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Mechanism of hydrolysis of phosphodiesters with ribonuclease T1

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Abstract - Ribonuclease T1 (RNase T1) specifically hydrolyzed the phosphodiester linkages of guanosine 3'-phosphate of single-stranded RNA and the cleavage occurs in a two-step reaction mechanism. It is considered that in the first step Glu 58 abstracts a proton from 2'-OH and His 40 or His 92 adds a proton to O-5' of ribose. We succeeded to express the chemically synthesized genes for RNase T1 and its several mutants at base-recognition site in E. coli and reported the structure-function relationship of this enzyme. In this paper, we changed Glu 58, His 40 and His 92 to alanine etc. and analyzed the activity of these mutant enzymes in order to clarify the role of catalytic residues. Gln 58 mutant and Ala 58 mutant still retained slight activity but both Ala 40 and Ala 92 mutants lost the activity almost completely. This result indicates that Glu 58 is not essential but His 40 and His 92 are indispensable for RNase T1 activity. We propose a new reaction mechanism, in which His 40 abstracts a proton from 2'-OH and His 92 protonates O-5' of ribose while Glu 58 enhances the basicity of His 40.

INTRODUCTION

By the chemical synthesis of oligonucleotides and recombinant DNA techniques, designed genes for relatively large peptides can be synthesized and expressed in bacteria (e.g. the human growth hormone (hGH), ref.1). Recently, we have succeeded to synthesize ribonuclease T1 (RNase T1, EC 3.1.27.3) in E. coli by using a totally synthesized gene (ref. 2). RNase T1 which hydrolyzed specifically the phosphodiester linkages of guanosine 3'-phosphate of single strand RNA, has been isolated from Aspergillus oryzae (ref. 3). Because of the high base specificity of RNase T1, it has been used extensively in the structural determination of RNA, which led to the first sequencing of the yeast alanine tRNA. It is also one of the most well-studied enzymes by biochemical- and biophysical- strategies (ref. 4,5). Recently the three-dimentional structure of RNase T1 complexed with an inhibitor, guanosine 2'-phosphate (G2'p, ref. 6,7) or G3'p (ref. 8) was determined by X-ray crystallography and the base-specific binding site was presented for the first time. The use of synthetic genes, in site-directed mutagenesis should become easier and thus provide further information concerning the structure-function relationship of enzymes.

PREVIOUSLY PROPOSED REACTION MECHANISM

The reaction of RNase T1 is composed of two steps and its reaction mechanism is considered to be the following (Fig. 2a). In the first, that is the transesterification step, the carboxyl group of Glu 58 abstracts a proton from the 2'-OH and either His 92 or His 40 protonates the 5'-O the adjacent ribose producing a 2',3'-cyclic phosphate intermediate. In the second step, either His 92 or His 40 abstracts a proton from a water molecule and Glu 58 adds a proton to the 2'-O, hydrolyzing the 2',3'-cyclic phosphate intermediate.

This mechanism was proposed from results of mainly chemical modification studies on RNase T1 (ref. 9). Some discrepancies are to be observed, however, between the results of chemical modification and NMR studies (ref. 10), and it is not known whether His 92 or His 40 acts as the general acid in the reaction. These catalytic residues are well conserved in guanine-specific ribonucleases from other microorganisms(ref. 4,5) and furthermore, recent crystallographic studies on an RNase T1-2'-GMP complex showed the catalytic residues to be located near the phosphate group (ref. 6,7).

ACTIVITIES OF CATALYTIC-SITE MUTANTS OF RNase T1

In order to clarify the relation between Glu 58, His 92 and His 40, for the present study we changed these residues to alanine etc. by replacing appropriate regions of the gene with synthetic oligonucleotide fragments (Fig. 1). By substituting a changed group by a methyl group, it may be possible to elucidate the function of these amino acid residues in the catalytic action.

The mutant enzymes were purified by column chromatographies to obtain a single band on SDS-polyacrylamide gel electrophoresis. The nucleolytic activity was analyzed using homo-chromatography system by measuring the amount of ^{32}pGp produced from substrate ^{32}pGpC. The table 1 summarized the relative activity of these mutant enzymes with respect to wild type enzyme.

Table 1. Activities of catalytic-site mutants of RNase T1.

Substituted positions	Relative activity
RNase T1 wild type	100 %
Glu 58 → Asp 58	10
Gln 58	1
Ala 58	7
His 40 → Ala 40	0.01
His 92 → Ala 92	0

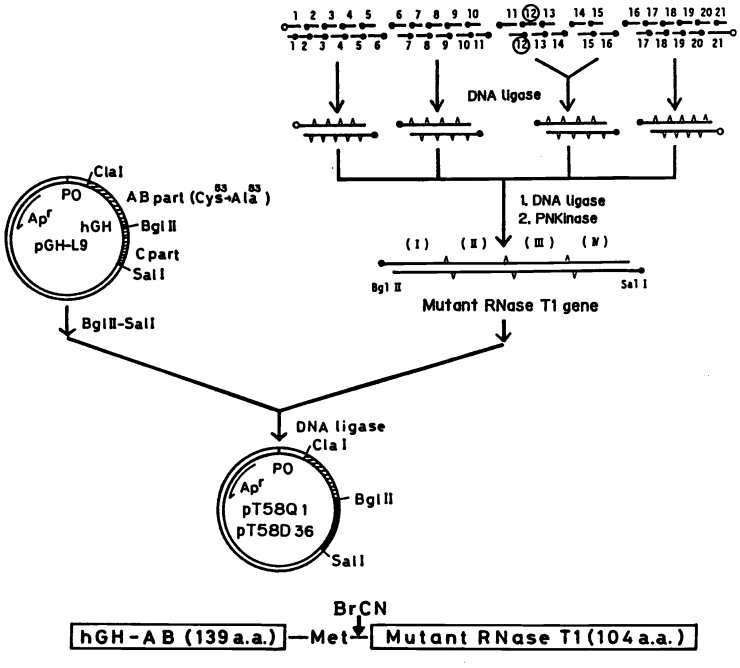

Fig. 1. Construction of the mutant RNase T1 gene and its insertion into the expressionvector. Dots indicate a 5'-phosphate and open circles indicate a 5'-hydroxyl group. The synthesized RNase T1 gene was inserted between the BglII and SalI sites of vector pGH-L9. The thich bleck line of the constructed plasmid indicates the mutant RNase T1 gene. Fusion proteins were cleaved with cyanogen bromide to generate mutant RNase T1.

A 90% decrease in activity for the Asp 58 mutant could be explained by the shorter carboxyl side-chain being less favourable for abstracting the 2'-OH proton (ref. 11). But, for the mutants having Gln 58 or Ala 58, where the carboxyl group is replaced by a carboxyamide group and the carboxyethyl group is replaced by a methyl group, respectively, a little activity is retained. On the other hand, substituting either of the histidine residues with an alanine residue caused a complete loss of activity. This result indicates that the Glu 58 residue is important but not essential and both histidine residues are indispensable for catalytic activity. The reaction mechanism proposed in Fig. 2a cannot explain this result and therefore we propose a new reaction mechanism in Fig. 2b.

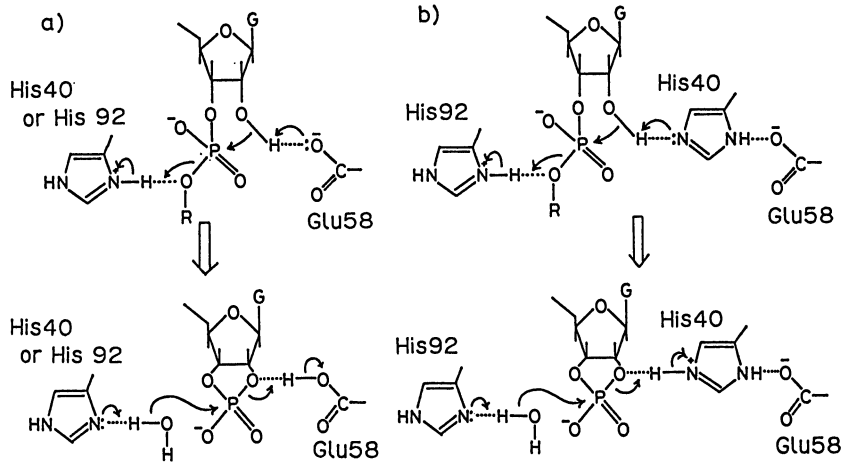

Fig. 2. Reaction mechanism for RNase T1. a) Previously proposed from the results of chemical modification and NMR studies (ref. 4). b) Newly proposed in this paper from the results of protein engineering stratedy.

In our newly proposed mechanism, His 40 abstracts a proton from the 2'-hydroxyl group and His 92 adds a proton to O-5' of the ribose in the first step. Also in this step, Glu 58 ensures an acidic environment around His 40 and enhances the basicity of His 40. In the second step, these residues play opposite roles to that of the first step. We have substituted His 92 with alanine so that the actual catalytic residue is deleted and the result is that activity is lost completely.

A couple of pieces of evidence support the proposal of this new mechanism.

i) The distance between 2'-O and His 40 NE2 is shorter than the distance between 2'-O and the Glu 58 OE2 in the complex of RNase T1-2'-GMP (ref. 5).

ii) Two hydrogen-bonds, 3'-GMP 2'-O-His 40 ND1 and His 40 NE2-Glu58 OE2 have been found in the complex of RNase T1-3'-GMP (Tomita & Hakoshima personal communication). This alignment is well suited to the new reaction model.

iii) Another support is obtained from the mechanism of RNase A. Its reaction is catalyzed by His 12 and His 119 (ref. 12) and is composed of two steps as that of RNase T1. Moreover, it has been demonstrated that the interaction between His 119 and Asp 121 is important for full activity in an experiment using a synthetic oligopeptide (ref. 13).

The newly proposed mechanism for RNase T1 is very similar to the reaction mechanism for RNase A. And it is very interesting, because RNase T1 is obtained from a lower eukaryote while RNase A is mammalian in fact bovine and no homology between their amino acid sequences and also no similarity in their tertiary structures. Convergent evolution (ref. 14) which found between Trypsin (mammalian) and Subtilisin (microorganism) may also be found in these two ribonucleases.

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