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BINDING OF 8-BROMOGUANYLIC ACID TO RIBONUCLEASE T_1 AS STUDIED BY ABSORPTION AND CIRCULAR DICHROISM SPECTROSCOPY

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8-Bromoguanosine 2'- and 3'-phosphates have been shown to bind to RNase T_1 with the same affinity as the corresponding guanosine phosphates, inducing difference absorption and circular dichroism spectra similar to those induced by the guanosine phosphates. Since the brominated ligands have reduced electron density on N-7 of the guanine ring and *syn*-fixed conformation due to a bulky, electron-withdrawing Br substituent on C-8, the difference spectra are not attributable to the protonation on N-7 and to the restriction of the ligand to *syn*-conformation as proposed previously.

Difference absorption (1-4) and circular dichroism (5,6) spectra are induced on binding of a guanine ligand to RNase T_1. Controversies exist, however, in interpretation of these difference spectra. The difference absorption spectrum was explained by protonation on N-7 of the guanine ring because of its similarity to the acid-induced difference spectrum for the ligand (2-4). Importance of N-7 as a hydrogen acceptor was supported by ^1H NMR study, although the complete protonation was not proven (7). On the other hand, Kyogoku *et al.*(8) and Heinemann and Saenger (9) argued against the protonation on N-7 based on their respective ^{15}N NMR and X-ray studies. As for the difference CD spectrum, Oshima and Imahori ascribed it to conformation change of the ligand from *anti* to *syn* (5), whereas Sander and Ts'o explained by exciton coupling interaction between the ligand and the aromatic residue(s) of the enzyme (6).

One of the approaches to these problems is to study the binding properties of modified ligands. 8-Bromoguanylic acid drew our attention, because it has reduced electron density on N-7 and *syn*-fixed conformation due to a bulky, electron-withdrawing Br substituent on C-8. In spite of these

The abbreviations used are: RNase, ribonuclease; BrG2'p and BrG3'p, 8-bromoguanosine 2'- and 3'-phosphates, respectively.

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changes, 8-bromoguanosine 2':3'-cyclic phosphate can be a substrate for RNase T_1 (10). Oshima and Imahori have described a preliminary account with a mixture of BrG2'p and BrG3'p, but have not given detailed discussion (11). This paper describes spectroscopic studies on the binding of each of these ligands to RNase T_1 and discusses their implications for the above controversies.

Materials and Methods

RNase T_1 was prepared as described previously (12) and purified further by the affinity and DEAE-cellulose chromatographies developed for purification of RNase F_1 (13). RNase T_1 was determined spectrophotometrically using a molar absorbance of 2.12×10^4 M^{-1} cm^{-1} at 278 nm (14). BrG2'(3')p was synthesized as described (10) and the 2'- and 3'-isomers were separated on a column of Dowex 1x4 (Cl^- form).

All measurements were carried out with the solvent 0.01 M sodium acetate buffer (pH 5.6) containing 0.1 M NaCl. Difference absorption spectra were determined using a Hitachi 220A spectrophotometer equipped with a tandem cell holder thermostatted at 25°C. Two 1-cm cells filled with 2.0 ml of RNase T_1 solution and of a ligand solution were placed in tandem at the sample side and the sum spectrum against a solvent reference was stored in the memory (background correction). The contents of the cells were mixed and put back into the cells at about equal portions, then the difference spectrum over the memorized sum spectrum was recorded directly. For the determination of the dissociation constant K_d of the enzyme-ligand complex and the difference molar absorbance $\Delta\varepsilon$, small volumes of a concentrated ligand solution were added stepwise to a given enzyme solution (*ca.* 20 μM) and the absorbances at two wavelengths λ_{max} and λ_o were determined after each addition. The wavelengths were chosen so that at λ_{max} and λ_o the difference absorbance reached a maximum and zero, respectively. The total ligand concentration $[L]_t = \{A(\lambda_o) - A_i(\lambda_o)\}/\varepsilon(\lambda_o)$ and the difference absorbance $\Delta A(\lambda_{max}) = A(\lambda_{max}) - A_i(\lambda_{max}) - \varepsilon(\lambda_{max})[L]_t$ were calculated from the observed absorbances. Here, $A(\lambda)$ represents absorbance at wavelength λ and suffix i indicates the initial value; $\varepsilon(\lambda)$ is the molar absorbance of the ligand at wavelength λ. The obtained set of data ($[L]_t$, $\Delta A(\lambda_{max})$) ($[L]_t$ = 0-100 μM) was processed essentially according to Walz and Hooverman (4) on the assumption of 1:1 complex formation to give K_d and $\Delta\varepsilon$. For the determination of the acid-induced difference spectrum, the standard buffer and 0.8 M $HClO_4$ (2.0 ml) were put into the cells at the sample and reference sides, respectively. Identical small volumes of the concentrated ligand solution were added to both solutions and the difference spectrum was recorded.

CD measurements were performed with a 1-cm cell using a JASCO J-40A spectropolarimeter equipped with a data processor J-DPZ. The CD spectra of RNase T_1 in the presence and absence of a ligand were recorded and the difference spectrum was obtained by use of the data processor. The CD intencity due to the free ligand was small under the conditions employed and thus was neglected.

Results and Discussion

Difference absorption spectra were induced on binding of BrG2'p and BrG3'p to RNase T_1 (Fig. 1). These spectra were almost the same as those observed with the parent ligands (1-4) except for a slight red-shift (4 nm), which was smaller than that between the absorption spectra of the free li-

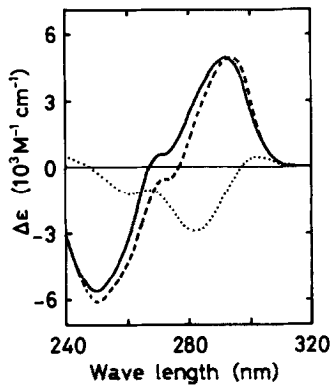

Fig. 1. The difference absorption spectra induced on binding of 8-bromoguanylic acids to RNase T_1 and the acid-induced difference spectrum for BrG3'p. The spectra were determined as described in Materials and Methods at final total concentrations of 15.6 μM RNase T_1 and 16.9 μM BrG2'p (——); 14.1 μM RNase T_1 and 22.5 μM BrG3'p (---); 36.4 μM BrG3'p and 0.8 M $HClO_4$ (···). The spectra are expressed in molar absorbance. For the RNase T_1-ligand complex, conversion to molar absorbance was performed by normalization of the difference spectrum to give the determined Δε value at 294 nm.

gands: absorption maxima of BrG2'p and G2'p were at 260 and 252 nm, respectively. Contrary to the situation for the parent ligands, the difference spectra induced by the brominated ligands were dissimilar to the acid-induced difference spectrum for BrG3'p (Fig. 1). Spectrophotometric titration of BrG3'p at 280 nm gave an estimated pK_a value below 0. Therefore, the spectrum shown in Fig. 1 was not taken after the complete protonation. When more concentrated acid solutions were used, a time-dependent change of absorbance became evident because of glycoside bond hydrolysis, making precise determination of pK_a difficult. The lower pK_a of BrG3'p than that of G3'p (2.3) reflects reduction of the electron density on N-7 by the bromination.

The dissociation constants K_d of the enzyme-ligand complexes were determined to be 5.1 and 22.5 μM for BrG2'p and BrG3'p, respectively. Control experiments gave K_d values of 4.6 and 21.3 μM for G2'p and G3'p, respectively, which agreed with the association constants (145 and 52 x 10^3 M^{-1}, respectively) determined by Walz and Hooverman under similar conditions (4). These results show that the brominated ligands have the same affinity to RNase T_1 as the parent ligands despite the reduced electron density on N-7.

The similarity of the difference spectra induced on binding of the brominated and parent ligands to RNase T_1 together with their dissimilarity

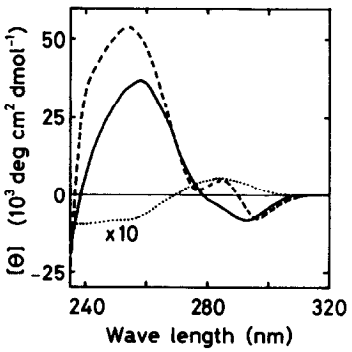

Fig. 2. The difference CD spectra induced on binding of 8-bromoguanylic acids to RNase T_1 and the CD spectrum of BrG2'p. The spectra were obtained as described in Materials and Methods at total concentrations of 34.1 μM RNase T_1 and 76.3 μM BrG2'p (——); 29.7 μM RNase T_1 and 45.7 μM BrG3'p (---); 87.1 μM BrG2'p(···). The spectra are expressed in molar ellipticity. The observed difference ellipticity was converted to difference molar ellipticity using the RNase T_1-ligand complex concentration calculated from the total concentrations and the determined K_d value. The spectrum of BrG2'p is multiplied by a factor of 10.

to the acid-induced difference spectrum for BrG3'p argues against the protonation on N-7 as the interpretation for the difference spectra. Proton abstraction from imidazolium (pK_a *ca.* 7) or even carboxylic acid (pK_a *ca.* 4) residue of the enzyme by such a weak base as BrG3'p is indeed unlikely. Non-importance of N-7 is also supported by the finding that the bromination does not affect the affinity of the guanine ligands to RNase T_1. The difference spectra induced on binding of the guanine ligands to RNase T_1 should, therefore, be reinterpreted with other perturbations. The hydrogen bond formation at N-1 and O-6 and/or the stacking on Tyr 45 revealed by the X-ray study (9) seem the most probable reasons for the difference spectra.

The difference CD spectra induced on binding of BrG2'p and BrG3'p to RNase T_1 are shown in Fig. 2 together with the CD spectrum of BrG2'p. The difference spectra have a peak around 255 nm, a shoulder at 285 nm and a trough around 295 nm. The shoulder is more prominent for BrG3'p, giving rise to a hump. These difference spectra resemble those induced by the parent ligands (5,6) except for changes in the trough: a red-shift of about 15 nm and decrease in magnitude were observed. The gross similarity suggests the common mechanism for the difference spectra induced by the brominated and parent ligands. 8-Bromoguanosine has been shown to adopt only *syn* con-

formation because of the bulky Br substituent on C-8 (15). Probably, the same situation holds for the brominated nucleotides. The mechanism for the difference spectra, therefore, should not be the *anti-syn* conformation change of the ligands. It should also be noted that the difference molar ellipticity is too large to be attributed only to the conformation change of the ligand.

Our results support, therefore, the interpretation by Sander and Ts'o that the difference CD spectra are induced by the exciton coupling interaction between the ligand and the aromatic residue(s) of the enzyme (6). Probably, Tyr 45 and/or Tyr 42 are the residues interacting with the ligands, if the results of the X-ray study (9) are taken into consideration.

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