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Effect of Mutagenic Replacement of the Carboxyl Terminal Amino Acid, Val124, on the Properties and Regeneration of Bovine Pancreatic Ribonuclease A

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An important role of C-terminal amino acid residues of bovine pancreatic ribonuclease A (RNase A) in the formation of the three-dimensional structure was previously implied. In this study, we replaced the C-terminal amino acid, Val124, with amino acid residues with different properties by site-directed mutagenesis. The recombinant mutant enzymes were purified and subjected to a refolding study after being converted to a fully reduced and denatured state. There was a significant difference among the mutant enzymes in the rate of recovery of the activity when air oxidation was performed: the rate decreased in the order of V124E, V124L, V124G, V124K, V124A, and V124W. On the other hand, the recovery rates for all the mutant RNase A in the presence of GSH and GSSG were almost the same. The recovered activity of V124E after 24 h incubation reached approximately 90% of that of the wild type enzyme, followed by V124L 80%, V124A and V124W 65%, and V124K and V124G 50%. The duration of the initial lag phase became shorter in the order of V124W, V124A, V124K or V124G, V124E, or V124L. The results imply that the C-terminal amino acid significantly influences the formation of correct disulfide bonds during the refolding process and that the hydrophobic interaction of Val124 is important for efficient packing of the RNase A molecule.

Key words: carboxyl terminus, refolding, RNase A.

Bovine pancreatic ribonuclease A (RNase A) [EC 3.1.27.5] is composed of 124 amino acid residues with a molecular weight of 13,683, and contains four disulfide bonds at positions 26–84, 40–95, 58–110, and 65–72. Fully reduced and enzymatically inactive RNase A prepared with 8 M urea or 6 M guanidine hydrochloride in the presence of a reducing agent such as 2-mercaptoethanol can regenerate a soluble protein with specific activity, and secondary and tertiary structures identical to those of the native enzyme after exposure to air oxidation (*1, 2*).

Taniuchi (*3*) found an interesting phenomenon: When RNase(1–120), with four amino acid residues deleted from its C-terminal by limited proteolysis, was fully reduced and reoxidized, the same number of disulfide bonds was formed as for the reduced native RNase A, but no enzymatic activity was recovered. This was due to the formation of wrongly paired disulfide bonds. Taniuchi's results suggest to us that refolding information is concentrated within the C-terminal tetrapeptide.

The importance of the C-terminal region of RNase A for the structure and function has also been shown by means of refolding experiments in which C-terminal–deleted enzymes regained activity in the presence of C-terminal peptide fragments (*4–6*). These results suggest that an interaction between the C-terminal region and another part of RNase A is required for construction of the active structure.

Four amino acid residues of the C-terminus comprise a β strand and are located on the surface of the enzyme, where the role of hydrophobic C-terminal amino acid Val124 is puzzling. This paper deals the enzymatic and refolding properties of mutant enzymes in which Val124 is replaced with alanine, tryptophan, lysine, glycine, glutamic acid, or leucine residue.

MATERIALS AND METHODS

Materials—Plasmid pETRN coding the RNase A gene was constructed according to the method of delCardayre *et al.* (*7*). An *Escherichia coli* strain, *Epicurian Coli XL-1Blue*, was used as host cells for mutagenesis. An *E. coli* strain, BL21(DE3), was used for overexpression of mutant RNase A. Commercial RNase A (Type III-A; Sigma, Missouri, USA) was used as the wild type enzyme. C>p was purchased from Seikagaku Kogyo (Tokyo). All other reagents were of analytical grade and purchased from Nacalai Tesque (Kyoto).

Site-Directed Mutagenesis—Mutant plasmids were constructed with a Quick Change Site-Directed Mutagenesis Kit (Stratagene, California, USA) using a double-stranded plasmid, pETRN, and two kinds of oligonucleotide primers (*8*). The sequences of the oligonucleotide primers designed to replace the valine codon with an alanine codon were 5′-CAC-TTT-GAC-GCC-AGT-GCC-TAA-CTA-GAT-AAG-CTT-GCG-3′ and 5′-CGC-AAG-CTT-ATC-TAG-TTA-GGC-ACT-

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Abbreviations: CD, circular dichroism; C>p, cytidine 2′,3′-cyclic monophosphate, C-terminal, carboxyl terminal; GSH, reduced glutathione, GSSG, oxidized glutathione; IPTG, isopropyl-1-thio-β-galactoside; MES, 4-morpholinoethanesulfonic acid

GGC-GTC-AAA-GTG-3′. The sequences of the oligonucleotide primers designed to replace the valine codon with a tryptophan codon were 5′-CAC-TTT-GAC-GCC-AGT-TGG-TAA-CTA-GAT-AAG-CTT-GCG-3′ and 5′-CGC-AAG-CTT-ATC-TAG-TTA-CCA-ACT-GGC-GTC-AAA-GTG-3′. The sequences of the oligonucleotide primers designed to replace the valine codon with a lysine codon were 5′-CAC-TTT-GAC-GCC-AGT-AAG-TAA-CTA-GAT-AAG-CTT-GCG-3′ and 5′-CGC-AAG-CTT-ATC-TAG-TTA-CTT-ACT-GGC-GTC-AAA-GTG-3′. The sequences of the oligonucleotide primers designed to replace the valine codon with a glycine codon were 5′-T-GAC-GCC-AGT-GGC-TAA-CTA-GAT-AAG-C-3′ and 5′-G-CTT-ATC-TAG-TTA-GCC-ACT-GGC-GTC-A-3′. The sequences of the oligonucleotide primers designed to replace the valine codon with a glutamic acid codon were 5′-CAC-TTT-GAC-GCC-AGT-GAG-TAA-CTA-GAT-AAG-3′ and 5′-CTT-ATC-TAG-TTA-CTC-ACT-GGC-GTC-AAA-GTG-3′. The sequences of the oligonucleotide primers designed to replace the valine codon with a leucine codon were 5′-CAC-TTT-GAC-GCC-AGT-CTC-TAA-CTA-GAT-AAG-3′ and 5′-CTT-ATC-TAG-TTA-GAG-ACT-GGC-GTC-AAA-GTG-3′. All the mutations introduced into pETRN were confirmed by DNA sequencing with an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, California, USA) using a Bigdye terminator sequencing kit (Perkin Elmer).

Expression and Purification of Mutant RNase A—Mutant RNase A was expressed by the method of Dodge and Scheraga (*9*) with the following minor modifications. Mutated plasmids were transformed into BL21(DE3). A five milliliter culture of BL21(DE3) harboring mutated pETRN after 16 h incubation was added to 500 ml LB medium with 25 μg/ml ampicillin and then the culture medium was incubated with shaking at 37°C. When the optical density at 570 nm reached 0.8, protein expression was induced by the addition of 1 ml of 10 mM IPTG. After 3 h incubation, the cells were collected by centrifugation and then suspended in 20 ml of 100 mM NaCl. The cells were sonicated for 5 min. The lysed cells were centrifuged and suspended in 5 ml of 4 M guanidine thiocyanate, 2 mM EDTA, 100 mM Tris-HCl, and 80 mM GSH, pH 8.0. The suspension was stirred for 2 h to solubilize the RNase A. Insoluble cell debris was removed by centrifugation, and the soluble portion was collected and diluted with 200 ml of 100 mM Tris-HCl containing 2 mM EDTA, pH 8.0. For the formation of four disulfide bonds, the solution of reduced RNase A was mixed with 62 mg of GSSG and then stirred for 72 h at 4°C. The mixture was concentrated using an ultrafiltration cell with a YM3 membrane (Amicon) and then adjusted to pH 6.0 with 0.1 N acetic acid. For purification, the concentrated solution was loaded onto a Mono S HR 5/5 cation-exchange column (Pharmacia Biotechnology, 7 × 54 mm) equilibrated with 25 mM sodium phosphate buffer, pH 6.5, and then the protein was eluted with a linear gradient of 0 to 0.2 M NaCl. Fractions exhibiting the highest specific activity were collected and then the phosphate buffer was exchanged for distilled water. The purified enzyme was concentrated and kept at 4°C.

Determination of Kinetic Parameters—The hydrolytic reaction for C>p was measured spectrophotometrically (*10*) in 0.2 M sodium acetate buffer, pH 5.5, at 25°C by recording the increase in absorbance at 296 nm. The extinction coefficient was taken as 516.4 M^{-1} cm^{-1}. The substrate concentration was in a range of 0.16 to 1.9 mM.

Circular Dichroism Spectroscopy—CD spectra, from 190 to 250 nm, were recorded at room temperature with a Jasco J-720W spectropolarimeter in 10 mM MES buffer, pH 6.0, in a cell of 0.1 cm optical path length. The concentrations of the wild type and mutant RNase A were 6.5 and 5 μM, respectively.

Thermal Denaturation—Changes in the [θ] value at 222 nm were recorded as a function of temperature. Temperature was continuously increased by 0.5°C per minute from 25 to 75°C in a jacketed cell of 1 cm optical path length. Temperature was monitored with a thermometer placed inside the cell compartment. The enzyme concentration was adjusted to 5.0 μM with 10 mM MES buffer, pH 6.0, containing 100 mM KCl.

Regeneration of the Denatured and Reduced Enzymes—RNase A was dissolved in 100 mM Tris-HCl, pH 8.0, containing 2 mM EDTA and 8 M urea, and then mixed with 10 μl of 2-mercaptoethanol. The solution was bubbled with nitrogen gas for 5 min, covered with a small sheet of parafilm, and then incubated at 25°C overnight. To isolate the denatured and reduced RNase A, the reaction mixture was loaded onto a Sephadex G-25 column (15 × 50 mm) equilibrated with 0.1 M acetic acid and then the protein was eluted with 0.1 M acetic acid at the flow rate of 2 ml per min. Fractions containing protein were collected. The protein solution was mixed with a 1/10 volume of 1 M Tris-HCl, pH 10.0, and then adjusted to pH 8.2 to start the refolding process. The solution was incubated at 25°C.

To use GSH and GSSG for regeneration, denatured and reduced RNase A prepared as above was mixed with a 1/10 volume of 1 M Tris-HCl, pH 10.0, containing 2 mM EDTA and then adjusted to pH 8.2. The solution was mixed with 2 mM GSH and 0.2 mM GSSG, and then incubated at 25°C. Regeneration of RNase A activity was monitored by measuring the activity toward C>p.

Protein Concentrations—At each purification step, protein concentrations were determined by the bicinchoninic acid method (*11*). For other experiments, the protein concentrations of unfolded and folded RNase A were determined using extinction coefficients of 8,500 M^{-1} cm^{-1} at 275 nm (*12*), and 9,800 M^{-1} cm^{-1} at 277.5 nm (*13*), respectively.

Sulfhydryl Group Determination—Sulfhydryl groups in reduced RNase A were titrated with Ellman's reagent [0.01 M 5,5′-dithiobis-(2-nitrobenzoic acid) in 0.05 M sodium phosphate buffer, pH 7.0] (*14*). An aliquot of 150 μl removed from the RNase A reoxidation solution was added to 450 μl of 0.1 M Tris-HCl, pH 8.0, containing 8 M guanidine hydrochloride and 10 mM EDTA. To the mixture, 25 μl of Ellman's reagent was added, and then the reaction mixture was incubated at 25°C for 10 min. The concentration of SH groups was determined using an extinction coefficient of 13,380 M^{-1} cm^{-1} at 412 nm (*15*).

RESULTS

Expression and Purification of V124 Mutant RNase A—Three to 10 mg of a mutant RNase A was obtained from approximately 6 g of *E. coli* cells (wet weight) collected from 1 liter culture. The purified mutant RNase A gave a single-stained band on SDS-PAGE (Fig. 1) and was used for further experiments.

Properties of V124 Mutant RNase A—Kinetic parameters of the wild type and mutant RNase A for the cleavage of

C>p were determined by means of Hanes-Woolf plots (Table I). K_m of the mutant RNase A was slightly higher than that of the wild type enzyme, while its k_{cat} was almost the same as that of the wild type enzyme; thus k_{cat}/K_m of mutant RNase A was slightly smaller than that of the wild type enzyme. Hydrolytic activity toward C>p of the mutant enzymes is almost the same as that of the wild type enzyme.

The wild type and mutant enzymes showed indistinguishable CD spectra (Fig. 2). The thermal denaturation profiles monitored as to changes in the [θ] value at 222 nm were almost identical for the wild type and mutant RNase A (Fig. 3). These profiles showed that thermal denaturation of all the mutant enzymes followed a two-state transition. The *T*m values obtained were nearly the same, whereas the V124K and G mutant RNase A exhibited a little lower values than the wild type enzyme.

Activity Regeneration with Air Oxidation or Glutathione—Activity regeneration by air oxidation in the absence of glutathione was compared for the fully denatured and reduced forms of the wild type and mutant RNase A (Fig. 4). The rate of recovery of the activity at an early period of refolding decreased in the order of V124E or V124L, V124G, V124K, V124A, and V124W. The fully recovered activity of V124E reached approximately 90% of that of the wild type enzyme, whereas those of other mutant enzymes were as follows: V124L 80%, V124A and V124W 65%, and V124K and V124G 50%. The duration of the initial lag phase of the regeneration became shorter in the order of V124W, V124A, V124K, or V124G, V124E or V124L. When free sulfhydryl groups were titrated during the refolding process, the sulfhydryl group number of the mutant enzymes decreased at the same rate as for the wild type enzyme, and no lag phase was observed for any mutant enzymes (data not shown).

The recovery of the activity in the presence of glutathiones for the fully denatured and reduced wild type and mutant RNase A is shown in Fig. 5. The most effective concentrations of glutathiones were 2 mM GSH and 0.2 mM GSSG for the refolding of RNase A (*16*). The recovery of the activity of both the wild type and mutant RNase A significantly increased when glutathione was present. The rates of recovery of activity of the mutant enzymes were almost the same as that of the wild type enzyme, and the recovered activity of the mutant enzymes reached the same level as in the case of the wild type enzyme except for the little lower yields of the V124G and V124K mutants.

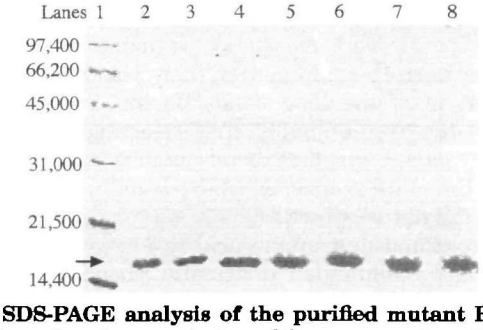

Fig. 1 **SDS-PAGE analysis of the purified mutant RNase A.** The polyacrylamide concentration of the running gel was 17% Lane 1, molecular weight standards (phosphorylase *b*, 97,400, serum albumin, 66,200, ovalbumin, 45,000, carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400); lane 2, wild type RNase A; lane 3 V124A; lane 4, V124W; lane 5, V124K; lane 6, V124G; lane 7, V124E; and lane 8, V124L

TABLE I. **Kinetic parameters of the wild type and mutant RNase A for the cleavage of C>p.**

	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (mM^{-1} min^{-1})
Wild type	0 46 ± 0 1	140 ± 10	300 ± 20
V124A	0 71 ± 0 1	180 ± 40	240 ± 20
V124W	0 63 ± 0 1	160 ± 60	250 ± 40
V124K	0 54 ± 0.1	130 ± 10	240 ± 20
V124G	0.62 ± 0.1	130 ± 10	220 ± 20
V124E	0.59 ± 0 1	140 ± 10	250 ± 20
V124L	0 57 ± 0 1	170 ± 10	300 ± 20

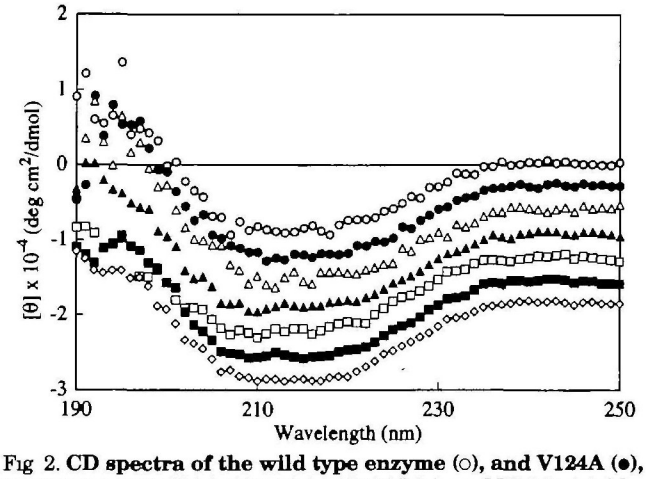

Fig 2. **CD spectra of the wild type enzyme (○), and V124A (●), V124W (△), V124K (▲), V124G (□), V124E (■), and V124L (◇).** The spectra of the mutant RNase A were obtained by subtracting the following [θ] values from each ordinate 3,000 for V124A, 6,000 for V124W, 9,000 for V124K, 12,000 for V124G, 15,000 for V124E, and 18,000 for V124L

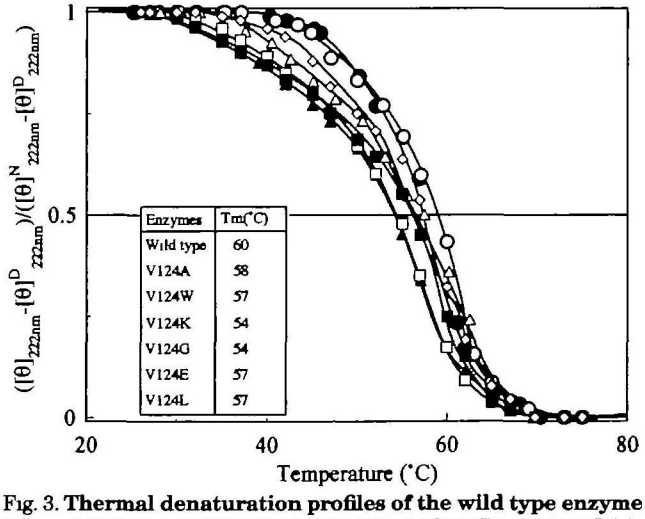

Fig. 3. **Thermal denaturation profiles of the wild type enzyme (○), and V124A (●), V124W (△), V124K (▲), V124G (□), V124E (■), and V124L (◇).**

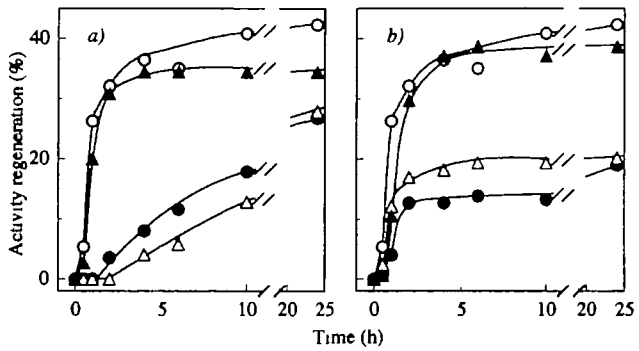

Fig 4. **Recovery of the activity on air oxidation in the absence of a glutathione.** Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. Hydrolytic activity toward C>p was measured at various times. The theoretical activity was taken as 100%. (a) Wild type enzyme (○), V124A (●), V124W (△), and V124L (▲). (b) Wild type enzyme (○), V124K (●), V124G (△), and V124E (▲).

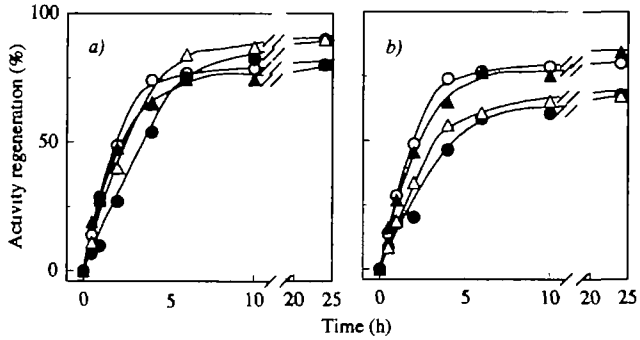

Fig. 5 **Recovery of the activity in the presence of GSH and GSSG.** Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. (a) Wild type enzyme (○), V124A (●), V124W (△), and V124L (▲) (b) Wild type enzyme (○), V124K (●), V124G (△), and V124E (▲) See Fig 4 for other conditions.

DISCUSSION

C-terminal Val124 in RNase A is conserved among mammalian pancreatic RNases. Replacement of this residue with alanine, tryptophan, lysine, glycine, glutamic acid, or leucine produced an active enzyme that is indistinguishable from the wild type enzyme, as judged from the activity and structural properties; almost the same kinetic parameters, CD spectra, T_m and two-state thermal denaturation profiles. These results suggest that the construction of the active center or domains of the mutant enzymes is not affected by mutation of Val124. Little influence of amino acid replacement on activity and structure is somewhat expected since the side chain of the C-terminal valine residue is located on the outside of the enzyme surface, far from the construction of the active site. There seems to be little influence on the spatial orientation of the side chain on amino acid replacement at Val124, since the α-carboxyl oxygen of Val124 is pretty much fixed with the α-carboxyl oxygen of His105 *via* a hydrogen bond (*17*).

However, the mutation of Val124 affected the recovery of activity for the fully denatured and reduced forms. The refolding rates for all mutant RNase A became slower than that of the wild type enzyme when air oxidation was performed. However, they became almost the same when GSH and GSSG were used as oxidizing agents. The probable effect of GSH and GSSG is the formation of correct disulfide bonds by exchanging disulfide bonds between free sulfhydryl groups or incorrectly paired disulfide bonds. In the absence of GSH and GSSG, Val124 mutant enzymes tend to give randomly formed disulfide bonds and, thus, incorrectly folded intermediate(s) may accumulate.

Val124 interacts with nearby hydrophobic residues, Ile106 and Ile107 (*17*). When Val124 is replaced with alanine or glycine, the hydrophobic interaction with isoleucine residues becomes weakened. The lack of this interaction may be a cause for the inability to form a compact structure around the C-terminal region.

Indeed, when a bulky tryptophan residue was introduced at the 124th position, the compact packing of V124W became difficult. Furthermore, the regeneration profile of V124L is similar to that of the wild type, since a leucine residue is comparable with a valine one in size and hydrophobicity. These results show that the size and hydrophobicity of the Val124 side chain must be an important element for the refolding process of RNase A.

There are two positively charged residues, Lys104 and His105, placed close to Val124 (*17*). It is most likely that the low regeneration rate obtained for V124K is due to the electric repulsion. In contrast, the glutamic acid mutant is the most favorable mutant because of its negative charge interacting with Lys104 and His105 during the regeneration reaction. This interaction contributes significantly to the formation of a compact structure of V124E.

For some mutant enzymes, there was a significant initial lag phase prior to the appearance of activity. It was suspected that the appearance of activity might be directly synchronized with the formation of disulfide bonds. When the disulfide bond formation rates of the wild type and mutant enzymes were measured, we found there was no relation between them. Moreover, there was an increase in the formation of disulfide bonds during the lag phase. Therefore, the results imply that rearrangement of the incorrectly paired disulfide bonds occurs during the lag phase for the mutant enzyme, which would perturb the formation of the native structure.

The C-terminal four amino acid residues are conserved among many mammalian pancreatic ribonucleases. They construct one of "chain folding initiation sites" (CFIS) (*18–20*), a native-like structure rapidly formed in an early stage of folding. The C-terminal region of RNase A has been shown to act as a CFIS in the folding pathway experimentally. The C-terminal 20 amino acid fragment of RNase A has a partially ordered structure that is stabilized through a hydrophobic interaction (*18*). It is quite likely that the C-terminal region of RNase A is one of the nuclei positions in the folding of the enzyme.

From our results, it is concluded that hydrophobic interaction of Val124 in RNase A is important for efficient packing of the RNase A molecule.

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