  Bull. Chem. Soc. Jpn., 62, 3488—3491 (1989) [Vol. 62, No. 11

Binding of Modified Guanine Nucleotides to Ribonuclease T_1 as Studied by Absorption Spectroscopy. 8-Aminoguanosine 2′-Phosphate, 8-Methylaminoguanosine 2′-Phosphate, and 8-Dimethylaminoguanosine 2′-Phosphate

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(Received May 6, 1989)

The title nucleotides have been synthesized and their binding to ribonuclease T_1 studied by means of absorption spectroscopy. Measurements were made at pH 5.5 and 4.1, where a major portion of the base moiety of the nucleotides was in a neutral form and a cationic form protonated at N7, respectively. At both pH's, difference absorption spectra were induced upon binding of the nucleotides to the enzyme. A comparison of the difference spectra with that for guanosine 2′-phosphate has led to the conclusion that perturbation in the bound nucleotide is the principal cause for the difference spectra. The difference spectra at pH 5.5 and 4.1 were similar, suggesting that the protonation at N7 of the nucleotides can be ruled out as the cause for the difference spectra. The dissociation constants at pH 5.5 and 4.1 of the enzyme–8-aminoguanosine 2′-phosphate complex were of the same magnitude, indicating that both the neutral and the protonated forms bind tightly to the enzyme. However, the cationic form of 8-methylamino- or 8-dimethylaminoguanosine 2′-phosphate has been shown to interact with the enzyme much more loosely than the neutral form.

Ribonuclease (RNase) T_1 is an endoribonuclease that specifically cleaves single-stranded RNA at the 3′ end of guanine nucleotides via 2′:3′-cyclic phosphate intermediates. Specific recognition of the guanine nucleotide is, therefore, a key step in its action mechanism. It can bind monomer guanine nucleotides, among which guanosine 2′-phosphate (**1**) shows the highest affinity.[1] The binding is detected in a number of ways. Absorption spectroscopy is a simple and sensitive method for detection. A difference absorption spectrum is induced upon the binding of a guanine nucleotide to RNase T_1: the spectrum of the RNase T_1–nucleotide complex is not equal to the sum of the component spectra measured separately.[2]

Interpretation of this phenomenon has been controversial. Oshima and Imahori,[3] followed by others,[4,5] have pointed out a gross similarity between the difference spectrum and the acid-induced spectral change of the guanine nucleotide. This led them to propose the hypothesis that the guanine ligand is protonated on N7 upon binding to the enzyme and that this protonation is the cause for the difference spectrum. However, Yoshida and Kanae argued against this hypothesis on the grounds of a spectroscopic study using 8-bromoguanosine 2′-phosphate (**2**).[6] With nucleotide **2**, the acid-induced spectral change was dissimilar to the difference spectrum. However, a possibility can be envisaged that the protonation site of **2** might be different from N7 due to the introduction of a strongly electron-withdrawing group at C8.

In the present study, we have synthesized 8-aminoguanosine 2′-phosphate (**3**), 8-methylaminoguanosine 2′-phosphate (**4**), and 8-dimethylaminoguanosine 2′-phosphate (**5**) and studied their interactions with RNase T_1. The introduction of an electron-donating group at C8 of the guanine base increases the pK_a considerably, thereby allowing a study of the binding properties of a guanine nucleotide protonated at N7. This provides a direct verification of the protonation hypothesis.

Experimental

RNase T_1. This enzyme was purified as described by Kanaya and Uchida[7] and was determined spectrophotometrically using a molar absorbance of 21200 $M^{-1}\,cm^{-1}$ (1 M=1 mol dm^{-3}).[8]

Chemical Syntheses. Nucleotides **3**, **4**, and **5** were synthesized from **2** by the application of published methods for the syntheses of similar, but not identical, compounds shown in parentheses: **3** according to Holmes and Robins (8-aminoguanosine),[9] **4** according to Long et al. (8-methylaminoguanosine)[10] and **5** according to Miller et al. (8-dimethylaminoguanosine 3′:5′-cyclic phosphate).[11] Nucleotide **2** was synthesized essentially according to Yuki and Yoshida.[12] In each synthesis, some modifications were made to the original method in order to improve the performance. Therefore, the whole synthetic procedures will be described. However, the products were identified only on the grounds of the absorption spectra, since the synthetic reactions were the same as the original ones. Generally, the absorption spectra of our products were in good agreement with the reported spectra of the corresponding compounds. The purity of the products was checked by HPLC, which was carried out under the following conditions: chromatograph, Tosoh HLC-803 A; column, TSKgel IEX-540 K 4 mm (i.d.) ×300 mm; eluent, 0.25 or 0.5 M KH_2PO_4; flow rate, 1.0 $cm^3\,min^{-1}$; detection, absorbance at 260 nm. Special attention was paid so that each product was free from its 3′-isomer, a possible by-product of phosphoryl transfer. The nucleotides were determined through quantitation of phosphorus, which was carried out by the method of Fiske and Subbarow.[13]

8-Bromoguanosine 2′-Phosphate (2). To guanosine 2′-

(3′)-phosphate (free acid, 813 mg, 2.23 mmol) dissolved in 4 cm^3 of *N,N*-dimethylformamide, was added 0.15 cm^3 of Br_2 in 1 cm^3 of the solvent. The reaction mixture was left standing at room temperature for 15 min, then added dropwise into 6 cm^3 of ice-cold water under stirring. The solution was adjusted to pH ca. 7 with concd aq. ammonia, then poured into 120 cm^3 of ice-cold acetone. The resulting precipitate was collected by centrifugation, washed twice with acetone and once with ether, and dried in vacuo (yield 933 mg). The crude product was purified on a column of DE 52 (HCO_3^- form, 1.8×70 cm) in three portions. One third of the product was dissolved in 10 cm^3 of water and loaded on the column equilibrated with 0.18 M NH_4HCO_3. The column was developed by a linear gradient of 0.18—0.35 M NH_4HCO_3 over 1 dm^3. Nucleotide **2** and its 3′-isomer were separated and recovered by lyophilization: total yield, **2** (1.17 mmol, 57%), the 3′-isomer (0.69 mmol, 34%). Retention time (t_R) in HPLC with 0.5 M KH_2PO_4 as the solvent, **2** (6.0 min), the 3′-isomer (7.25 min).

8-Aminoguanosine 2′-Phosphate (3). Nucleotide **2** (21.4 μmol) was dissolved in 0.5 cm^3 of water containing 6 mm^3 of hydrazine monohydrate. The solution was heated at 95 °C for 26 h in a sealed glass tube, cooled to room temperature, and diluted with 10 cm^3 of water. After being adjusted to pH ca. 7, the solution was loaded on a column of DE 52 (formate form, 1.6×35 cm). After unadsorbed material was washed off with water, the column was developed by a linear gradient of 0—0.1 M formic acid over 0.6 dm^3. The product **3** (7.1 μmol, 33%) was separated from a small amount of the 3′-isomer (1.0 μmol, 5%) and recovered by lyophilization: t_R (0.25 M KH_2PO_4), 4.2 min (the 3′-isomer, 5.0 min); UV (10 mM HCl), 250 (ε 16700), 288 (10600); (H_2O), 255 (16300), 291 (9600); (10 mM NaOH), 258 (14600), ca. 280 (sh, 11800).

8-Methylaminoguanosine 2′-Phosphate (4). Nucleotide **2** (23.2 μmol) was suspended in 0.3 cm^3 of 40 wt% methylamine in methanol; the suspension was heated at 95 °C for 39 h in a sealed glass tube. The work up and the chromatography as described for **3** afforded **4** (15.2 μmol, 66%): t_R (0.25 M KH_2PO_4), 4.1 min; UV (10 mM HCl), 252 (ε 17500), 289 (10200); (H_2O), 259 (16500), 295 (9200); (10 mM NaOH), 261 (15000), ca. 280 (sh, 12550). Only a small amount (<1 μmol) of the 3′-isomer was detected in the chromatography: t_R, 4.9 min.

8-Dimethylaminoguanosine 2′-Phosphate (5). Nucleotide **2** (156 μmol) was suspended in a mixture of anhydrous dimethylamine (2.2 cm^3) and methanol (10 cm^3). The suspension was heated at 120 °C for 5 h in a steel bomb, then evaporated. The residue was dissolved in 10 cm^3 of water and loaded on a column of DE 52 (HCO_3^- form, 1.8×50 cm) equilibrated with 0.05 M NH_4HCO_3. The product **5** (84.6 μmol, 54%) was eluted from the column by a linear gradient of 0.05—0.35 M NH_4HCO_3 over 1 dm^3 and recovered by lyophylization: t_R (0.5 M KH_2PO_4), 4.8 min; UV (10 mM HCl), 264 (ε 17500), ca. 290 (sh, 11100); (H_2O), 262 (16500), ca. 290 (sh, 8700); (10 mM NaOH), 269 (15100).

Measurement of Difference Spectra. This was carried out as described previously[6] in a solvent, 10 mM sodium acetate buffer (pH 5.5 or 4.1) containing 0.1 M NaCl, with a Hitachi 220 A spectrophotometer equipped with a tandem cell holder thermostatted at 25 °C. A typical experiment was run with 2.0 cm^3 of 39.0 μM RNase T_1 and 2.0 cm^3 of 38.2 μM **3** giving rise to a difference absorbance of 0.128 at a maximum wavelength (λ_{max}) of 308 nm in an optical path of 2.0 cm.

Determination of the Dissociation Constant K_d and the Maximum Difference Molar Absorbance $\Delta\varepsilon(\lambda_{max})$ for a RNase T_1-Nucleotide Complex. This was carried out as described previously.[6] A typical run was as follows. To 2.5 cm^3 of 12.2 μM RNase T_1, was added 8.11 mM **3** in 0.2 mm^3 portions up to a total of 13 mm^3. After each addition, the absorbances at 263 nm (a crossover point of the difference spectrum) and 308 nm (λ_{max}) were measured. The data, thus obtained, were processed to give the K_d and $\Delta\varepsilon(\lambda_{max})$.

Results and Discussion

Spectrophotometric Titration of the 8-Substituted Guanine Nucleotides. Guanosine undergoes spectral changes with pH, showing acid dissociation constants (pK_a) of 2.3 and 9.4. Miles et al. have established that the lower dissociation constant (pK_{a1}) corresponds to protonation at N7 and that the higher one (pK_{a2}) corresponds to deprotonation from N1-H. The pK_a's of **3**, **4**, and **5** were examined by spectrophotometric titration. As shown in Table 1, the introduction of an electron-donating group at C8 increased the pK_{a1} considerably and also the pK_{a2} to a lesser extent. These changes are as expected from the protonation and deprotonation sites described above. Therefore, the base moiety of these nucleotides is supposed to be in a cationic form protonated at N7 in a sufficiently acidic solution.

Interaction of the 8-Substituted Guanine Nucleotides with RNase T_1. The difference absorption spectrum induced upon binding of the parent nucleotide **1** to RNase T_1 at pH 5.5 has been shown to have one major trough at 245 nm and one major peak at 290 nm.[2-5] In addition, it has a minor peak at ca. 260 nm and a minor trough at ca. 270 nm in-between. Sato and Egami examined the difference absorbance at 290 nm as a function of pH and found that it remained nearly constant between pH 4 and 5.5, falling off on both acidic and alkaline sides.[2] This suggests that RNase T_1 binds the ligand equally well in a pH range 4—5.5. We studied the binding of **1** to RNase T_1 at pH 4.1 in detail and found that the entire difference spectrum was unchanged from that at pH 5.5. Moreover, the K_d was determined to be 4.0 μM, in good agreement with that (4.6 μM) at pH 5.5. Since the ionization state of the guanine base is the same at pH 5.5 and 4.1, these results indicate that the nucleotide binding property of the enzyme remains the same at these pH's. Having confirmed this fact, we studied the interaction of **3**, **4**, and **5** with RNase T_1 at these pH's.

Table 1. Acid Dissociation Constants of the 8-Substituted Guanine Nucleotides

Nucleotide	pK_{a1}	pK_{a2}
3	4.9	10.4
4	5.0	10.6
5	3.8	10.2

At pH 4.1, these nucleotides are expected to be mainly in the form protonated at N7, due to the elevated pK_{a1}. Therefore, the effect of protonation on the difference spectrum or the binding can be assessed directly.

The base moiety of **3** was calculated from its pK_{a1} to be 80% in the neutral form at pH 5.5 and 86% in the cationic form at pH 4.1. A change in absorption spectrum takes place with the change of ionization state (see UV characteristics shown in the Experimental section). A blue-shift is the main feature accompanying protonation. In spite of this spectral change, the difference spectra induced upon binding of **3** to RNase T_1 were quite similar at pH 5.5 and 4.1 (Fig. 1A). At pH 5.5, two distinct peaks were observed at 270 and 308 nm and two troughs at 248 and 286 nm. At pH 4.1, a slight blue-shift was seen for the shorter wavelength trough and peak. Compared to the difference spectrum for the parent nucleotide **1**, a large red-shift by 18 nm of the longer wavelength peak is noteworthy. This red-shift corresponds almost exactly to the red-shift between 275 and 290 nm of the longer wavelength absorption bands of **1** and **3**, respectively. This suggests that a perturbation of the nucleotide and not a protein chromophore(s) is the principal cause for the difference spectrum. Clearly, the perturbation cannot be protonation of the ligand, since even the protonated nucleotide causes a similar difference spectrum. It should also be noted that the spectral change accompanying the protonation and the difference spectrum are dissimilar. A blue-shift is the characteristic for the former, whereas the latter can be regarded as being due to a red-shift of the absorption bands of the nucleotide.

The K_d for **3** was slightly lower than that for **1** at both pH 5.5 and 4.1 (Table 2). Especially noteworthy is the low K_d, thus the tight binding at pH 4.1, which means that **3** protonated at N7 can bind tightly to the enzyme. This precludes a binding mechanism in which N7 plays an important role as a hydrogen acceptor. The X-ray analysis of the RNase T_1–**1** complex has indicated the probable hydrogen bonds to N7.[14,15] A ^1H NMR study has also proposed hydrogen bond interaction between N7 of the guanine base and a histidine residue in the active site of the enzyme.[16] However, in the tertiary structure determined by the X-ray analysis, the distance between N7 and any of the suggested hydrogen donors is large (>3 Å). Moreover, an ^{15}N NMR study showed no sign of the participation of N7 in the binding.[17] In accordance with these latter observations, our result presents evidence for the dispensability of the hydrogen bond to N7 in the formation of the enzyme–nucleotide complex. In fact,

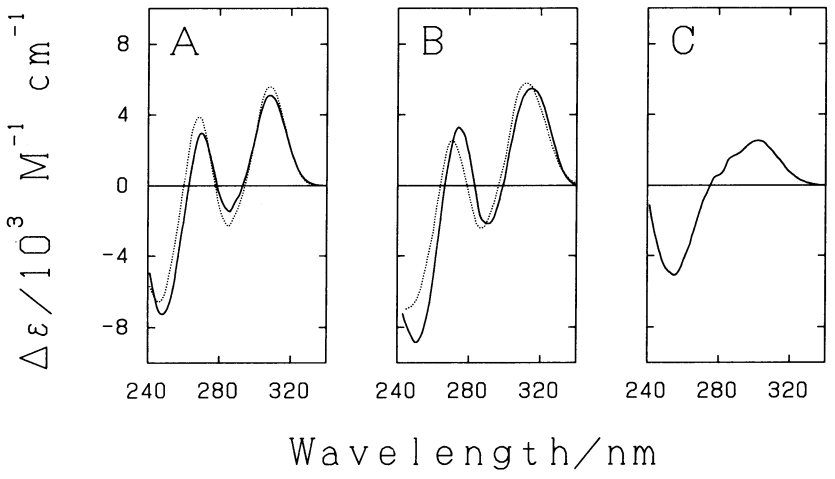

Fig. 1. Difference spectra induced upon binding of the 8-substituted nucleotides to RNase T_1. A: **3**, B: **4**, C: **5**. —: at pH 5.5, ------: at pH 4.1.

Table 2. Dissociation Constants and Related Parameters for RNase T_1–8-Substituted Guanine Nucleotide Complexes

Nucleotide	pH 5.5			pH 4.1		
	λ_{max}	$\Delta\varepsilon(\lambda_{max})$	K_d	λ_{max}	$\Delta\varepsilon(\lambda_{max})$	K_d
	nm	$10^3\ M^{-1}\ cm^{-1}$	μM	nm	$10^3\ M^{-1}\ cm^{-1}$	μM
3	308	5.1	2.7	308	5.6	1.3
4	315	5.5	26	312	5.8	80
5	302	2.5	15	ca. 302	nd[a]	nd[a]

a) Not determined.

Seela et al. have shown that RNase T_1 is able to hydrolyze poly(7-deazaguanylic acid) lacking N7.[18]

The base moiety of **4** is 72% in the neutral form at pH 5.5 and 91% in the cationic form at pH 4.1. As for the difference spectra shown in Fig. 1B, qualitatively the same argument holds as that described for **3**. The major differences are a larger red-shift of the longer wavelength peak to 315 nm and a shift observed between the spectra at pH 5.5 and 4.1 over the entire wavelength region. In spite of the qualitative similarity, an important quantitative difference emerged: the K_d was much higher at pH 4.1 than at pH 5.5 (Table 2). This shows that nucleotide **4** protonated at N7 binds to the enzyme much more loosely than its neutral form. At pH 5.5, the observed K_d is substantially higher for **4** than for **3**. It should be kept in mind, however, that 28% of **4** is still in the cationic form with a large K_d at pH 5.5. The intrinsic K_d for the neutral form of **4** is, therefore, estimated to be much lower than 26 μM actually observed and to be at a level similar to that for **3**. Accordingly, we conclude that the neutral form of **4** binds to the enzyme tightly, whereas the protonated form binds loosely. This is in contrast to the situation for **3**, whereby both forms interact with the enzyme equally well. We have no explanation for this discrepancy at present. However, it is possible that concomitant occurrence of protonation at N7 and a bulky substituent at C8 may seriously hinder the nucleotide from binding to the enzyme.

Nucleotide **5** is almost entirely (98%) in the neutral form at pH 5.5, but only 33% in the cationic form even at pH 4.1. General properties, e.g. absorption spectrum or pK_a, of **5** cannot be simply extrapolated from those of **3** and **4**. Likewise, the difference spectrum induced upon binding of **5** to RNase T_1 at pH 5.5 (Fig. 1C) differed considerably from those for **3** and **4**. The spectrum has only one trough at 255 nm and one peak at 302 nm, both of relatively small magnitude. At pH 5.5, the K_d is several times higher than that for the parent nucleotide **1** (Table 2), but is equivalent to that for the 3′-isomer of **1** (21.3 μM, our own measurement). Accordingly, nucleotide **5** can be regarded to bind to the enzyme still tightly in spite of the bulky dimethylamino group introduced at C8. This shows the considerable freedom of space in the vicinity of C8 of the bound guanine base. Nucleotide **5** can only adopt a syn conformation about the glycosyl bond rotation, because of the bulky substituent at C8.[19] Relatively tight binding of **5** is in accord with the X-ray structure in which the nucleotide bound at the active site of RNase T_1 assumes a syn conformation.[14,15] At pH 4.1, we were unable to determine the K_d for **5** because of the small magnitude of the difference spectrum. However, the attenuation of the difference spectrum suggests that the protonated form of **5** interacts with the enzyme very weakly, like the protonated form of **4**.

In conclusion, the present study shows that a perturbation of the nucleotide base is the main cause for the difference spectrum induced upon its binding to RNase T_1. The contribution of a protein chromophore(s) may be small, if any. Protonation at N7, which used to be assumed to cause the difference spectrum, should be ruled out. It is also clear that the protonation per se does not interfere a nucleotide from binding to the enzyme. This argues against the participation of N7 in the specific binding as a hydrogen acceptor.

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