interaction of the adjacent nucleotide at the 5' side or nucleoside at the 3' side is thought to destabilize the enzyme-nucleotide complex. It has been known from kinetic studies that cytidine at the 3' side of G-3'-*P* accelerates the enzyme reaction[11]. The destabilization of enzyme- substrate complex may be part of the driving force of the reaction. We have shown that non-standard type of substrates such as ApA can be hydrolyzed very slowly by RNase F1[11]. Likewise, the dinucleotides studied here were hydrolyzed. Chromatographic examination as described earlier[11] revealed that 9.6 %, 27.3 % and 32.4 % of ApGp, CpGp and UpGp, respectively, were degraded after the spectrophotometric titration had been completed. Therefore, the parameters reported here should be evaluated with some reservation for these dinucleotides. Nevertheless, we conside that our conclusion should still be valuable. GfpC, on the other hand, was shown to be completely resistant to the enzyme.

As stated earlier, abundant data have been accumulated on interaction between RNase T1 and various nucleotides. For the guanine nucleotides, the following order of affinities was reported: G-2'-*P* > G-3'-*P* > dG-3'-*P* > G-5'-*P* ≈ dG-5'-*P*[2]. This corresponds almost precisely to the order for RNase F1. As for 8-substituted guanine nucleotides, the order was: 8-NH_2G-2'-*P* > 8-BrG-2'-*P* (≈G-2'-*P*) > 8-Me_2NG-2'-*P* > 8-MeNHG-2'-*P*[4,5]. Again, overall tendency was similar for both the enzymes except for inversion of 8-Me_2NG-2'-*P* and 8-MeNHG-2'-*P*. Quantitatively, however, $\Delta\Delta G^o$ between the binding of 8-NH_2G-2'-*P* and G-2'-*P* was 1.0 and 0.3 kcal/mol for RNase F1 and RNase T1 (calculated from the previously reported data), respectively. Therefore, the binding affinity of 8-NH_2G-2'-*P* is much more strengthened for RNase F1 than for RNase T1. Thus, we can conclude that the guanine nucleotide binding sites of these enzymes have very similar but not completely the same properties. Although no study on the binding of dinucleotides to RNase T1 has so far been reported, Walz and Terenna measured the binding of ApG, CpG and UpG, which lacked 3'-phosph ate of our corresponding dinucleotides[3]. They found that the addition of a nucleoside to the 5' side of G-5'-*P* did weaken the binding affinity, which was in accord qualitatively with our present results. The role played by nucleotides adjacent to the guanine nucleotide bound to the active site presents intriguing problem with respect to the enzyme mechanism and needs to be studied further.

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