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Biochemistry

Inquiries into the structure–function relationship of ribonuclease T1 using chemically synthesized coding sequences

(phosphotriester solid-phase synthesis/*trp* promoter/fusion protein/cyanogen bromide cleavage)

M. IKEHARA*, E. OHTSUKA†, T. TOKUNAGA*, S. NISHIKAWA*, S. UESUGI*, T. TANAKA*, Y. AOYAMA*, S. KIKYODANI*, K. FUJIMOTO*, K. YANASE*, K. FUCHIMURA*, AND H. MORIOKA*

*Faculty of Pharmaceutical Sciences, Osaka University, Osaka, Japan; and †Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan

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ABSTRACT **The genes for ribonuclease T1 and its site-specific mutants were chemically synthesized and introduced to *Escherichia coli*. All enzymes were fusion products produced by joining the synthetic gene at specific restriction sites to the synthetic gene for human growth hormone in a plasmid containing the *E. coli trp* promoter. The fusion protein from this plasmid contained 66% of the amino-terminal sequences of the human growth hormone, which were recognizable immunologically. RNase T1 or its mutants were cleaved from the fusion protein with cyanogen bromide. The synthetic RNase T1 endowed with the revised wild-type triad Gly-Ser-Pro, residues 71–73, was fully functional, readily hydrolyzing pGpC bonds, whereas a mutant enzyme having the originally reported, erroneous triad Pro-Gly-Ser was totally inactive. Various amino acid substitutions were also introduced to the guanosine recognition region comprised of residues 42–45, Tyr-Asn-Asn-Tyr. Substitution of either of the tyrosine residues noted above with phenylalanine had no dramatic effect on the enzyme's function. Replacement of asparagine-43 with arginine or alanine also caused only a small change in the hydrolyzing activity—a mutant enzyme maintained greater than 50% of the wild-type activity. In sharp contrast, when aspartic acid or alanine was substituted for asparagine-44, the activity was dramatically reduced to a few percent of the wild-type activity.**

By combining the chemical synthesis of oligonucleotides with recombinant DNA technology, genes designed for relatively large peptides can be synthesized and expressed in bacteria; e.g., the human growth hormone (hGH) (1). We decided to introduce pertinent amino acid substitutions to ribonuclease T1 (RNase T1, EC 3.1.27.3) by the use of totally synthesized genes. RNase T1, which specifically hydrolyzes the phosphodiester linkages of guanosine 3′-phosphate, has been isolated from *Aspergillus oryzae* (2). Because of the high base specificity of RNase T1, it has been used extensively for the structural determination of RNA—i.e., the first sequencing of the yeast alanine tRNA (3). The three-dimensional structure of RNase T1 complexed with an inhibitor, guanosine 2′-phosphate (4, 5) or guanosine 3′-phosphate (6) was determined by x-ray crystallography, and further information as to the base-specific binding site and the phosphodiester-cleavage site have been obtained by chemical modifications of this enzyme (7) as well as by physical studies (8, 9). The use of synthetic genes, with amino acids substituted by site-specific mutations should provide even more information concerning the structure–function relationship of this enzyme. The sequence of 104 amino acids for the wild-type RNase T1 has been reported (10) but subsequently revised (11) to reveal the Gly-Ser-Pro (residues 71–73) tripeptidic sequence instead of the originally reported Pro-Gly-Ser. We have reported a synthesis of an RNase T1 with the originally reported, but erroneous, Pro-Gly-Ser sequence encoded by a chemically synthesized gene (12). The expressed protein was not enzymatically active. It was pointed out that other RNases from fungi have the sequence of Ser-Pro for residues 72 and 73, as seen in RNase U2 (13–15), and Gly-Ser-Pro as found in RNase Ms (16) and RNase F (17). In this paper we report that the wild-type RNase T1 expressed from a synthetic gene encoding the revised sequence Gly-Ser-Pro indeed hydrolyzed pGpC. Other synthetic RNase T1 mutants with substituted amino acid residues at the recognition site for guanylic acid have also been synthesized by replacement of synthetic gene fragments. The catalytic activities of these products are also discussed.

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METHODS

Oligonucleotides. Oligodeoxyribonucleotides were synthesized by the triester method as described (1). The protected diribonucleotide (GpC) was deblocked (18) and phosphorylated by polynucleotide kinase (19).

Enzymes. DNA ligase and various restriction enzymes were obtained from either Takara Shuzo or Boehringer Mannheim. RNase T1 was a gift from Sankyo. DNase I was purchased from Boehringer Mannheim. Lysozyme was purchased from Sigma, and all other enzymes were obtained from Takara Shuzo.

Construction and Expression of Genes for RNase T1 and Its Designed Mutants. Oligonucleotides containing 4–11 bases were phosphorylated and successively joined by T4 DNA ligase (see Fig. 2) using conditions described (1).

Plasmid pGHL-9 (10 μg in 100 μl of buffer) (1) was treated with 20 units each of *Bgl* II and *Sal* I at 37°C for 3 hr. These enzymes were removed from the DNA by phenol extraction followed by an ether wash. The cleaved vector was precipitated with ethanol and dissolved in 50 μl of 10 mM Tris·HCl (pH 7.5) and 1 mM EDTA. The vector fragment (0.67 pmol) and the synthetic gene (2–5 pmol) were incubated in a total volume of 20 μl with T4 DNA ligase (1.2 units) overnight at 20°C. Plasmids were phenol extracted, precipitated, and then used to transform *Escherichia coli* HB101 in L broth containing ampicillin (20 μg/ml).

Plasmid preparation and expression were performed using M9-casamino acid medium containing ampicillin at 20 μg/ml. The DNA sequence was determined by the dideoxynucleoside triphosphate method (20).

E. coli cells were grown in 100 ml of the above media for 1–1.5 hr. The gene expression was induced by the addition of 3-indoleacrylic acid (40 μg/ml) when the OD_{660} = 0.01–0.02. Incubation was continued for 7 hr, at which time the cells were collected and washed with 10 mM Tris·HCl, 1 mM EDTA, and 0.1 M NaCl (pH 8.0).

Abbreviation: hGH, human growth hormone.

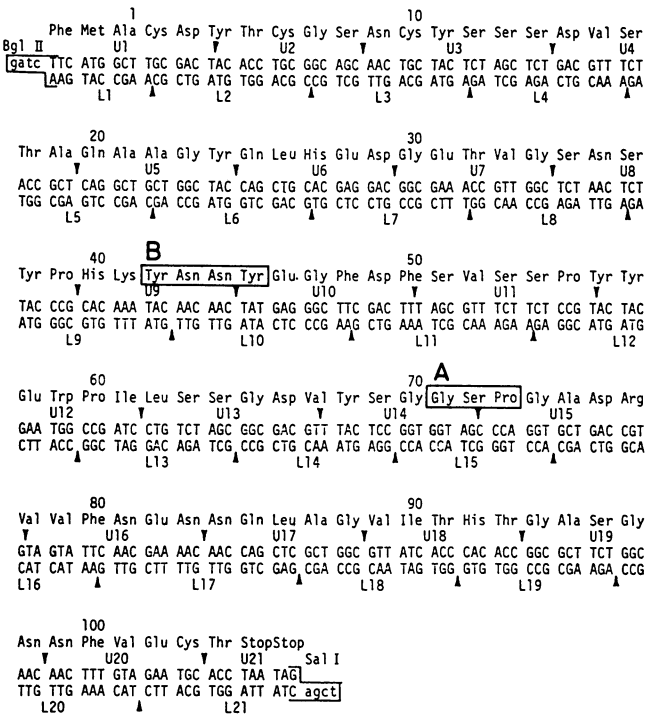

FIG. 1. The amino acid sequence of RNase T1 and its nucleic acid sequence. Ligation points are marked with arrowheads. Oligonucleotides U14, U15, and L15 were used to code Gly-Ser-Pro as indicated by box A. U9, U10, L9, and L10 were replaced to mutate Tyr-Asn-Asn-Tyr indicated by box B. The following codons for italicized amino acids were used; *Phe*-Asn-Asn-Tyr, UUC; Tyr-Asn-Asn-*Phe*, UUU; Tyr-*His-Asp*-Tyr, CACGAC; Tyr-*Arg*-Asn-Tyr, CGC; Tyr-*Arg-Ala*-Tyr, CGCGCU; Tyr-*Ala*-Asn-Tyr, GCA; Tyr-Asn-*Ala*-Tyr, GCU.

Estimation of Amounts of Induced Protein. Aliquots of *E. coli* cells [*ca.* 1 mg (wet weight)] were treated with lysozyme (0.1 mg) in 0.1 ml of 50 mM Tris·HCl (pH 8.0), 50 mM EDTA, 15% sucrose, and 0.025% $NaDodSO_4$ at 0°C for 30 min. The mixture was then digested by 0.02 ml of DNase I (0.1 mg/ml) in 150 mM Tris·HCl (pH 7.5), 280 mM $MgCl_2$, and 4 mM $CaCl_2$ at 0°C for 30 min and then diluted with 1 ml of 7 M guanidine·HCl in a dilution buffer (21) [50 mM Tris·HCl (pH 7.5), 0.1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, and contained bovine serum albumin at 100 μg/ml, and 20% (vol/vol) glycerol]. The mixture was kept at room temperature for 20 min and then diluted 1:50 with the above dilution buffer. The induced protein was subjected to a radioimmunoassay for hGH with a Phadebas hGH PRIST kit (22). The amino-terminal two-thirds of the hGH protein alone (hGH-AB), as well as the fusion hGH-AB protein, were immunologically recognized by the antibody at 5% efficiency relative to the intact hormone.

Protein analysis was conducted using $NaDodSO_4$/12.5% PAGE (23).

Cleavage with Cyanogen Bromide. Cells [250 mg (wet weight)] were lysed by using lysozyme (5 mg) and DNase (0.1 mg) under the conditions described above. The protein content of the lysate was assayed by the method of Lowry, and the solution was lyophilized. The residue was treated with a solution of cyanogen bromide (10 mg per 3 mg of protein per ml) in 70% HCOOH and 10 mM 2-mercaptoethanol at 4°C for 20 hr, and then dialyzed against 50 mM sodium bicarbonate (five changes of 5 liters each). The extent of cleavage was confirmed by $NaDodSO_4$/12.5% PAGE.

Assay for RNase T1 Activity. 5'-[^{32}P]pGpC (*ca.* 200 cpm/pmol) was used as a substrate, and RNase T1 content was measured by radioimmunoassay before treatment of an extract with cyanogen bromide.

RNase T1 activity was assayed by initial measurements of the rate of appearance of 5'-[^{32}P]pGp from the radioactive substrate 5'-[^{32}P]pGpC (200 cpm/pmol) using a standard homochromatogram procedure (24, 25).

RESULTS

Synthesis and Expression of the Coding Sequence for RNase T1 Containing the Wild-Type Gly-Ser-Pro Triad. A gene for RNase T1 having the revised, correct tripeptidic sequence was constructed using synthetic oligodeoxyribonucleotides that had codons preferred by the highly expressed genes of *E. coli*. The three incorrect fragments U14, U15, and L15, which were previously synthesized in accordance with the reported incorrect tripeptide sequence, were replaced to yield Gly-Ser-Pro, residues 71–73 (Fig. 1). Oligonucleotides for the correct U14, U15, and L15, d(TTACTCCGGTGGTAG), d(CCCAGGTGCTGACCGTG), and d(ACCTGGGCTACCACC) were synthesized by the solid-phase phosphotriester method using dinucleotide units (1). Since RNase T1 does not

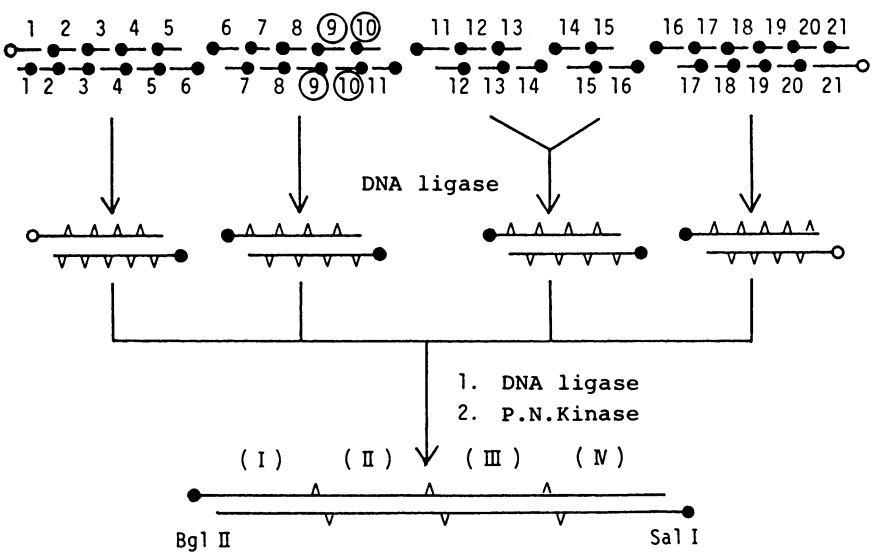

FIG. 2. Ligation of the chemically synthesized oligonucleotides to construct the RNase T1 gene. Dots indicate 5' phosphates and open circles indicate free 5'-hydroxy groups. Oligonucleotides 9 and 10 (circled) were replaced for mutation of Tyr-Asn-Asn-Tyr, residues 42–45.

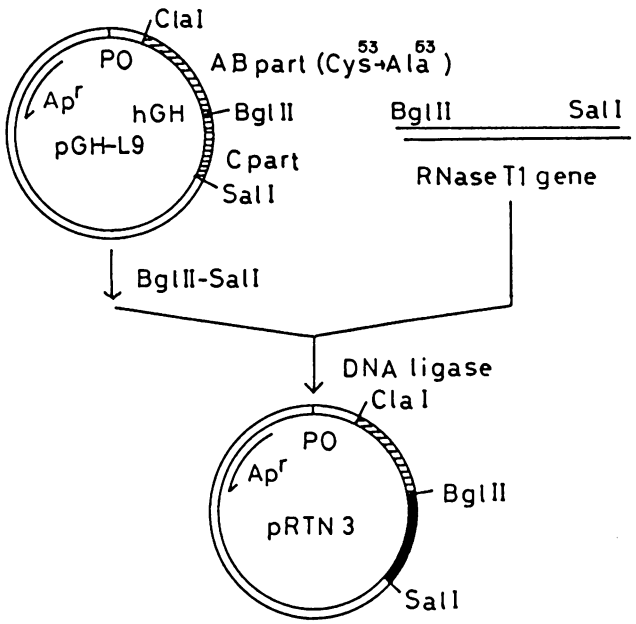

FIG. 3. Construction of fusion genes for RNase T1 and its mutants. Fusion proteins were cleaved with cyanogen bromide to generate RNase T1 and mutants.

contain methionine, it can be expressed as a fusion protein and then liberated by cleavage of the amino-terminal methionine with cyanogen bromide (26, 27). We have found that a synthetic gene for hGH could be expressed efficiently, in a stable form under the control of the *E. coli trp* promoter. Direct expression of the RNase T1 gene using the *E. coli trp* promoter was not satisfactory (data not shown); therefore, the gene was modified to fuse it with a segment of the hGH gene. A restriction site *Bgl* II was incorporated upstream of the amino-terminal methionine to ligate the RNase T1 gene to a *Bgl* II site located at two-thirds of the distance from the 5′-coding sequence and of the hGH gene in the expression plasmid pGHL-9 (1). After joining the synthetic fragments by T4 ligase as shown in Fig. 2, the gene was ligated to the *Bgl* II–*Sal* I site to yield the plasmid pRTN3 (Fig. 3). The pRTN3 plasmids were expressed by induction with indoleacrylic acid (28). The fact that the amino-terminal two-thirds of hGH (the AB part) was found to be recognized by anti-hGH antibodies with 5% of the efficiency of the total hGH enabled us to estimate the amount of expressed protein by radioimmunoassay (22). Cell lysates were treated with cyanogen bromide and then assayed for RNase T1 activity. The results are

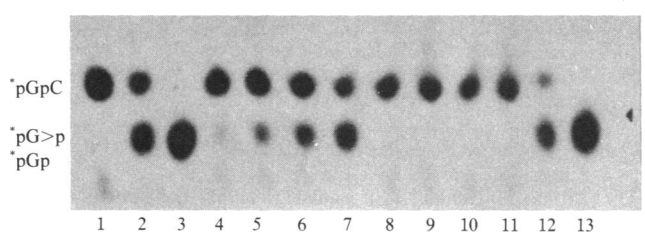

FIG. 4. Homochromatography of products in the reaction of [5′-^{32}P]pGpC (*pGpC) with RNase T1 and its mutant. Lane 1, *pGpC; lanes 2 and 3, natural RNase T1 (7.8 pg and 78 pg); lanes 4 and 5, fused protein hGHAB-RNase T1 (30 pg and 90 pg as RNase T1); lanes 6 and 7, CNBr-treated hGH-AB-RNase T1 (30 pg and 90 pg as RNase T1); lanes 8 and 9, fused protein hGHAB-RNase T1 (770 pg and 2310 pg as RNase T1); lanes 10 and 11, CNBr-treated hGHAB-RNase T1 (770 pg and 2310 pg as RNase T1); lanes 12 and 13, natural RNase T1 (the same amount in lanes 2 and 3) in the presence of the whole HB 101 lysate. The reaction was incubated at 37°C for 30 min in 10 μl of 50 mM Tris·HCl (pH 7.5), 1 mM EDTA, and 4 μM *pGpC.

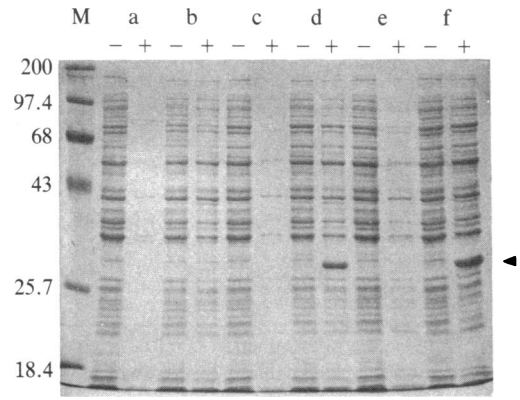

FIG. 5. NaDodSO$_4$/12.5% PAGE analysis of expressed proteins from *E. coli* clones containing genes for RNase T1 and mutants in the presence (+) or absence (−) of 3-indoleacrylic acid. Lane M, size markers; lanes a, YNNY; lanes b, FNNY; lanes c, YNNF; lanes d, YHDY; lanes e, YRNY; lanes f, YRAY. Position of the fusion proteins is indicated by an arrowhead.

shown in Fig. 4. The fused product expressed from the gene in pRTN3, containing the revised and correct tripeptidic sequence Gly-Ser-Pro, showed RNase T1 activity after treatment with cyanogen bromide. Even without cyanogen bromide treatment, the fusion product cleaved pGpC to a limited extent. In contrast, no activity was detected with the fusion product obtained from the pRT3 plasmid, which encoded the erroneous tripeptidic sequence Pro-Gly-Ser.

Synthesis of Mutant RNase T1 at the Recognition Site for GMP. Seven mutants of RNase T1 at tetrapeptidic recognition site for guanylic acid, Tyr-Asn-Asn-Tyr (YNNY; residues 42–45) were obtained by the expression of genes that were prepared by replacement of U9, U10, L9, and L10 with oligodeoxyribonucleotides having appropriate amino acid codons. Total proteins from cells were analyzed by gel electrophoresis as shown in Fig. 5. Fused products containing hGH-AB and the mutant RNase T1 were detected as a protein of 2.7 kDa. The amount of fused RNase T1 was quantified by radioimmunoassay. Understandably, genes that encoded mutant RNase T1s with high activities were found to be expressed rather ineffectively.

RNase T1 Activity of Mutant Enzymes. Initial velocities of various RNase T1 mutants were compared with the wild-type RNase T1 with the sequence Gly-Ser-Pro (residues 71–73). The results are listed in Table 1. Four mutants having altered tyrosine-42, asparagine-43, and tyrosine-45 (FNNY, YNNF, YRNY, and YANY) demonstrated fairly high activities. Mutants involving substitutions at position 44 (YHDY, YRAY, and YNAY) were characterized by low activities. Kinetic constants for these mutants are shown in Table 2.

Table 1. Initial velocity of pGpC cleavage by RNase T1 and its mutants

Substituted sequence, positions 42–45	V_0, pmol per min per μl	% RNase T1 activity
YNNY	3.5 ± 0.4*	100
FNNY	2.2 ± 0.3*	63
YNNF	2.7 ± 0.3*	77
YHDY	0.17 ± 0.08*	5
YRNY	1.8 ± 0.1*	51
YRAY	0.09 ± 0.06*	3
YANY	2.4 ± 0.3*	69
YNAY	0.35†	1.3
YNNY	0.006‡	0.17

*Reaction mixture: substrate, 150 μM; enzyme, 0.5 ng/μl (45.1 nM).
†Reaction mixture: substrate, 150 μM; enzyme, 2.5 ng/μl (225 nM).
‡Reaction mixture: substrate, 100 μM; enzyme, 0.5 ng/μl.

Table 2. Kinetic constants for RNase T1 and its mutants

Substituted sequence, positions 42–45	K_m, μM	k_{cat}, min^{-1}	k_{cat}/K_m, min$^{-1}\cdot\mu$M^{-1}
YNNY	350 ± 10	790 ± 60	2.3 ± 0.2
FNNY	200 ± 40	320 ± 100	1.6 ± 0.2
YNNF	300 ± 80	710 ± 220	2.4 ± 0.1
YHDY	1020 ± 200	190 ± 70	0.19 ± 0.04
YRNY	200 ± 80	160 ± 70	0.80 ± 0.06
YRAY	1150 ± 200	75 ± 17	0.065 ± 0.003
YANY	180 ± 50	230 ± 70	1.3 ± 0.1
YNAY	300 ± 40	25 ± 3	0.084 ± 0.001

K_m and k_{cat} values were determined from initial rate measurements for hydrolysis of ^{32}P-labeled pGpC over a substrate concentration range of 0.1–1 mM.

These results indicated that replacement of either one of the two tyrosine residues at position 42 or 45 by phenylalanine did not affect K_m values, but a substitution of tyrosine-42 with phenylalanine produced a smaller k_{cat} value. A similar result was obtained when asparagine-43 was substituted with arginine. Substitution of asparagine-44 produced large effects on the kinetic constants as seen in YRAY and YHDY.

DISCUSSION

A gene for RNase T1 was synthesized by assembling 42 chemically synthesized oligodeoxyribonucleotides and expressing the product as a fusion protein in *E. coli*. The expression efficiency of the present gene coding for the active enzyme with Gly-Ser-Pro, residues 71–73, was lower than a previous one coding for the inactive enzyme having Pro-Gly-Ser (12). This may be explained by the fact that the correct enzyme even as the fusion protein demonstrated a recognizable RNase T1 activity. Even the slight nucleolytic activity could be toxic to *E. coli* cells.

Amino acid substitutions at positions 71–73 resulted in the greatly reduced activity, although the region is known to be involved neither in substrate binding nor in catalytic activity as suggested by studies of chemical modifications and x-ray crystallography. Substitutions at positions 71–73 may, therefore, cause a change in the overall protein conformation. The presence of a proline residue in this region is suggestive of the above (29).

The crystallographic study suggested that the tetrapeptidic position Tyr-Asn-Asn-Tyr (YNNY, positions 42–45) may be involved in recognition of the guanine base (5, 6). Therefore, the sequence of this area has been changed to FNNY, YNNF, YRNY, YRAY, YHDY, YANY, and YNAY. Enzyme activities of these mutants were measured using pGpC as substrate. Mutant enzymes having phenylalanine either at position 42 or 45 maintained nearly the same activity as the wild type, thus indicating that tyrosine residue can be substituted by phenylalanine with no obvious alteration in the substrate recognition. Nevertheless, tyrosine-42 may play a more important role than tyrosine-45 due to its contribution in forming the hydrophobic cavity, whereas tyrosine-45 is located on the surface of the enzyme. Substitutions of asparagine at positions 43 and 44 caused a decrease in the enzyme activity; of the two, however, asparagine-44 appeared more critical than asparagine-43. Effects of substitutions at position 43 and 44 may be explained by interactions illustrated in Fig. 6. Asparagine-43 may be interacting only weakly with N-7 of the guanine residue and, therefore, can be replaced by alanine or arginine. On the other hand, the side chain of asparagine-44 is involved in binding to a water molecule in the loop as well as the backbone nitrogens of glycine-47 and phenylalanine-48. One mutant which had histidine-43 but still maintained aspartic acid-44 gave 5% of the wild-type activity. Incidentally the tetrapeptidic sequence YHDY has been found in RNase Ms (16), which has an affinity toward adenosine as well as guanosine. Investigations of the affinity of the mutant YHDY to oligonucleotides containing adenylic acid may be of interest.

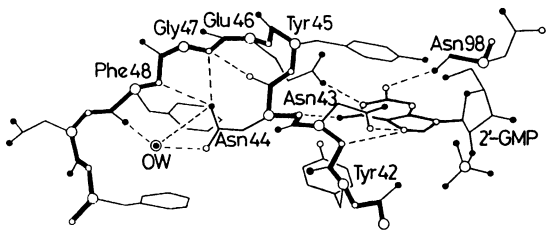

FIG. 6. Three-dimensional structure of the guanosine 2'-phosphate recognition site of RNase T1 traced from x-ray analysis data. The bold black lines indicate main chains, and thin black lines indicate side chains. Broken lines indicate hydrogen bonds.

The present method of obtaining site-specific amino acid-substituted enzymes involves the synthesis of genes by successive ligation of DNA fragments containing appropriate codons. This technique should prove to be a powerful tool for the analysis of structure function relationships of all proteins.

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