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GLU 46 OF RIBONUCLEASE T_1 IS AN ESSENTIAL RESIDUE FOR THE RECOGNITION OF GUANINE BASE

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The Glu 46 of ribonuclease T_1, which is assumed to interact with N1 of the guanine residue in RNA by a hydrogen bond from the result of X-ray analysis, was changed to alanine by site-directed mutagenesis and its function examined. The nucleolytic activity of the Ala 46 mutant enzyme against pGpC decreased to 0.4 % of that of the wild-type enzyme, on the other hand its activity against pApC increased. This result suggests that the Glu 46 is essential for the recognition of the guanine base but that it also interferes with the recognition of the adenine base.

To elucidate enzyme function at molecular levels is very important not only in the field of pure science but also in the field of applied science. Ribonuclease T_1 (RNase T_1) is a guanosine specific microbial ribonuclease (1) which is very useful for sequence analysis of RNA. It is very stable and can be obtained in large quantities. It has been extensively studied using biochemical and biophysical approaches (2,3). We have been studying it using protein engineering techniques, and have obtained much information about its correct amino acid sequence (4), interactions at the binding pocket of the guanine base (4), and a new reaction mechanism (5,6).

Many guanosine specific ribonucleases have been found in microorganisms but no adenosine specific ribonuclease has been found so far. Only RNase U_2 (7) can recognize adenosine with a relatively high selectivity.

How does RNase T_1 recognize guanine base? Protein engineering is one of the most powerful methods used to study such interactions between an enzyme and a substrate. Recent crystallographic study on a RNase T_1-2'-GMP complex

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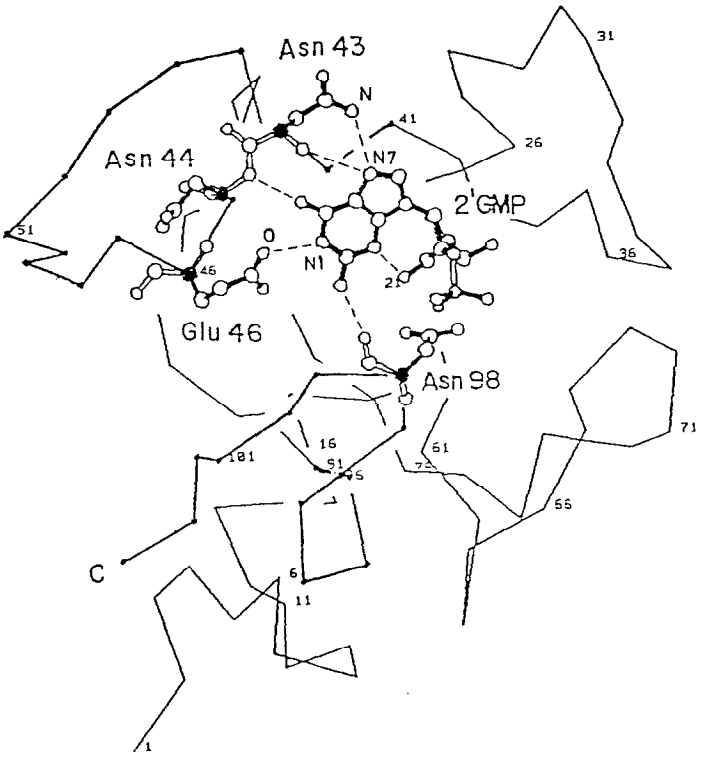

Fig. 1 Tertiary strucutre of the complex of RNase T_1 and 2'-GMP in the binding pocket. The 2'-GMP molecule and the interactive amino acid residues are represented by ball-and-stick drawing with wire drawing of Cα-carbon atom tracing of the protein. Dashed lines indicate hydrogen-bonding interactions.

provided a clue to understanding their interactions (8,9). These results revealed that the guanine base has hydrogen bonds with Asn 43, Asn 44, Glu 46 and Asn 98 of RNase T_1 (Fig. 1), and is stacked with Tyr 42 and Tyr 45 (9). In these four residues which have hydrogen bonds with the guanine base, Asn 43 and Glu 46 have interactions with the guanine base through their side chains but Asn 44 and Asn 98 have interactions through their main chains.

The function of the former residues can be studied by replacing them with other amino acids but it is difficult to study the latter residues with hydrogen-bonds through the main chains by substitution. In the previous paper (4), when Asn 43 has been changed to Ala in order to remove the hydrogen bond with N7 of guanine base, the nucleolytic activity of Ala 43 mutant RNase T_1, which was partially purified, was considerably high even though one hydrogen-bond has been lost. In this paper we have focused on Glu 46, which had a

hydrogen bond with N1 of the guanine base, and have clarified the function of the hydrogen bond in the binding pocket by changing it to Ala 46.

Materials and Methods

Synthesis of the gene for mutant RNase T_1 and construction of a expression plasmid

The mutant gene was synthesized by cassette mutagenesis as reported previously (4). To change Glu 46 to Ala 46, two deoxyoligonucleotides, d(TATGCTGGCTTCGACTT) and d(AAGCCAGCATAGTTGTT) (underlines indicate changed codons), were used to construct a gene instead of U10 and L10 (4). The gene was inserted at the BglII-SalI site of pGH-L9 (10) to be expressed as a fusion protein under the control of the *E. coli trp* promoter as reported previously (4). The plasmid pT46A16 which encodes the mutant protein was introduced into *E. coli* HB101 and the nucleotide sequence of the mutant gene was verified by the dideoxy method (11).

Expression of the mutant gene and purification of mutant enzyme.

The recombinant cells harboring the plasmid pT46A16 were induced with 3-indoleacrylic acid and, Ala 46 mutant RNase T_1 was purified by the procedures as described previously (5,6). The mutant enzyme at each step in purification was analyzed by SDS-PAGE. The protein concentration was determined by Lowry's method or by using an extinction coefficient, $A^{1\%}_{278}$=19 (12), in the case of the purified mutant enzyme.

Analysis of nucleolytic activity and enzyme kinetics

Enzymatic activity was measured by analysis of the cleavage of [5'-^{32}P]pGpC using the system of homochromatography as described previously (5,6). The standard reaction mixture consisted of 150 μM [5'-^{32}P]pGpC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA and 90 nM of Ala 46 mutant RNase T_1 in a volume of 10 μl and was incubated at 37°C. The enzymatic activity was linear at least within 10 min under the above conditions. Enzyme kinetics were measured under the standard conditions, in mixture containing 100-1000 μM (133, 200, 333, 500 and 1000 μM) [5'-^{32}P]pGpC. Kinetic constants were obtained from analysis of Lineweaver-Burk plots (several times repeated).

When [5'-^{32}P]pApC was used as a substrate, the concentrations of the substrate and enzyme were 20 μM and 90 μM, respectively. The reaction mixture (total 10 μl), containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA or 50 mM sodium acetate (pH 4.6), 1 mM EDTA, was incubated at 37°C. After 2.5, 5 and 10 minutes, reactions were stopped by addition of 2 μl of 1N HCl and the products were analyzed by homochromatography.

Results and Discussion

The gene for Ala 46 mutant of RNase T_1 was prepared by cassette mutagenesis using chemically synthesized deoxyoligonucleotides, the nucleotide sequence was verified by the dideoxy method (data not shown). The gene was expressed as a fusion protein under the control of *trp* promoter in *E. coli* in the same way as described previously (4,5,6). The fused protein was treated by cyanogen bromide to cleave at the Met junction and the product was purified by column chromatography as reported previously (5,6).

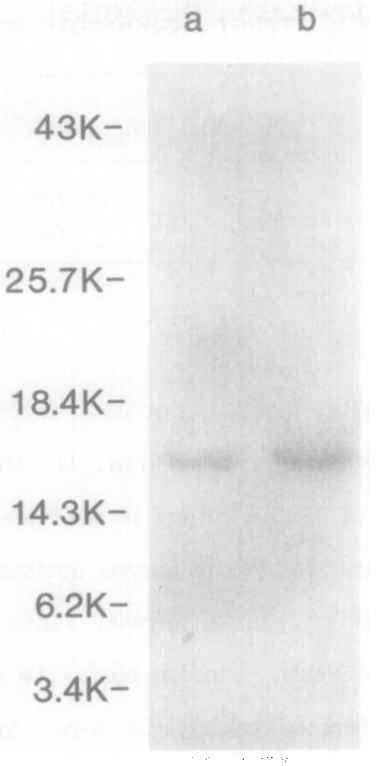

Fig. 2 Gel electrophoresis (15% SDS-PAGE) of purified Ala 46 mutant RNase T_1. Lane a; Ala 46 mutant RNase T_1, lane b; RNase T_1.

The finally purified Ala 46 mutant enzyme obtained by HPLC (TSK-gel, DEAE-2SW) showed a single band on 15% SDS-PAGE (Fig. 2) and migrated slightly faster than RNase T_1. This result may be due to the loss of one negative charge by substitution of Glu by Ala. 135 μg of the Ala 46 mutant enzyme was obtained from 4 l of culture. The CD pattern and the thermal denaturation curve monitored by CD ellipticity at 240 nm of the Ala 46 mutant enzyme were very similar to those of the wild type (data not shown), suggesting very little change in the folding structure.

The nucleolytic activity of the mutant enzyme against pGpC was drastically reduced. Table 1 summarizes steady state kinetic data. The affinity constant (K_m) slightly increases, while the catalytic constant (k_{cat}) of the enzyme reduces to 1/150 of that of the wild type. Catalytic efficiency (k_{cat}/K_m), which reflects the enzyme activity, is less than 1% of that of the wild type. When we compare these values with those of purified Ala 43 mutant

Table 1. Kinetic constants for wild-type and mutant RNase T_1

Mutation	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
Wild type	536	75,700	142.5
Glu46 → Ala46	819	496	0.6

enzyme (unpublished results, Nishikawa et al.) which lacks the hydrogen bond between Asn 43 and N7 of guanine base (Fig. 1), the K_m value is almost the same but the k_{cat} value of Ala 43 mutant is over 20-fold higher than that of Ala 46 mutant, and consequently the enzyme activity of Ala 43 mutant is 20-fold higher than that of Ala 46 mutant. This result suggests the difference in the role of each hydrogen bond. We can raise two points for explanation of this difference taking the X-ray data of RNase T_1-2'-GMP complex into account:

i) The distances between N1H of guanine and carboxy oxygen of Glu 46, and between N7 of guanine and nitrogen of amino of Asn 43 are 2.6 Å and 3.0 Å respectively, i.e. the former hydrogen bond is stronger than the latter.

ii) The side chain of Glu 46, carboxy group, is negatively charged at pH 7.5 of the reaction condition and consequently the hydrogen bond of N1H-Glu 46 may be stronger than the nonionized hydrogen bond of N7-Asn 43.

These two evidences suggest that the hydrogen bond of N1H-Glu 46 is more important than that of N7-Asn 43 for the recognition of the guanine base and the activity is reduced more drastically by removing this important hydrogen bond.

N1 of the guanine base has a proton at pH 7.5 while N1 of the adenine has only lone pairs. Thus the situation of each N1 is different for the guanine base and the adenine base. Accordingly, Glu 46 of RNase T_1, which has strong interaction with N1 of the guanine, must have a key role for recognition of the guanine base and substitution of Glu 46 by Ala 46 is assumed to reduce the high substrate specificity of RNase T_1.

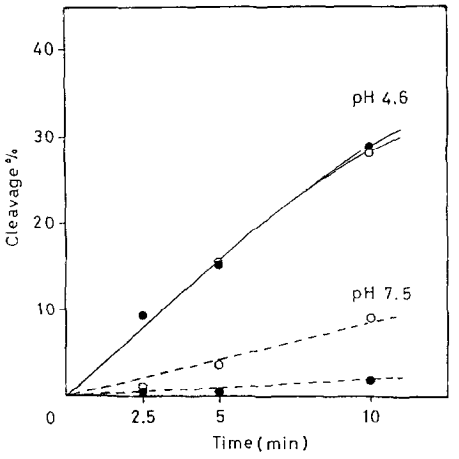

Fig. 3 Time course of hydrolysis of [5'-^{32}P]pApC by RNase T_1 and Ala 46 mutant RNase T_1. Open circles, Ala 46 mutant RNase T_1; closed circles, RNase T_1. Solid lines, at pH 4.6; dashed lines, at pH 7.5.

The relative rate of cleavage of ApC vs. GpC is 1/150000 (13) for RNase T_1. To detect this low activity against pApC, we have run the reactions under the artificial conditions, such as higher concentration of enzyme, other than that of substrate. The nucleolytic activity against [5'-^{32}P]pApC of Ala 46 mutant enzyme is similar to that of RNase T_1 at pH 4.6, but is 4-fold higher than that of RNase T_1 at pH 7.5 as shown in Fig. 3. At pH 7.5, negative charge of the carboxy anion of Glu 46 may be repulsive to the lone pairs of N1 of the adenine base, while methyl group of Ala 46 does not have such repulsion, accordingly the binding pocket may be slightly changed to accept pApC. The relative rate of cleavage of pApC vs. pGpC by Ala 46 mutant becomes 1/200 at pH 7.5, suggesting that the substrate specificity has drastically changed although the absolute activity against pGpC is very low. When we changed Tyr 45 of RNase T_1, which acts as a "lid" for the binding pocket of guanine base, to Trp 45 for enhancing the stacking effect, the Trp 45 mutant acquires a slightly higher nucleolytic activity than the wild type enzyme against pGpC and also against pApC (14). The combination of double mutation to Ala 46 and Trp 45 may produce more profound change in the base specificity of RNase T_1. We are now constructing these doubly mutated RNase T_1 for further study.

Acknowledgements

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