An exposed tyrosine residue of RNase T_1 and its involvement in the interaction with guanylic acid

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A photo-CIDNP spectrum of RNase T_1 showed that 4 out of the total 9 tyrosines are accessible to the photosensitive dye, while none of the histidine and tryptophan residues are accessible. By comparison with the results of nitration of tyrosine side chains followed by peptide analysis, it can be concluded that Tyr 45 is mostly exposed on the surface of RNase T_1. On addition of 2′-GMP, the signal of Tyr 45 shifts upfield and is remarkably broadened, which suggests that the phenyl ring of Tyr 45 stacks on the guanine ring of 2′-GMP. Similar phenomena were observed on addition of 3′-GMP and 3′-dGMP.

Photo-CIDNP *RNase T_1* *Guanylic acid* *Nitration* *NMR* *Enzyme-substrate binding*

1. INTRODUCTION

Ribonuclease T_1 (RNase T_1; EC 3.1.27.3) is a highly specific endonuclease from *Aspergillus oryzae* which cleaves at the 3′-phosphodiester bond of guanylic acid residues in single-stranded RNA [1,2]. The enzyme is a small acidic protein which is composed of 104 amino acid residues [2]. From chemical modifications and physicochemical studies it has been suggested that His 40, Glu 58, Arg 77 and His 92 are essential for catalysis [1–6]. Besides these residues, the participation of some main chain peptide groups and other side chain groups in substrate binding was demonstrated by recent X-ray analyses of the RNase T_1-2′-GMP complex [7,8]. These analyses also revealed that the guanine base is sandwiched by two tyrosine residues, Tyr 42 and Tyr 45.

Here photo-CIDNP (photochemically induced dynamic nuclear polarization) [9] has been employed to clarify whether or not tyrosine, histidine and tryptophan residues of RNase T_1 are involved in inhibitor binding. The resonances due to 4 out of the total of 9 tyrosine residues were identified in the photo-CIDNP spectrum of RNase T_1. We were able to assign one of them to a specific surface residue by nitration followed by peptide analysis. Based on the assignment, Tyr 45 was shown to protrude into the solvent in the free form of RNase T_1, and to be involved in the interaction with guanylic acid.

2. MATERIALS AND METHODS

RNase T_1 was a generous gift from Dr H. Tamaoki of Sankyo Co. Ltd. Guanosine 2′-monophosphate (2′-GMP), guanosine 3′-monophosphate (3′-GMP) and 2′-deoxyguanosine 3′-monophosphate (3′-dGMP) were from Sigma. TPCK-trypsin was obtained from Worthington and *Staphylococcus aureus* V8 protease from Miles.

Nitration of tyrosine residues was performed by adding a 30-fold molar excess of tetranitromethane dissolved in a small amount of ethanol to a 1% RNase T_1 solution in 50 mM Tris-HCl buffer, pH 8.1 (final ethanol concentration, ~2%) and allowing to stand at 25°C for 6 h. The resultant nitrated

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RNase T_1 was separated by reverse-phase HPLC (Synchropak RP-P C_{18}). The location of the nitrated tyrosine residue was determined by a successive digestion with trypsin and *S. aureus* V8 protease followed by amino acid analysis and Edman degradation. Trypsin digestion was performed according to Tamaoki et al. [10]. The tryptic peptide containing the nitrated tyrosine separated by reverse-phase HPLC (peptide T-1) was digested with *S. aureus* V8 protease in 50 mM phosphate buffer, pH 7.8 (the molar ratio of substrate to enzyme was 25 to 1) for 24 h at 37°C. The digest was separated by reverse-phase HPLC. The composition of the peptide in each peak was determined by amino acid analysis and the nitrotyrosine-containing peptide was sequenced by an automated sequencer (Applied Biosystems, Model 470A). The analysis of PTH-amino acids was performed by isocratic reverse-phase HPLC [11].

^{1}H-photo-CIDNP spectra were taken at 500 MHz on a JEOL GX-500s NMR spectrometer by using a specially designed probe with a 3 mm quartz rod to introduce laser light. RNase T_1 was dissolved in D_2O and heated to 60°C for 2 min and then lyophilized to replace exchangeable protons. The RNase T_1 was redissolved alone or with inhibitor in D_2O with 0.2 M NaCl at p*D* 5.5, to which 0.2 mM of a flavin dye (3-*N*-carboxymethyl lumiflavin) was added. The sample tube was irradiated in the probe for 100 ms by 488 nm light from an NEC GLC-3300 argon ion laser prior to data acquisition. Alternating light and dark free induction decays were collected and subtraction yielded the photo-CIDNP difference spectrum. All the spectra were taken at 23°C.

3. RESULTS

The proton magnetic resonance spectrum of RNase T_1 and the photo-CIDNP difference spectrum are shown in fig. 1a and b. The resonances observed in the aromatic region are due to 9 tyrosine, 4 phenylalanine, 3 histidine and one tryptophan residue. Of these, the C2 and C4 proton resonances from His 27, His 40 and His 92 were already assigned by using the deuterium exchange technique and pH titration [3,4]. In the photo-CIDNP spectrum we could identify 3,5 ring proton resonances from 4 tyrosine residues as A, B, C and D at 6.84, 6.76, 6.66 and 6.48 ppm respectively (fig.1b). The positive peak at 7.14 ppm may be due to the cross-polarization on the 2,6 protons of Tyr A. Also the positive peaks around 3 ppm are due to the C_β protons of the tyrosine residues. Sharp positive and negative peaks at 7.99, 4.16 and 2.61 ppm arise from flavin dye judged from the resonance position of flavin itself. No signals corresponding to histidine and tryptophan could be observed.

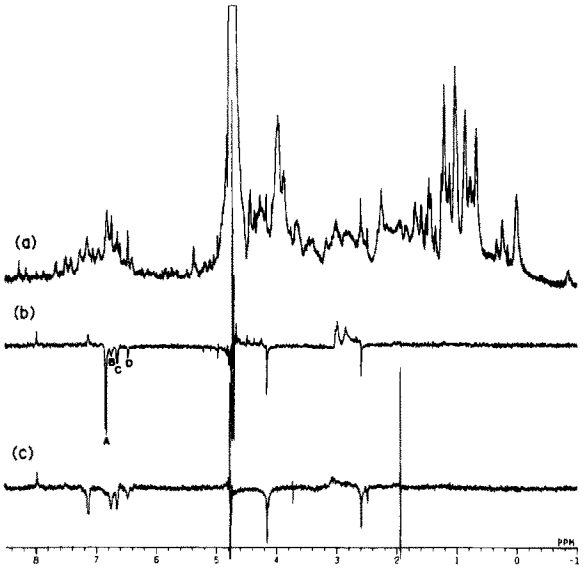

Fig.1. Photo-CIDNP spectra of intact and nitrated RNase T_1. (a) Dark spectrum and (b) photo-CIDNP difference spectrum of 0.6 mM RNase T_1; (c) photo-CIDNP difference spectrum of 0.4 mM nitrated RNase T_1. Each solution is in D_2O with 0.2 M NaCl at p*D* 5.5. The peaks at 1.94 and 3.73 ppm in (c) are due to uncompensated signals of buffers which could not be eliminated completely from the reaction mixture.

After nitration with tetranitromethane under mild conditions, the signals at 6.84 and around 3 ppm in the photo-CIDNP spectrum decreased remarkably, while the other resonances did not change much and a new negative peak appeared at 7.13 ppm (fig.1c). This decrease must arise from the nitration of Tyr A and the new peak must be due to the nitrated tyrosine residue.

The nitrated RNase T_1 purified by reverse-phase HPLC was submitted to amino acid analysis and only one tyrosine was found to be nitrated (not shown). Reverse-phase HPLC after tryptic diges-

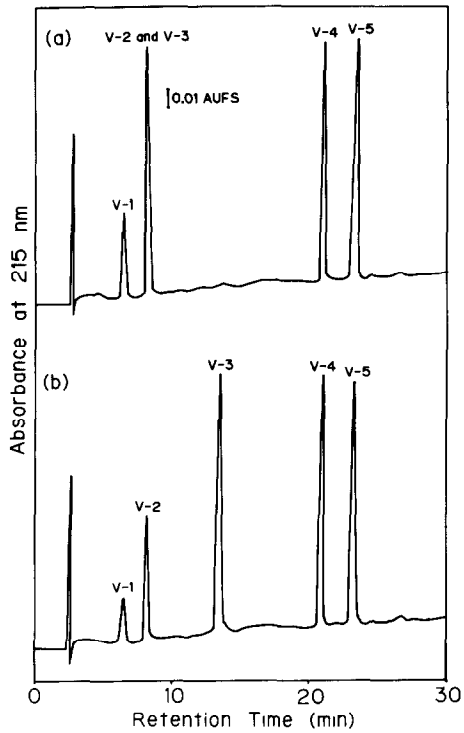

Fig.2. Separation of *S. aureus* V8 protease digest of peptide T-1 from nitrated RNase T_1. The digest of peptide T-1 from (a) intact or (b) nitrated RNase T_1 was chromatographed on Synchropak RP-P C_{18} with a linear gradient of acetonitrile from 10 to 35% in 0.1% TFA for 30 min. Peptides identified are shown by residue numbers: V-1, residues 47–49; V-2, residues 67–77; V-3, residues 42–46; V-4, residues 50–58 and V-5, residues 59–66. In the peaks not marked, none of the peptides and amino acids were detected.

tion of the nitrated RNase T_1 gave two peptides cleaved at both Lys 41 and Arg 77. One was peptide T-1 (residues 42–77, 62% yield) and the other was peptide T-2 (residues 1–41 connected by a disulfide bond to residues 78–104, 48% yield). Since nitrated tyrosine was only detected in peptide T-1, the peptide was further digested with *S. aureus* V8 protease. Among the 5 shorter peptides which were thus obtained and resolved by reverse-phase HPLC (fig.2), peptide V-3 (residues 42–46, 51% yield) was found to contain the nitrated tyrosine residue (amino acid composition in molar ratio: Asp 2.0, Glu 1.0, Tyr 0.9 and NO_2-Tyr 1.0). PTH-NO_2-Tyr, which was eluted between PTH-Trp and diphenylthiourea in our chromatography [11], was not detected at the first cycle of the Edman degradation but at the fourth. These results show that Tyr 45 was exclusively nitrated under the present conditions. By comparison of this result with the intensity decrease of the tyrosine resonance in photo-CIDNP spectrum, we could assign the resonance of Tyr A at 6.84 ppm to Tyr 45.

To obtain information on the involvement of these residues in the guanine recognition, product-type inhibitors (2′-GMP, 3′-GMP or 3′-dGMP) were added to the RNase T_1 solution. On addition of an equimolar amount of 2′-GMP, we were no longer able to see the strong negative CIDNP signal at 6.84 ppm of Tyr 45, while the other 3 weak negative signals were retained. At the same time, a new negative photo-CIDNP signal appeared at 6.50 ppm and became stronger as the amount of 2′-GMP was increased. It showed slow exchange behavior and it was hard to trace its original position in the free form of RNase T_1. On addition of 3′-dGMP, which is weakly bound to RNase T_1, the peak at 6.84 ppm shifted continuously to 6.50 ppm. Therefore we could conclude that the negative CIDNP signal at 6.50 ppm arose from Tyr 45 in the enzyme-inhibitor complex (fig.3). It is hard to follow the shifts of the tyrosine signals in the dark spectrum because of complicated behavior in the overlapped signal patterns. When we compare the signal intensity in fig. 3a and c under the same conditions, it is obvious that the negative peak from Tyr 45 is reduced and

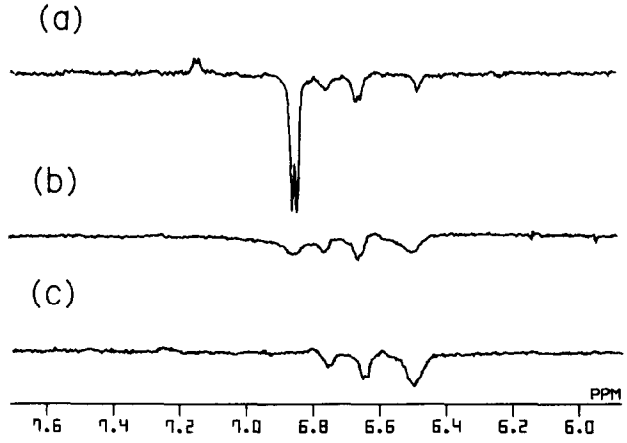

Fig.3. The aromatic regions of photo-CIDNP difference spectra of RNase $T_1 \cdot 2'$-GMP complex. Each solution contains 0.6 mM RNase T_1 with 0.2 M NaCl at p*D* 5.5. (a) RNase T_1 alone, (b) 1:0.5 RNase $T_1/2'$-GMP, (c) 1:1 RNase $T_1/2'$-GMP.

broadened on complex formation. Therefore it is safe to say that the state of Tyr 45 is affected by the inhibitor binding and the access of the dye is hindered. On addition of 3′-GMP or 3′-dGMP, a similar upfield shift and decrease in intensity of Tyr 45 as in the case of 2′-GMP was observed. In the spectrum of the complex in the presence of an excess amount of 3′-dGMP, however, the signal at 6.50 ppm is sharper than those of the complexes with 2′-GMP and 3′-GMP. This indicates weaker binding of 3′-dGMP.

The guanine H8 resonance itself shows a positive photo-CIDNP signal at neutral pH [12], but in our experimental conditions at pH 5.5 there was no photo-CIDNP signal even without RNase T_1. By raising the pH of the solution to 7.0, we could clearly see the photo-CIDNP signal of the guanine H8 proton. On addition of RNase T_1, the signal was extremely broadened, which may indicate restriction in the motion of the guanine ring in complex formation.

4. DISCUSSION

In the present study the photo-CIDNP experiment has been employed to detect the resonances of 3,5 ring protons of tyrosine residues on the surface of RNase T_1. The spectrum demonstrated that 4 out of the total 9 tyrosine residues were detected. Among them, the Tyr 45 resonance at 6.84 ppm shows a strongly polarized signal, like the free amino acid. This suggests that its phenolic ring is exposed and rotates freely on the surface of RNase T_1.

Also photo-CIDNP experiments have shown that the Tyr 45 signal shifted upfield and diminished in intensity on complex formation. This phenomenon can be explained by assuming that the phenolic ring of Tyr 45 stacks on the guanine plane and thus the resonance of its 3,5 ring protons shifts upfield by the ring-current effect. It is also suggested that the mobility of this phenolic ring is restricted and the ring is partly shielded from access by the dye on account of the steric effect of the guanine base. It is shown in X-ray analyses of the complex that the phenolic side chain of Tyr 45 hangs over the guanine base which sits on the side chain of Tyr 42 [7,8]. The present result showed agreement between the complex structure in solution and in the crystal.

Similar phenomena were observed on addition of 3′-GMP and 3′-dGMP, respectively. In both cases the phenolic ring of Tyr 45 also stacks on the guanine base. The geometry of the guanine base and tyrosine residues at the active center seems to be almost the same in 3 inhibitors, 2′-GMP, 3′-GMP and 3′-dGMP.

In previous NMR studies [3,4], His 40 and His 92 were shown to be involved in complex formation with 3′-GMP. The present photo-CIDNP experiment has shown that the imidazole groups of both residues are not exposed on the surface of RNase T_1 nor involved in hydrogen bonding to some parts of the enzyme. This is in contrast to the case of RNase A, where His 119, which is essential to the catalytic activity, is accessible to the flavin dye [13].

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