J. Biochem. 131, 193–200 (2002)

Significance of the Four Carboxyl Terminal Amino Acid Residues of Bovine Pancreatic Ribonuclease A for Structural Folding

Takahiro Fujii, Hiroshi Ueno,[1] and Rikimaru Hayashi[2]

Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Sakyo, Kyoto 606-8502

Received September 28, 2001; accepted November 23, 2001

The C-terminal amino acid residues of bovine pancreatic ribonuclease A (RNase A) form a core structure in the initial stage of the folding process that leads to the formation of the tertiary structure. In this paper, roles of the C-terminal four amino acids in the structure, function, and refolding were studied by use of recombinant mutant enzymes in which these residues were deleted or replaced. Purified mutant enzymes were analyzed for their secondary structure, thermal stability, and ability to regenerate from the denatured and reduced state. The C-terminal deleted mutant enzymes showed lower hydrolytic activity for C>p and nearly identical CD spectra compared with the wild-type enzyme. The rate of recovery of activity was significantly different among the C-terminal deleted mutant enzymes when air oxidation was employed in the absence of GSH and GSSG: the rates decreased in the order of des-124-, des-(123-124)-, and des-(122-124)-RNase A. It is noteworthy that the regeneration rates of mutant RNase A in the presence of GSH and GSSG were nearly the same. Des-(121-124)-RNase A failed to recover activity both in the presence and absence of glutathione, due to the mismatched formation of disulfide bonds. The mutant enzyme in which all of the C-terminal four amino acid residues were replaced by alanine residues showed lower hydrolytic activity and an indistinguishable CD spectrum compared with the wild-type enzyme, and also recovered its activity from the denatured and reduced state by air oxidation. The D121 mutant enzymes showed decreased hydrolytic activity and identical CD spectra compared with the wild type. The recovery rates of activity of D121A and D121K were determined to be lower than that of the wild-type enzyme, while the rate of recovery of D121E was comparable to that of the wild type. The C-terminal amino acids play a significant role in the formation of the correct disulfide bonds during the refolding process, and the interaction of amino acid residues and the existence of the main chain around the C-terminal region are both important for achieving the efficient packing of the RNase A molecule.

Key words: C-terminus, folding, RNase A, unfolding.

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 denatured and reduced RNase A, prepared in 8 M urea or 6 M guanidine hydrochloride in the presence of a reducing agent such as 2-mercaptoethanol, leads to the regeneration of a soluble protein after exposure to air oxidation. Refolded protein exhibits a specific activity and secondary and tertiary structures which are identical to those of the native enzyme (*1, 2*).

The findings reported by Taniuchi (*3*) are interesting in this respect. He prepared des-(121-124)-RNase A, in which four amino acid residues from its C-terminus were deleted by pepsin digestion. When des-(121-124)-RNase A was denatured and reduced, followed by air oxidation, four disulfide bonds were formed, but no enzymatic activity was recovered. He concluded that this was due to the formation of wrongly paired disulfide bonds and suggested that folding information was concentrated within the C-terminal tetrapeptide. Puett (*4, 5*) showed that des-(121-124)-RNase A with the correct disulfide bonds has the conformational free energy reduced by about 30% and became unstable, but reversibly unfolds/refolds to form a structure indistinguishable from the native RNase A. That is, des-(121-124)-RNase A also has the information needed for regenerating the native conformation if the correct disulfide bonds are retained. Both sets of experiments using des-(121-124)-RNase A lead to the conclusion that the native conformation of RNase A cannot be formed during biosynthesis until the polypeptide chain has been extended from the amino terminus beyond residue 120 and tertiary structures of proteins are stabilized by disulfide bonds.

The importance of the C-terminal region of RNase A in terms of structure and function has also been demon-

[1] Present address: Laboratory of Applied Microbiology, Department of Food Science and Nutrition, Faculty of Human Life and Environment, Nara Women's University, Nara 630-8506.

[2] To whom correspondence should be addressed. Tel: +81-75-753-6110, Fax: +81-75-753-6128, E-mail: hayashi@kais.kyoto-u.ac.jp

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; MALDI-TOF/MS, matrix assisted laser desorption ionization time of flight mass spectrometry; MES, 2-(*N*-morpholino)-ethanesulfonic acid.

strated by means of refolding experiments in which C-terminal–deleted enzymes regained activity in the presence of the corresponding C-terminal peptide fragments (*6–8*). These experiments suggest that an interaction between the C-terminal region and another part of RNase A is required for the formation of the active structure.

Our previous study (*9*) demonstrated the importance of the hydrophobic interaction of Val124 with nearby hydrophobic residues, Ile106 and Ile107, for achieving efficient packing. To investigate the role of the C-terminal region (Asp^{121}-Ala-Ser-Val^{124}), deletion mutant enzymes, des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A, and amino acid substitution mutant enzymes, D121A, D121E, and D121K RNase A were prepared and their refolding was examined. Ala(121-124) RNase A, in which all of the C-terminal four amino acid residues were replaced with alanines, was also prepared and its refolding was compared with that of the C-terminal deleted mutant enzymes.

MATERIALS AND METHODS

Materials—A plasmid pETRN carrying the RNase A gene was constructed according to the method of delCardayre *et al.* (*10*). An *Escherichia coli* strain, *Epicurian coli XL-1Blue*, was used as a host cell for mutagenesis. Another *E. coli* strain, BL21(DE3), was used for expression 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 for the construction of Asp121 mutant enzymes or pETRN-V124A coding mutant RNase A in which Val124 was replaced with alanine (*9*) for the construction of C-terminal deletion and Ala(121-124) mutant enzymes and two types of oligonucleotide primers (*11*). Mutant plasmid pETRN-V124A was used in order to optimize the GC content of the primers. All the oligonucleotide primers were synthesized by Japan Bio Service (Saitama). The sequences of the oligonucleotide primers designed to construct mutant enzymes are summarized in Table I. All mutations introduced into the plasmids 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 (*12*) with the following minor modifications. BL21(DE3) was transformed with mutated plasmids. After a 16-h incubation, a 5-ml culture of BL21(DE3) containing mutated pETRN was added to 500 ml of LB medium along with 25 μg/ml ampicillin, then the culture 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 a 3-h incubation, the cells were collected by centrifugation, suspended in 20 ml of 100 mM NaCl, and sonicated for 5 min. The resulting 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 at 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 at pH 8.0. For the formation of four disulfide bonds, 62 mg of GSSG was added to the solution of reduced RNase A, which was then stirred for 72 h at 4°C. The mixture was concentrated using an ultrafiltration cell with a YM3 membrane (Amicon), 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 the protein was eluted with a linear gradient of 0 to 0.2 M NaCl. Fractions exhibiting the highest specific activity were collected, and the phosphate buffer was exchanged for water. The purified enzyme was concentrated and kept at 4°C.

Determination of Kinetic Parameters—The hydrolytic reaction for C>p was measured spectrophotometrically (*13*) in 0.2 M sodium acetate buffer, pH 5.5, at 25°C by measurement of the increase in absorbance at 296 nm. The extinc-

TABLE I. **Sequences of oligonucleotides.**

Enzymes		Sequences
des-124	F:	5′-C-TTT-GAC-GCC-AGT-TGA-TAA-CTA-GAT-AAG-CTT-GCG-3′
	R:	5′-CGC-AAG-CTT-ATC-TAG-TTA-TCA-ACT-GGC-GTC-AAA-G-3′
des-(123-124)	F:	5′-CAC-TTT-GAC-GCC-TGA-GCC-TAA-CTA-GAT-AAG-3′
	R:	5′-CTT-ATC-TAG-TTA-GGC-TCA-GGC-GTC-AAA-GTG-3′
des-(122-124)	F:	5′-CT-GTC-CAC-TTT-GAC-TGA-AGT-GCC-TAA-CTA-G-3′
	R:	5′-C-TAG-TTA-GGC-ACT-TCA-GTC-AAA-GTG-GAC-AG-3′
des-(121-124)	F:	5′-CCT-GTC-CAC-TTT-TAA-GCC-AGT-GCC-TAA-C-3′
	R:	5′-G-TTA-GGC-ACT-GGC-TTA-AAA-GTG-GAC-AGG-3′
Ala(121-124)	F:	5′-GTC-CAC-TTT-GCC-GCC-GCC-GCC-TAA-CTA-G-3′
	R:	5′-C-TAG-TTA-GGC-GGC-GGC-GGC-AAA-GTG-GAC-3′
D121A	F:	5′-CCT-GTC-CAC-TTT-GCC-GCC-AGT-GTT-TAA-C-3′
	R:	5′-G-TTA-AAC-ACT-GGC-GGC-AAA-GTG-GAC-AGG-3′
D121E	F:	5′-CCT-GTC-CAC-TTT-GAG-GCC-AGT-GTT-TAA-C-3′
	R:	5′-G-TTA-AAC-ACT-GGC-CTC-AAA-GTG-GAC-AGG-3′
D121K	F:	5′-CCT-GTC-CAC-TTT-AAG-GCC-AGT-GTT-TAA-C-3′
	R:	5′-G-TTA-AAC-ACT-GGC-CTT-AAA-GTG-GAC-AGG-3′

F: forward primer, R: reverse primer.

tion coefficient was taken as 516.4 M^{-1} cm^{-1}. The substrate concentration was in the range of 0.16 and 1.9 mM. Kinetic parameters for the cleavage of C>p were determined by means of Hanes-Woolf plots.

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 in optical path length. The concentrations of the wild-type and mutant RNase A were 6.5 and 5 μM, respectively. The content of the secondary structures in the wild-type and mutant RNase A was estimated by the method of Chen *et al.* (*14*). Estimation of the secondary structure was confirmed by the method of Andrade *et al.* (*15*) to test the validity of our results.

Thermal Denaturation—Changes in [θ] value at 222 nm were recorded as a function of temperature. The temperature was continuously increased at rate of 0.5°C per minute from 25 to 75°C with a jacketed cell of 1 cm in optical path length. The values at 25 and at 75°C were taken as the ellipticity values of the native and unfolded state respectively. The temperature was monitored with a thermometer placed inside the cell. 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, then mixed with 10 μl of 2-mercaptoethanol. Nitrogen was bubbled through the solution for 5 min, then it was sealed with a small sheet of parafilm and 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 100 mM acetic acid, and 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, then the pH was adjusted to 8.2 to initiate the refolding process under normal conditions. The solution was incubated at 25°C.

In the case of regeneration in the presence of GSH and GSSG, denatured and reduced RNase A prepared as described 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 incubated at 25°C. Regeneration of RNase A activity was monitored by measuring the activity with respect to C>p.

Protein Concentrations—At each purification step, protein concentrations were determined by the bicinchoninic acid method (*16*). 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 (*17*) and 9,800 M^{-1} cm^{-1} at 277.5 nm (*18*), respectively.

Sulfhydryl Group Determination—Sulfhydryl groups in the reduced RNase A were titrated with Ellman's reagent [10 mM 5,5′-dithiobis-(2-nitrobenzoic acid) in 50 mM sodium phosphate buffer, pH 7.0] (*19*). An aliquot of 150 μl was removed from the reoxidation solution of RNase A and added to 450 μl of 0.1 M Tris-HCl, pH 8.0, containing 8 M guanidine hydrochloride and 10 mM EDTA. After addition of 25 μl of Ellman's reagent, 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 (*20*).

Mass Spectrometry Analysis—Mass spectra were measured with a Voyager RP Biospectrometry Workstation (PerSeptive Biosystems, Massachusetts, USA). Samples were mixed with the same quantity of matrix reagent, composed of α-cyano-4-hydroxy-cinnamic acid at 5 mg/ml and trifluoroacetic acid at a concentration of 0.05% in 50% acetonitrile. A 2-μl portion of the mixture was applied to a sample tray, dried at room temperature, and used for the analysis.

Cyanogen Bromide Cleavage—A 300-μl portion of the protein solution was mixed with 700 μl of formic acid. After additional of 100 μl of 10 mg/ml cyanogen bromide, the solution was incubated at 37°C overnight. The solution was evaporated to dryness with an Automated Environmental SpeedVac System AES1010 (Savant, New York, USA), and the residue was dissolved in 50 μl of distilled water. The resulting solution was then subjected to mass spectrometric analysis in order to investigate the fragmentation of RNase A. The yields of resultant peptides were calculated on the basis of peak areas derived from MALDI-TOF-MS.

Amino Acid Analysis—Protein was hydrolyzed in 6 M HCl at 110°C for 22 h. The solution was evaporated to dryness in a SpeedVac AES1010, and the residue was dissolved in 0.2 M sodium citrate, pH 2.2, for amino acid analysis. Amino acid analyses were performed with a JEOL JLC-500/V amino acid analyzer (JEOL, Tokyo).

RESULTS

Expression and Purification of Mutant RNase A—Yields of 2–8 mg of C-terminal deleted mutant enzymes [des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A], Ala(121-124) RNase A, and D121 mutant RNase A were obtained from approximately 6 g of wet *E. coli* cells collected from a 1 liter culture. All of the purified mutant RNase A samples gave a single-stained band on SDS-PAGE and had the expected amino acid composition confirmed by amino acid analysis (Table II).

Properties and Refolding of C-Terminal Deleted and Ala(121-124) Mutant RNase A—CD spectra of the wild type and C-terminal deleted [des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A] and Ala(121-124) mutant enzymes are shown in Fig. 1. The helix contents of the C-terminal deleted and Ala(121-124) mutant enzymes were comparable to that of the wild-type, but a decrease in sheet content was observed for des-(122-124)- and des-(121-124)-RNase A (Fig. 2). The thermal denaturation profiles monitored as changes in the [θ] value at 222 nm showed that all the mutant enzymes followed a two-state transition (Fig. 3). The T_m values of des-124-, des-(123-124)-, and des-(122-124)-RNase A were lower by 3–6°C than that of the wild type. In contrast, des-(121-124)-RNase A exhibited much lower value (14°C) than the wild-type enzyme. This result is consistent with the previous report (*21*). The determined T_m value of Ala(121-124) RNase A was comparable to that of the wild-type enzyme.

Kinetic parameters of the wild-type, C-terminal deleted and Ala(121-124) mutant RNase A for the cleavage of C>p are summarized in Table III. The K_m values of des-124-, des-(123-124)-, and Ala(121-124) RNase A were slightly higher and the k_{cat} of these mutant enzymes was slightly lower than that of the wild-type enzyme; thus the k_{cat}/K_m of

TABLE II. **Amino acid compositions of wild-type and mutant RNase A.**

	des-124-	des-(123-124)-	des-(122-124)-	des-(121-124)-	Ala(121-124)	D121A	D121E	D121K	Wild type
Asp	15.7	15.7	16.0	14.6	14.5	14.5	14.6	14.9	15.8
Thr	10.0	9.5	10.2	9.1	10.5	10.4	9.2	10.7	9.7
Ser	15.4	14.3	14.4	13.7	13.6	15.3	14.8	14.8	14.7
Glu	12.8	12.7	13.7	12.9	13.5	13.4	14.4	13.5	13.5
Pro	3.5	3.3	3.0	4.2	3.4	2.7	3.2	2.7	4.2
Gly	2.7	2.6	2.5	3.6	2.6	2.5	3.2	2.6	2.6
Ala	12.2	11.6	10.2	10.5	14.7	12.7	11.6	11.5	11.6
1/2Cys	2.5	2.4	1.8	2.3	2.7	2.5	1.5	2.4	2.2
Val	7.2	7.2	7.0	7.1	7.1	7.9	8.0	8.3	8.4
Met	3.6	3.3	3.0	3.3	3.2	3.3	3.2	3.3	3.5
Ile	1.7	1.5	1.4	1.9	1.3	1.3	1.5	1.4	2.0
Leu	1.8	1.7	1.6	2.4	1.8	1.7	2.1	1.8	2.1
Tyr	5.1	4.8	3.9	5.2	4.1	4.6	3.4	4.7	4.9
Phe	2.6	2.3	2.2	3.1	2.0	2.0	2.2	2.0	2.9
His	3.5	3.2	3.7	3.6	3.4	3.2	3.2	3.3	3.7
Lys	10.9	10.0	10.4	9.8	10.4	10.9	9.9	11.3	10.1
Arg	3.5	3.2	3.4	3.8	4.0	3.7	3.6	3.8	3.6

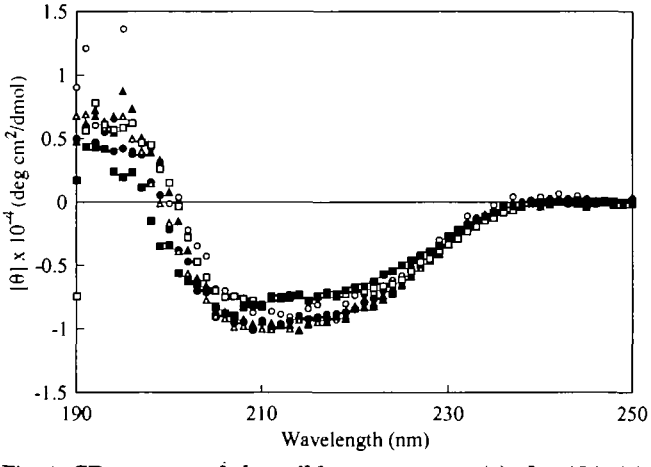

Fig. 1. **CD spectra of the wild-type enzyme** (○), **des-124-** (●), **des-(123-124)-** (△), **des-(122-124)-** (▲), **des-(121-124)-** (□), **and Ala(121-124) RNase A** (■).

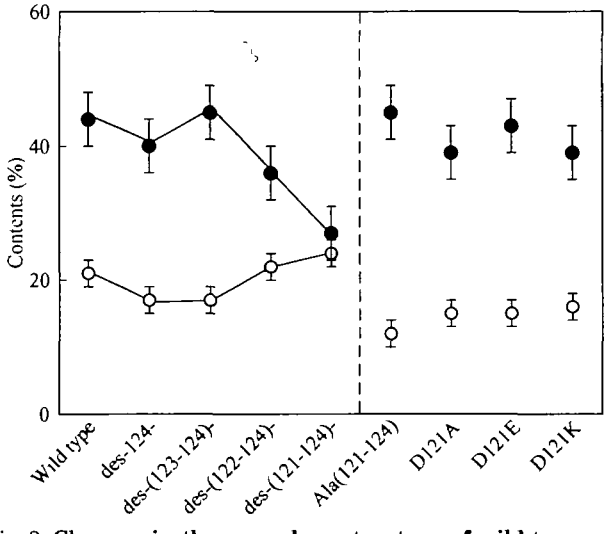

Fig. 2. **Changes in the secondary structure of wild-type and mutant RNase A.** The open circles represent the amounts of helix and the closed circles represent amounts of the sheet.

these mutants was somewhat smaller than that of the wild-type enzyme. The fact that des-124- and des-(123-124)-RNase A showed almost the same activity suggests that Ser123 has little effect on the formation of the active center. The K_m values of des-(122-124)- and des-(121-124)-RNase A were higher than that of the wild type, and the k_{cat} of these mutant enzymes was lower than that of the wild-type enzyme; thus the k_{cat}/K_m of these mutant RNase A was smaller than that of the wild-type enzyme. Ala122 and Asp121 are located near His119, the catalytic residue, and the deletion of these residues consequently affects the hydrolytic activity of RNase A.

Activity regeneration by air oxidation in the absence of glutathione was compared for the fully denatured and the reduced forms of the wild-type and mutant RNase A (Fig. 4). The rate of recovery of activity at an early period of folding decreased in the order of des-124-, des-(123-124)-, Ala-(121-124), des-(122-124)-, and des-(121-124)-RNase A. The fully recovered activity of des-124-RNase A was approximately 90% that of the wild-type enzyme, whereas the recovered activity of the other mutant enzymes were as follows: 80% for des-(123-124)-RNase A, 70% for des-(122-124)-RNase A, 0% for des-(121-124)-RNase A, and 90% for Ala(121-124) RNase A. The duration of the initial lag phase of the regeneration became shorter in the order of Ala(121-124), des-(122-124)-, des-(123-124)-, and des-124-RNase A. When free sulfhydryl groups were titrated during the refolding process, identical results were observed for both the wild-type and the mutant enzymes. A lag phase was not observed in the case of sulfhydryl group titrations (Fig. 4).

The recovery of activity in the presence of glutathione for the fully denatured and reduced wild-type and mutant RNase A is shown in Fig. 5. The recovery of activity of both the wild-type and all the mutant RNase A samples was significantly increased when glutathione was present. The most effective concentration of the glutathiones corresponded to 2 mM GSH and 0.2 mM GSSG as previously reported (22). The rates of recovery of the activity at an early folding period decreased in the order of des-(122-124), des-124-, Ala(121-124), des-(123-124)-, and des-(121-124)-RNase A. The recovered activity of the mutant enzymes, except for des-(121-124)-RNase A, reached the level achieved by the wild-type enzyme. No activity was recovered from dena-

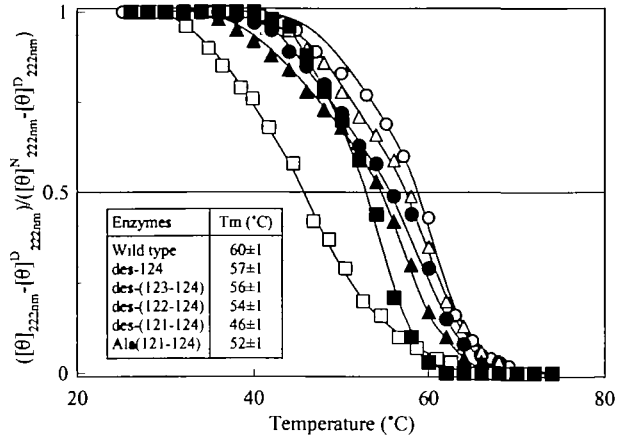

Fig. 3. **Thermal denaturation profiles of the wild-type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■).**

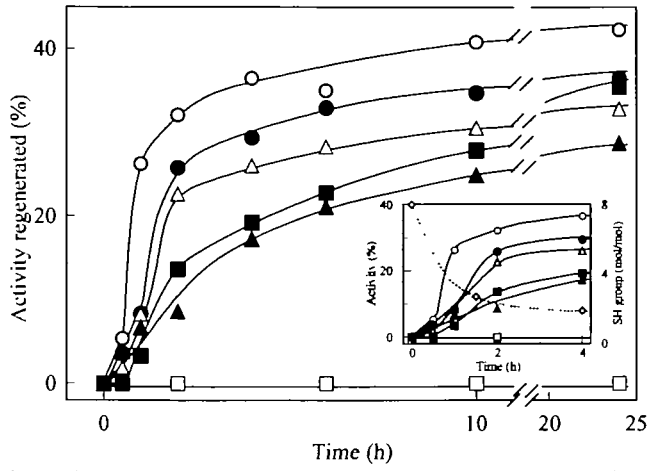

Fig. 4. **Activity regeneration of the wild-type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■) on air oxidation in the absence of glutathione.** Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. Hydrolytic activity with respect to C>p was measured at the times indicated. The activity of each native enzyme was taken as 100%. The inset shows the activity regeneration and disulfide bond formation (SH disappearance, ◇) in the early period of the regeneration process.

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

Enzyme	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\cdot min^{-1}$)
Wild type	0.46 ± 0.02	140 ± 10	300 ± 40
des-124	0.66 ± 0.02	63 ± 2	95 ± 5
des-(123-124)	0.64 ± 0.02	92 ± 2	140 ± 10
des-(122-124)	3.1 ± 0.1	11 ± 2	3.6 ± 0.7
des-(121-124)	2.0 ± 0.1	0.34 ± 0.01	0.17 ± 0.01
Ala(121-124)	0.58 ± 0.02	28 ± 2	49 ± 5
D121A	0.52 ± 0.02	32 ± 2	62 ± 7
D121E	0.59 ± 0.02	21 ± 1	36 ± 3
D121K	0.31 ± 0.02	7.1 ± 0.2	23 ± 2

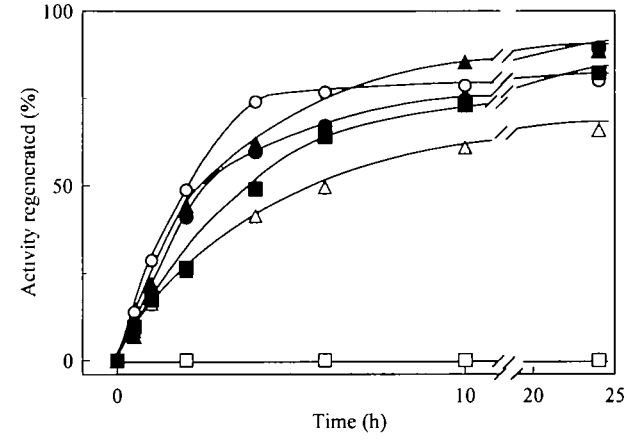

Fig. 5. **Activity regeneration of the wild-type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■) in the presence of 2 mM GSH and 0.2 mM GSSG.** See Fig. 4 for other details.

tured and reduced des-(121-124)-RNase A, because the incubation time for refolding was too short (in these experiments, the incubation time was 24 h, but the incubation for refolding was performed for 72 h in the purification steps).

After air oxidation for 24 h, the C-terminal deletion mutant enzymes were subjected to cleavage by cyanogen bromide to determine whether disulfide bonds were correctly formed. If disulfide bonds were formed correctly, only two peptide fragments, having molecular weights of about 1,500 and 12,000, respectively, would be expected to be produced by cyanogen bromide cleavage (*3*). However, if disulfide bonds were formed in a completely random manner, two additional peptide fragments with molecular weights of 5,500 and 6,500, respectively, would also be expected (*3*). Six of the 105 sets of four disulfide bonds produce these 5,500- and 6,500-Da peptide fragments. Of the four C-terminal deletion mutant enzymes, only des-(121-124)-RNase A gave rise to peptide fragments with molecular weights of 5,500 and 6,500 after air oxidation for 24 h. The yield of these fragments was approximately 6% of that of the fragment of molecular weight 12,000. These data show that the disulfide bonds of des-(121-124)-RNase A were randomly paired.

Properties and Refolding of D121 Mutant RNase A—CD spectra of the wild-type and D121 mutant RNase A are shown in Fig. 6. The estimated amounts of the secondary structures of the mutant enzymes were also similar to that of the wild type (Fig. 2). The thermal denaturation profiles, as estimated by changes in [θ] value at 222 nm, showed that all mutant enzymes followed a two-state transition (Fig. 7). The T_m value of D121E was nearly the same as that of the wild type, but the T_m values of D121A and D121K were slightly lower.

The kinetic parameters of the D121 mutant RNase A for the cleavage of C>p are summarized in Table III. The K_m value of the mutant RNase A was nearly the same as that of the wild type, but the k_{cat} was lower than that of the wild type; thus, k_{cat}/K_m of the mutant RNase A was smaller than that of the wild-type enzyme. This result shows that the hydrolytic activity with respect to C>p of the mutant enzymes became lower.

Activity regeneration by air oxidation in the absence of glutathione was compared for the fully denatured and reduced forms of the wild-type and the D121 mutant

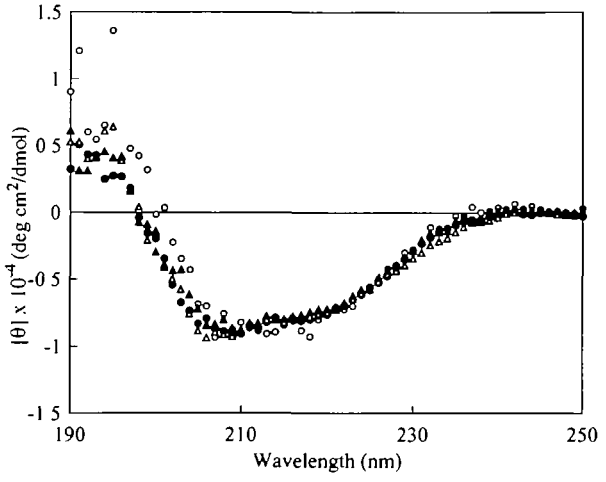

Fig. 6. **CD spectra of the wild-type enzyme (○), D121A (●), D121E (△), and D121K (▲).**

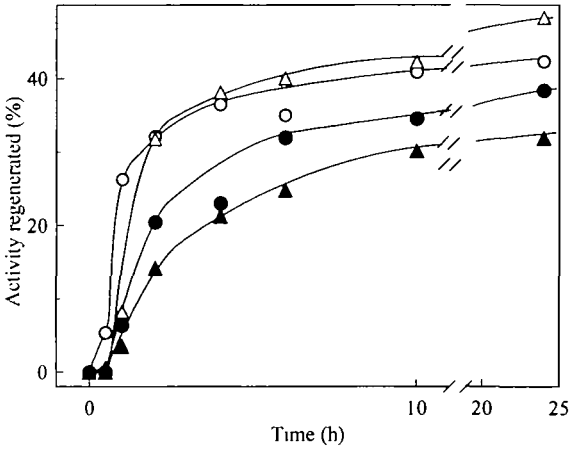

Fig. 8. **Activity regeneration of the wild-type enzyme (○), D121A (●), D121E (△), and D121K (▲) on air oxidation in the absence of glutathione.** See Fig. 4 for other details.

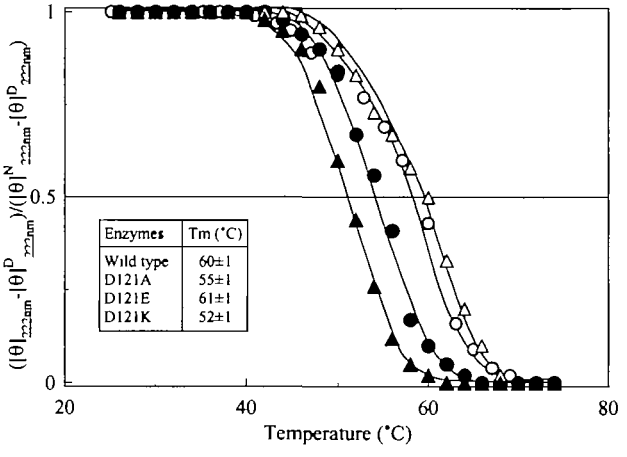

Fig. 7. **Thermal denaturation profiles of the wild-type enzyme (○), D121A (●), D121E (△), and D121K (▲).**

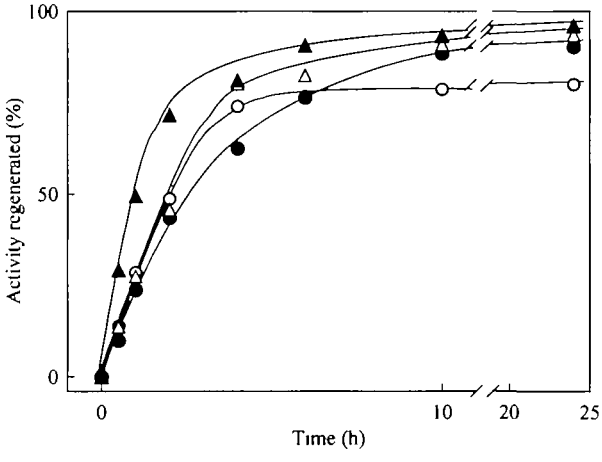

Fig. 9. **Activity regeneration of the wild-type enzyme (○), D121A (●), D121E (△), and D121K (▲) in the presence of 2 mM GSH and 0.2 mM GSSG.** See Fig. 4 for other details.

RNase A (Fig. 8). The rate of recovery of activity during the early period of refolding decreased in the order of D121E, D121A, and D121K. The fully recovered activity of D121E reached approximately the same level as observed for the wild-type enzyme, whereas those of the other mutant enzymes were as follows: 91% for D121A and 76% for D121K. All D121 mutant enzymes showed an initial lag phase during the regeneration.

The recovery of the activity in the presence of glutathione for the fully denatured and reduced wild-type and D121 mutant RNase A is shown in Fig. 9. The recovery of activity for both the wild type and the mutant RNase A significantly increased in the presence of glutathione. The recovery rates of activity for the mutant enzymes were nearly the same as that of the wild-type enzyme, and the recovered activity of the mutant enzymes reached the same level as that of the wild-type enzyme.

DISCUSSION

The four C-terminal amino acid residues are conserved among many mammalian pancreatic ribonucleases. The C-terminal region of RNase A is comprised of a β strand (*23*), which interacts with the surrounding amino acid residues to maintain the rigid tertiary structure of RNase A. The present study demonstrates that the deletion of one or two residues or a mutation in this region gives rise to an enzyme in which the secondary structure is nearly the same as that of the wild-type enzyme as judged from CD spectra, but that it has adverse effects on both the activity and the thermal stability.

A decrease in the sheet content in des-(122-124)- and des-(121-124)-RNase A appears to be the direct result of the disappearance of the C-terminal amino acid residues. Decrease in sheet content of 10–20% should correspond to the loss of about 12–24 amino acid residues in the β sheet, that is, the integrity of the β sheets which contain the C-terminal strand becomes loose. However, the core structures of these two mutant enzymes are correctly formed, as evidenced by the observation that the CD spectra are quite similar to that of the wild-type enzyme.

Des-124-RNase A is a mutant enzyme in which the C-terminal amino acid, Val124, of the wild-type RNase A is

absent. Val124 interacts with nearby hydrophobic residues, Ile106 and Ile107 (*23*). When Val124 is deleted, these hydrophobic interactions are lost and, thus, the protein is no longer able to form a compact structure around the C-terminal region. This may explain the slight reduction in hydrolytic activity and regeneration efficiency of des-124-RNase A.

Val124, Ala122, and Asp121 in the C-terminal four amino acid residues interact with His105 and Ile107 *via* hydrogen bonds (*23*). However, Ser123 does not enter into such interactions. This might be because the hydrolytic activity of des-(123-124)-RNase A was not significantly reduced from that of the wild-type, and the activity regeneration rate of this mutant enzyme was similar to that of des-124-RNase A. The results herein show that the formation of these hydrogen bonds is a very important element in the correct folding of RNase A.

The hydrolytic activity and the regeneration rate of des-(122-124)-RNase A were reduced. Since Ala122 interacts with His105 and Ile107, the deletion of the C-terminal three amino acid residues causes a lack of interaction that keep the C-terminal region compact and produce the fully active enzyme.

The replacement of Asp121 causes slight reduction of the hydrolytic activity relative to C>p. This result seems to be natural because Asp121 is located near His119 and has an influence on the activity of RNase A (*24–26*). The replacement of this residue also effects the regeneration of activity from the fully denatured and reduced form. However, D121E showed nearly the same folding profile as the wild-type enzyme. This result implies that the negative charge of Asp121 is an important element for the refolding of RNase A to form a compact structure via ionic interactions with surrounding residues during the regeneration.

Des-(121-124)-RNase A failed to recover any activity. This is because the C-terminal region of this mutant enzyme has lost its ability to participate in side chain interactions and, as a result, the protein is no longer able to keep the compact structure. Without these amino acid residues at 121 to 124, RNase A is not able to form a core structure in the early period of refolding, and the efficiency of the regeneration is reduced because of the formation of random paired disulfide bonds. Our results show that the formation of β sheets around the C-terminus is essential for the formation of an active RNase A structure.

The deletion or mutation of the C-terminal amino acid residues had an effect on the regeneration of activity from the fully denatured and reduced forms. The regeneration rates of the C-terminal deleted, Ala(121-124) and D121 mutant RNase A were slower than that of the wild-type enzyme when oxidation was performed in the absence of glutathione. However, they became nearly the same when GSH and GSSG were used as redox agents, except for des-(121-124)-RNase A. It is likely that GSH and GSSG affects the formation of correct disulfide bonds by exchanging disulfide bonds between the free sulfhydryl groups or incorrectly paired disulfide bonds. In the absence of GSH and GSSG, the C-terminal deletion mutant enzymes, especially des-(121-124)-RNase A, tended to give randomly formed disulfide bonds and, thus, incorrectly folded intermediate(s) accumulated.

In the regeneration of the denatured and reduced forms, of some C-terminal deleted [des-(123-124)-, des-(122-124), des-(121-124)-], Ala(121-124), and D121 mutant RNase A in the absence of glutathione, a significant initial lag phase was observed before any activity appearved. We suspect that this appearance of activity might be directly synchronized to the formation of disulfide bonds. Since the disulfide bond-forming rates of the wild-type and mutant enzymes were the same and no lag phase was observed in the formation of disulfide bonds (Fig. 4), the activity regeneration might be brought about by rearrangement of disulfide bonds. That is, incorrectly paired disulfide bonds accumulated during the initial regeneration period followed by rearrangement to the correct pairing during the lag phase of the mutant enzymes.

Our previous results showed that the formation of a compact structure is initiated by hydrophobic interactions by the C-terminal amino acid residue, Val124, and leads to the efficient packing of the RNase A molecule (*9*). The findings reported in this paper suggest that a negative charge of Asp121 is also involved in this process. Therefore, the unique characteristics of the side chains in the C-terminal four residues certainly contribute to the formation of the compact structure, but the length of the protein main chain is also an important factor in the refolding of RNase A. This is clearly demonstrated by the finding that the Ala(121-124) mutant enzyme, in which all of the C-terminal four amino acid residues have been replaced with alanine residues, also led to the regeneration of activity from the fully denatured and reduced forms, even though the intrinsic side chain interactions in the C-terminal region must have been weakened.

The C-terminal region of RNase A is a "chain folding initiation site" (CFIS) (*27–29*), in which a native-like structure is rapidly formed during the 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 synthesized C-terminal 20 amino acid peptide of RNase A sequence has a partially ordered structure, which is stabilized via hydrophobic interactions (*23*). It is quite likely that the C-terminal region of RNase A is one of the nuclei positions involved in folding of the enzyme.

We conclude, based on the data herein, that the C-terminal region of RNase A plays an important role in refolding by providing a suitable environment for the formation of a compact structure, which is stabilized by interactions of side chains of amino acid residues and the existence of the main chain in the C-terminal region. The findings herein indicate that, while structure-function relationships for these mutants are important, additional studies need to be done on other mutants, so that the role of individual amino acids in folding and the formation of the active enzyme can be completely understood.

REFERENCES

1. White, F.H., Jr. (1961) Regeneration of native secondary and tertiary structures by air oxidation of reduced ribonuclease. *J. Biol. Chem.* **236**, 1353–1360
2. Anfinsen, C.B. and Haber, E. (1961) Studies on the reduction and re-formation of protein disulfide bonds. *J. Biol. Chem.* **236**, 1360–1363
3. Taniuchi, H. (1970) Formation of randomly paired disulfide bonds in des-(121-124)-ribonuclease after reduction and reoxidation. *J. Biol. Chem.* **245**, 5459–5468
4. Puett, D. (1972) A study on the conformation and conforma-

tional stability of ribonuclease A and its peptic derivative, des-(121-124)-ribonuclease. *Biochemistry* **11**, 1980–1990
5. Puett, D. (1972) Chain folding in ribonuclease A derivatives lacking five and six carboxyl-terminal residues. *Biochemistry* **11**, 4304–4307
6. Gutte, B., Lin, M.C., Caldi, D.G., and Merrifield, R.B. (1972) Reactivation of des(119-, 120, or 121-124) ribonuclease A by mixture with synthetic COOH-terminal peptides of varying lengths. *J. Biol. Chem.* **247**, 4763–4767
7. Lin, M.C., Gutte, B., Caldi, D.G., Moore, S., and Merrifield, R.B. (1972) Reactivation of des(119-124) ribonuclease A by mixture with synthetic COOH-terminal peptides; the role of phenylalanine-120. *J. Biol. Chem.* **247**, 4768–4774
8. Hayashi, R., Moore, S., and Merrifield, R.B. (1973) Preparation of pancreatic ribonuclease 1-114 and 1-115 and their reactivation by mixture with synthetic COOH-terminal peptides. *J. Biol. Chem.* **248**, 3889–3892
9. Fujii, T., Doi, Y., Ueno, H., and Hayashi, R. (2000) Effect of mutagenic replacement of the carboxyl terminal amino acid, Val124, on the properties and regeneration of bovine pancreatic ribonuclease A. *J. Biochemistry* **127**, 877–881
10. delCardayre, S.B., Ribo, M., Yokel, E.M., Quirk, D.J., Rutter, W.J., and Raines, R.T. (1995) Engineering ribonuclease A: production, purification, and characterization of wild-type enzyme and mutant at Gln11. *Protein Eng.* **8**, 261–273
11. Braman, J., Papworth, C., and Greener A. (1996) Site-directed mutagenesis using double-stranded plasmid DNA templates. *Methods Mol. Biol.* **57**, 31-44
12. Dodge, R.F. and Scheraga, H.A. (1996) Folding and unfolding kinetics of the proline-to-alanine mutants of bovine pancreatic ribonuclease A. *Biochemistry* **35**, 1548–1559
13. Boix, E., Nogues, M.V., Schein, C.H., Benner, S.A. and Cuchillo, C.M. (1994) Reverse transphosphorylation by ribonuclease A needs an intact p2-binding site Point mutations at Lys-7 and Arg-10 alter the catalytic properties of the enzyme. *J. Biol. Chem.* **269**, 2529–2534
14. Chen, Y.-H., Yang, J.T., and Martinez, H.M. (1972) Determination of the secondary structures of proteins by circular dichroism and optical rotatory dispersion. *Biochemistry* **11**, 4120–4131
15. Andrade, M.A., Chacon, P., Merelo, J.J., and Moran, F. (1993) Evaluation of secondary structure of proteins from UV circular dichroism spectra using an unsupervised learning neural network. *Protein Eng.* **6**, 383–390
16. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C., (1985) Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, 76–85
17. Thannhauser, T.W. and Scheraga, H.A. (1985) Reversible blocking of half-cystine residues of proteins and an irreversible specific deamidation of asparagine-67 of S-sulforibonuclease under mild conditions. *Biochemistry* **24**, 7681–7688
18. Sela, M., and Anfinsen, C.B. (1957) Some spectrophotometric and polarimetric experiments with ribonuclease. *Biochim. Biophys. Acta* **24**, 229–235
19. Ellman, G.L. (1959) Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* **82**, 70–77
20. Gething, M.J. and Davidson B.E. (1972) The molar absorption coefficient of reduced Ellman's reagent: 3-carboxylato-4-nitrothiophenolate. *Eur. J. Biochem.* **30**, 352–353
21. Lin, M.C. (1970) The structural roles of amino acid residues near the carboxyl terminus of bovine pancreatic ribonuclease A. *J. Biol. Chem.* **245**, 6726–6731
22. Lyles, M.M. and Gilbert, H.F. (1991) Catalysis of the oxidative folding of ribonuclease A by protein disufide isomerase: Dependence of the rate on the composition of the redox buffer. *Biochemistry* **30**, 613–619
23. Birdsall, D.L. and McPherson, A. (1992) Crystal structure disposition of thymidylic acid tetramer in complex with ribonuclease A. *J. Biol. Chem.* **267**, 22230–22236
24. Schultz, L.W., Quirk, D.J., and Raines, R.T. (1998) His...Asp catalytic dyad of ribonuclease A: structure and function of the wild-type, D121N, and D121A enzymes. *Biochemistry* **37**, 8886–8898
25. Quirk, D.J., Park, C., Thompson, J.E., and Raines, R.T. (1998) His...Asp catalytic dyad of ribonuclease A: conformational stability of the wild-type, D121N, D121A, and H119A enzymes. *Biochemistry* **37**, 17958–17964
26. Quirk, D.J., and Raines, R.T. (1999) His...Asp catalytic dyad of ribonuclease A: histidine pKa values in the wild-type, D121N, and D121A enzymes. *Biophys. J.* **76**, 1571–1579
27. Nemethy, G. and Scheraga, H.A. (1979) A possible folding pathway of bovine pancreatic RNase. *Proc. Natl. Acad. Sci. USA* **76**, 6050–6054
28. Beals, J.M., Haas, E., Krausz, S., and Scheraga, H.A. (1991) Conformational studies of a peptide corresponding to a region of the C-terminus of ribonuclease A: Implications as a potential chain-folding initiation site. *Biochemistry* **30**, 7680–7692
29. Haas, E., Montelione, G.T., McWherter, C.A., and Scheraga, H.A. (1987) Local structure in a tryptic fragment of performic acid oxidized ribonuclease A corresponding to a proposed polypeptide chain-folding initiation site detected by tyrosine fluorescence lifetime and proton magnetic resonance measurements. *Biochemistry* **26**, 1672–1683