Study of structural formation of bovine pancreatic ribonuclease A: Role of the carboxyl terminal region

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Contents

Abbreviations

CD	circular dichroism
C>p	cytidine 2`, 3`-cyclic monophosphate
C-terminal	carboxyl terminal
DTT	dithiothreitol
GSH	reduced glutathione
GSSG	oxidized glutathione
IPTG	isopropyl-1-thio-β-D-galactoside
MALDI-TOF/MS	matrix assisted laser desorption ionization time of flight mass spectrometry
MES	2-(N-morpholino) ethanesulfonic acid

Introduction

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 des-(121-124)-RNase A, with four amino acid residues deleted from its C-terminus by limited proteolysis, was fully reduced and oxidized, 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. This important result suggests 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 refolding experiments in which C-terminal-deleted enzymes regains 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 the construction of the active structure.

The purpose of this study is to understand the role of the C-terminal amino acid residues of RNase A. The C-terminal residues are deleted or replaced with a site-directed mutagenesis technique, and the enzymatic and refolding properties of the obtained mutant enzymes are investigated.

Chapter 1. Folding studies of RNase A

I. Introduction

Protein folding, or how the amino acid sequence determines the three dimensional structure of a protein, remains an unsolved problem. This is a difficult problem because it requires the determination of the interactions at the residue level to account for the structures of the initial unfolded state and the final native state. Structure of all the intermediates and the transition states encountered in the folding reaction also must be determined. As intermediates in the folding pathway are usually transient species, equilibrium structural analysis and kinetic information will be required for a complete characterization of the folding pathway [7].

In recent years, studies of folding pathway has progressed on the basis of new experimental approaches, such as a multiple-jump stopped-flow technique with detection by UV absorbance, CD or fluorescence, a quench-flow of NH/ND exchange combined with two dimensional NMR analysis, and site-directed mutagenesis or the protein engineering method [8] used to evaluate interactions in intermediates and transition states. These methods have been applied to a few model proteins and precise information about folding pathways has been obtained [9-14].

Bovine pancreatic ribonuclease A (RNase A) [EC 3.1.27.5] composed of 124 amino acid residues with molecular weight of 13,683 contains four disulfide bonds at positions 26-84, 40-95, 58-110, and 65-72. RNase A is the first enzyme whose amino acid sequence is determined, and the third one whose three dimensional structure is solved by X-ray crystallography [15-18]. Because of the large amount of functional and structural informations at the early stage of the investigation, this protein has been served as a model protein for studies of folding, stability, and enzymology [19-21].

In this chapter, the author describes folding studies focusing of

pancreatic RNase A.

II. Early studies

A. RNase S system

Limited proteolysis of RNase A by subtilisin results in the cleavage of a single peptide bond between residues 20 and 21 [22]. The derived protein, RNase S, retains enzymatic activity although the N-terminal peptide of 20 amino acids (S-peptide, residues 1-20) is no longer covalently attached to the residual protein (S-protein, residues 21-124). Removal of S-peptide from S-protein leads to loss of enzymatic activity, but the activity is rapidly restored upon mixing of S-protein and S-peptide. This shows that amino acid residues occupying different positions on the primary structure could form the active center, thus non-covalent interactions are important for forming the active center. The finding of S-protein and S-peptide system also has shown that non-covalent interactions of amino acid side chain are important in retaining the three-dimensional structure of a protein. Derivatives of S-peptide have been prepared by means of chemical peptide synthesis in which amino acids 1-20 are deleted or mutated, and used to study how each residue of RNase A relates to enzymatic activity and the three dimensional structure. The discovery of RNase S system has been appreciated to evaluate roles of amino acid residues in protein.

B. Amino acid residues of carboxyl terminal region

When RNase A is denatured by 8 M urea or 6 M guanidine hydrochloride and reduced with reducing agent such as 2-mercaptoethanol, all disulfide bonds are cleaved and the protein forms a random coil. Fully reduced and enzymatically inactive RNase A is oxidized by the air to produce a soluble protein with a specific activity close to that of the native RNase A. Air-oxidized RNase A possesses the identical secondary and tertiary structures to

the native one. This experiment is very famous for building up the concept that all of the information needed for establishing the structural features is involved in the primary structure [1, 2].

Taniuchi [3] found an interesting phenomenon: when des-(121-124)-RNase A, in which four amino acid residues were deleted from its C-terminus by a pepsin digestion, was denatured and reduced, followed by oxidation, four disulfide bonds were formed, but no enzymatic activity was recovered, due to the formation of wrongly paired disulfide bonds. This result suggests that folding information is concentrated within the C-terminal tetrapeptide.

Puett [23, 24] 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 the 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 results from the 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.

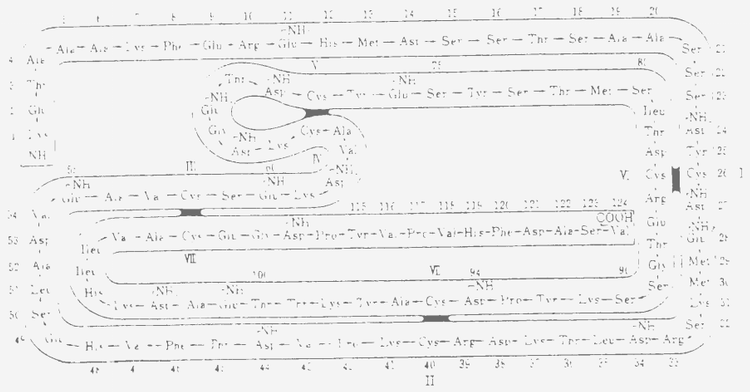


Fig. 1. Primary structure of RNase A. (Smyth *et al.*, (1963) *J. Biol. Chem.* **238**, 227)

III. Formation of local structure in RNase A folding

Many theoretical and experimental evidences obtained from folding/unfolding studies of RNase A have accumulated to support the view that thermodynamically stable local structures play an important role in the initial stage of protein folding [25-29]. These studies are summarized as follows.

A. Principles of protein folding

RNase A folding from disulfide-intact and disulfide-reduced unfolded states appear to follow the same general principle that the amino acid sequence contains all information required for the protein to attain its biologically active conformation [30].

1. Thermodynamic vs. kinetic control

Anfinsen and co-workers [30] first demonstrated the general principle that the native structure of RNase A is thermodynamically most stable, and showed that disulfide-reduced RNase A upon oxidation and thermally- or solvent-unfolded disulfide-intact RNase A refold to the same biologically active structure. Disulfide-reduced unfolded RNase A and disulfide-intact unfolded RNase A exhibit different properties [31-33], but these different initial states give rise to an indistinguishable refolded structure.

Thermally- or solvent-unfolded disulfide-intact RNase A is an equilibrium mixture of fast- and slow-folding species due to disulfide-bond isomerization [34] and proline *cis*/*trans* peptide-bond isomerization [35]. The folding mechanism itiself involves kinetic control, while the distribution of refolded products of RNase A is thermodynamically controlled. However, kinetic control of the distribution of folding pathways doesn't imply kinetic control of the folded product(s), because isomerization can take place in predominantly folded intermediates. On the basis of biological activity, all of these kinetically distinguishable folding pathways lead to the same thermodynamically

determined native conformation.

2. Dominance of short-range interactions

In the initial stages of folding in aqueous solution, the nonpolar side chains of the unfolded protein are exposed to water and are first driven to associate by short-range hydrophobic interactions [36]. Initial folding intermediates are generally less compact than fully folded structures and therefore less influenced by long-range interactions. This concept provides the basis for a procedure in which protein structures are built up from successively larger fragments in theoretical calculations of protein structure and for experimental studies of the conformations of protein fragments. For example, the concept that short-range interactions dominate is supported by conformational energy calculations of oligopeptides [37-41]. These studies indicate that short-range interactions play an essential role in determining protein structures.

3. Multistate folding pathways

Protein folding mechanisms are distinguished by their degree of cooperativity, i.e., the degree to which the folding of one part of the protein is coupled with folding of the remainder of the protein. A two-state model involves the highest cooperativity. In this case, both short- and long-range interactions would be involved in the folding mechanism. On the other hand, the cooperativity of the folding process is reduced if short-range interactions dominate in the initial stages of folding since short-range interactions would define rapidly forming local structures in partially folded intermediates. The formation of these structures should be independent of the conformations adopted by the rest of the protein. In later stages of folding, these locally ordered structures coalesce under the influence of longer range interactions. This is not a two-state but a multistate pathway. A two-state model is actually a special case of a multistate mechanism in which the populations of the

intermediate states are negligibly small.

For disulfide-intact RNase A, the folding mechanism(s) are highly cooperative [42-44]. This led early workers to conclude that the folding is best described as a two-state phenomenon involving an equilibrium between native, folded RNase A and an ensembles of statistically coiled unfolded conformations with little or no formation of partially folded species. But the inadequacies of the two-state model were already evident in data from early thermodynamic studies of the thermal unfolding transition. Within the thermal transition region, a multistate folding mechanism involves many species in equilibrium with one another [45]. Because the equilibrium constants, Ki, between locally folded and unfolded regions are different in different segments, i, of the polypeptide chain, these partially folded species, in principle, can be characterized by thermodynamic measurements.

One useful probe to distinguish native from nonnative conformations of RNase A is the susceptibility of specific buried peptide bonds to hydrolysis by proteolytic enzymes. The fully folded protein is essentially resistant to hydrolysis by trypsin, chymotrypsin, aminopeptidase, and carboxypeptidase, while fully thermally unfolded RNase A is cleaved by all of these proteases at sequence-specific peptide bonds. The degree of proteolytic susceptibility of these peptide bonds can therefore be used as a rough measure of the degree of local unfolding. In the absence of partially folded intermediates, the proteases should access to all the cleavage sites to an equal degree as the temperature is increased through the transition region. However, it was found that the individual proteolytic cleavage sites are cleaved to different degrees through the thermal transition [46-51]. These observations are consistent with the presence of partially folded structures in which some peptide bonds are in an environment accessible to proteases while others remain in folded environment inaccessible to proteolytic cleavage.

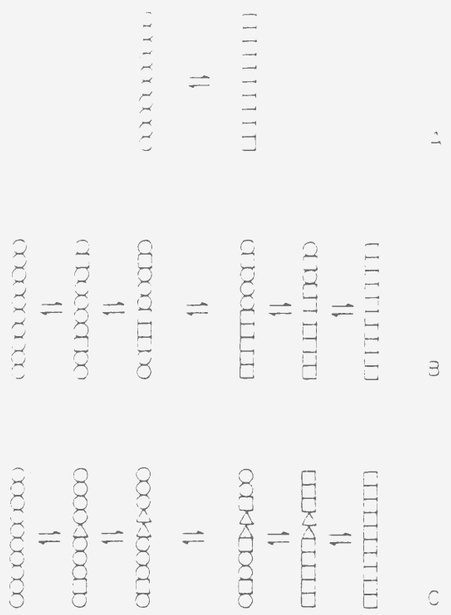


Fig. 2. Schematic representations of different classes of protein folding mechanisms. These three classes are distinguished by the types of folding intermediates involved. The symbols ○, □ and △ denote locally desordered, locally ordered native, and locally ordered nonnative protein structures, respectively. (Montelione and Scheraga, (1989) *Acc. Chem. Res.* **22**, 70)

4. Chain-folding initiation structures

The ideas of (1) the dominance of short-range interactions and (2) a multistate folding mechanism is associated with the concept that sequence-specific interactions define local structures that can form independently in several different regions of the polypeptide chain in the initial stages of the folding process. Such early-folding intermediates are an essential feature of hierarchical folding mechanisms and have been referred to as "nuclei" [36, 52, 53], "locally independently nucleating continuous segments" (LINKS) [54], "chain-folding initiation structures" (CFIS) [55], and so on. These structures limit the conformational space accessible to the protein through specific short- and medium-range interactions, thereby directing subsequent folding events, and provide stable core structures around which the rest of the polypeptide

chain can fold.

B. Experimental approaches to folding of RNase A

Experimental attempts have been made to develop a more detailed picture of the kinds of structures that RNase A adopts in the initial stages of folding. This work has been directed primarily along three avenues: (1) studies of polypeptide fragments, (2) studies of disulfide-reduced derivatives, and (3) studies of kinetic folding intermediates.

If short-range interactions indeed define local structures in the initial stages of folding, it follows that properly chosen peptide fragments of RNase A should adopt some degree of ordered structure by themselves in solution. Accordingly, a large part of work has focused on characterizing the conformations adopted by polypeptide fragments of RNase A under conditions of solvent and temperature at which the disulfide-intact protein is fully folded. During the course of these studies, it became clear that further information could be obtained from structural studies of disulfide-reduced RNase A. Using these model systems, spectroscopic probes have been applied to characterize local structures under equilibrium conditions. These probes have begun to be used to identify locally ordered structures in kinetic folding intermediates.

1. Studies of fragments of RNase A

Initiation structures of chain-folding form native or native-like conformations, and candidate initiation sites can be identified from an analysis of the three-dimensional structure of the native protein. Such conformations for chain-folding initiation include α-helical and β-bend conformations that can be stabilized by local interactions alone. Such structures are identified either by model building, by interactive molecular graphics, or by more sophisticated theoretical methods. Contact maps derived from the three-dimensional structure of the native protein are especially useful in this regard [56-59].

Assuming that local native-like conformations are formed before those that involve long-range interactions, this contact map is useful to identify six initiation sites: residues 4-11 (site A), residues 25-34 (site B), 51-57 (site C), 53-79 (site D), 71-111 (site E), and 103-124 (site F) (Fig. 3).

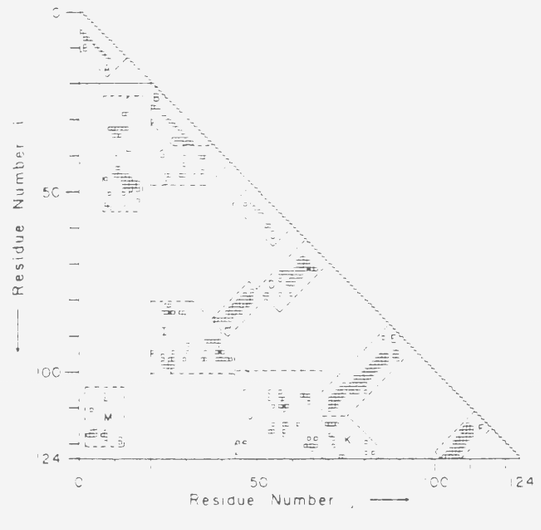


Fig. 3. Contact map of RNase S. Each point of the map represents presence (square) or absence (no marking) of a contact between amino acid residues *i* and *j*. Contacts between residues are omitted from the figure whenever $|i-j|<4$. (From ref. 59)

Refolding studies of reduced RNase A with antibody showed that the antigenic determinant was first formed at segment 80-124, followed by the nucleation at residues 106-118, the C-terminal hydrophobic core [60]. Later, this core region was shown not to unfold completely even at 70°C [61]. In addition, a 63-residue analog of RNase A containing the hydrophobic core region and two buried disulfide bonds (26-84, 58-110) was synthesized with solid-phase peptide synthesis method [62]. It bound on an affinity column specific for the active site of RNase A and showed 8 and 14% transphosphorylation activity toward poly(C) and poly(U), respectively. Thus, the analog independently folds native-like structure without outer shell portion

and N-terminal peptide. These results indicate that the C-terminal hydrophobic core is one of independent nucleation sites and at least a candidate for the folding initiation site.

The native structure of RNase A contains three α-helices in 1-13, 24-34, and 50-60. Peptide fragments, 1-19, 21-42, and 50-61, were shown to fold to helical structures in helix-stabilizing agent (trifluoroethanol) at low temperature. The 21-42 fragment adopted 14% of the native-like α-helical structure even in water solution at pH 5.4 and 22°C [63]. The 50-61 fragment adopted 18% of the native-like α-helical structure in 30% TFE solution at pH 5.4 and 22°C [64]. The fragment 50-79, which contains three cysteine residues (Cys58, 65, and 72) prefers the correct disulfide bond, 65-72, to the incorrect disulfide bond, 58-65 [65] though the entropic energy loss is the same in forming both correct and incorrect disulfide bonds. NMR study of the fragment 65-72 and 61-74 showed that the 65-72 peptide formed native-like β-turn structure [66]. Thus, the local interaction between Cys65 and Cys72 favors the native disulfide bond.

According to the experimental results described above, five parts in RNase A are candidates of the folding initiation sites; a C-terminal hydrophobic core, three α-helical structures, and the loop between 65-72.

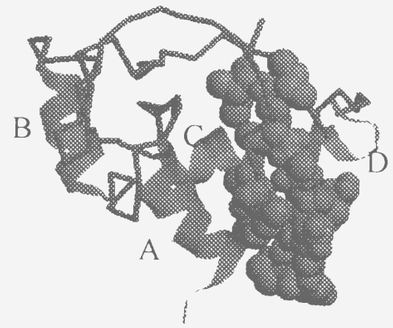


Fig. 4. The candidates of folding initiation sites of RNase A. Three helices, 1-12 (A), 24-34 (B), and 50-60 (C), and one disulfide loop, 65-72 (D) are shown as strand model. The C-terminal hydrophobic core is shown as space-filling model.

2. Disulfide-reduced derivatives of RNase A

Although reconstitution of protein fragments is potentially of great value in identifying local interactions in folding process, they are limited to the degree that they rely on predictive schemes for deciding which sequences to isolate, clone, or synthesize. In view of the shortcomings of these strategies for identifying chain-folding initiate structures, systems in which long-range interactions responsible for the high cooperativity are suppressed while short- and medium-range interactions are relatively unperturbed has been developed. One such model system for disulfide-containing proteins is the reduced RNase A under folding conditions. Significant amounts of local structure, identified by CD [67-69] and Raman [69, 70] spectroscopy, are present in disulfide-reduced RNase A. Binding of antibodies to disulfide-reduced RNase A indicates that polypeptide segments of residues 1-31, 31-79, 80-124 have trace amounts of native structure at 4 °C [32]. The most convincing evidence for formation of local structure in disulfide-reduced RNase A comes from 1H NMR [71].

IV. Folding intermediates

Many efforts have been made to detect and characterize intermediates during refolding. The attempts should provide prominent structural information about folding processes. In order to find kinetic intermediates, refolding of RNase A having intact disulfide bonds (S-S intact) have been extensively studied. Stopped-flow methods with CD, UV, and NMR spectroscopies are improved to describe the refolding of S-S intact RNase A which proceeds in a very short time. On the other hand, refolding studies of fully reduced RNase A have been also performed, in which intermediates are trapped as one-, two-, or three-disulfide species.

A. Refolding of S-S intact RNase A

The generally accepted model for unfolding of S-S intact RNase A is a single pathway,

$$N \xrightarrow{\text{fast}} U_f \underset{\text{slow}}{\rightleftharpoons} U_S^{I,II} \qquad (1)$$

and refolding are maultiple pathways,

$$U_f \longrightarrow N \qquad (2)$$

$$U_S^{II} \longrightarrow I_I \longrightarrow I_N \longrightarrow N \qquad (3)$$

$$U_S^{I} \longrightarrow (I') \longrightarrow N \qquad (4)$$

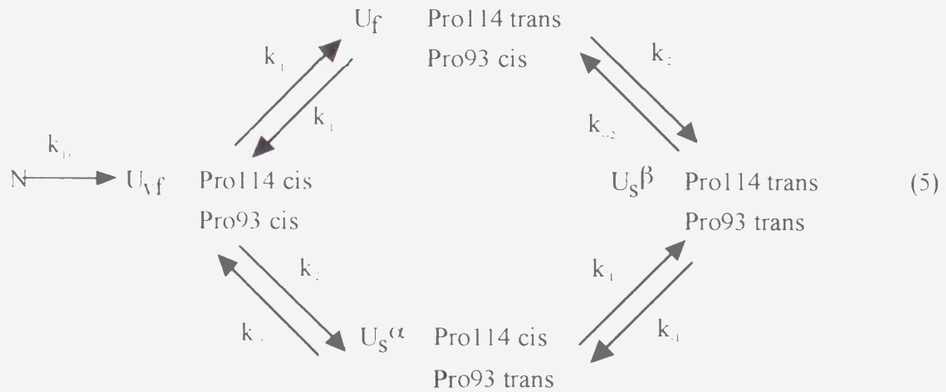
where U_f, fast refoldingspecies, U_S^I, slow ones, U_S^{II}, very slow ones representing 20, 60-70, and 10-20% of all unfolded proteins, respectively.

The nature of U_S^{II} has been explored most precisely among all unfolded species, since one of the intemediates, I_I, can be structurally characterized. I_I is an intermediate in which amide protons are protected against solvent water [72]. The second and third helices, and β-sheet region between 79-118 including a hydrophobic core are loosely formed I_I. Additionally, since 2'-CMP binds to I_I, the active site structure is partly formed, while the buried tyrosine side chain (Tyr25, 73, and 97) are still exposed. I_N is a native-like intermediate which has presumably *trans* X-Pro93 peptide bond [73]. Since *cis-trans* isomerizations of X-Pro (Pro93 and Pro114) peptide bonds [74] occur during refolding, it has been difficult to distinguish the true structural refolding from isomerization processes. Thus, it is necessary to resolve the *cis-trans* isomerization problem to simplify the refolding pathway.

1. X-Pro isomerization

The investigation with X-Pro isomerization problem has concentrated in *cis* X-Pro peptide bonds, Tyr92-Pro93 and Asn113-Pro114. Recent site-directed mutagenesis [75, 76] and kinetic studies [77] gave important information about X-Pro isomerization during refolding. Especially, Houry *et*

al. found a new very fast refolding species, U_{vf} containing only native X-Pro iso-forms, in addition to the usual fast and slow phases, by choosing appropriate refolding conditions (1.5 M guanidine hydrochloride, pH 3.0, at temperatures< 15°C) to slow down the refolding process. Thus, refolding of U_{vf} does not include X-Pro isomerizations. A new model for the unfolding pathway and interconversion among unfolded species is proposed based on two indepedent isomerization processes at two *cis* X-Pro bonds (equation (5)).



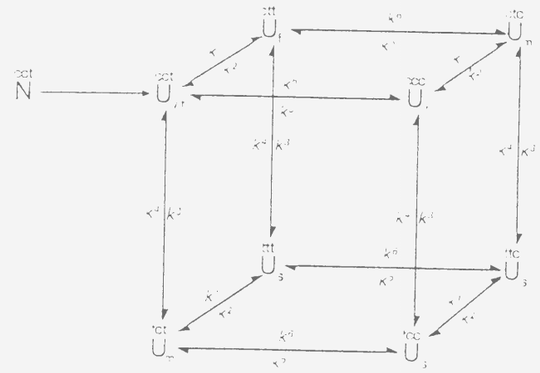
(5)

There still exists one discrepancy between exprimental results and the model in the equation (5). In the model, the relative concentrations of the unfolded species at equilibrium are

$[U_{vf}]:[U_f]:[U_s^{\alpha}]:[U_s^{\beta}]=6.25:18.75:18.75:56.25$

The ratio $[U_s^{\alpha}]:[U_s^{\beta}]=25:75$ is close to the value of $[U_s^{I}]:[U_s^{II}]=20:80$ [78]. Experimental results show that the major species U_s^{II} folds faster than the minor species U_s^{I}. In contrast, according to the equation (5), the major species U_s^{β} with two non-native X-Pro isoforms would fold slower than the minor species U_s^{α} with one non-native ones. A possible explanation for the discrepancy is that a third essential isomerization process exists in the refolding reaction. This means that complicated refolding kinetics cannot be explained only by *cis* X-Pro. Thus, *trans* X-Pro bonds have to be taken into consideration.

In the site-directed mutagenesis studies exchanging *trans* proline with alanine residues, P42A and P117A [79, 80], P42A showed the same refolding kinetics as the wild type RNase A, while P117A showed a single slow folding species. This indicates that Val116-Ala117 peptide bond, being not in *cis* conformation during the unfolded state, reduces one isomerization step. Thus, Val116-Pro117 peptide bond causes the third essential isomerization process. The folding pathway of S-S intact RNase A includes three X-Pro isomerization steps as shown in the equation (6). In this model, species with three wrong isomers, U_{ctt}, would correspond to the slower refolding species U_s^{I}, and the other three species (U_{ctc}, U_{ttc}, U_{ttt}) would correspond to U_s^{II}. The ratio $[U_{ctt}]:([U_{ctc}]+[U_{ttc}]+[U_{ttt}])=19:81$ is similar to the experimentally determined value of $[U_s^{I}]:[U_s^{II}]=20:80$.



(6)

2. Refolding of U_{vf}

Investigations about the refolding process of U_{vf}, containing only native X-Pro bonds, have just started. Double jump technique which can control unfolding time, combined with UV, CD, and NMR spectroscopies suggests the refolding model [81, 82].

$$[U_{vf} \longrightarrow I_u] \longrightarrow I_\phi \longrightarrow N \qquad (7)$$

At the initiation of folding, U_{vf} is converted to a largely unfolded intermediate,

termed I_u, which then results in the molten-globule-like intermediate, I_ϕ. Kinetic rates and the thermodynamic equilibrium constants of the refolding reaction show that the process from U to I_ϕ proceeds predominantly by hydrophobic interactions [83]. I_ϕ is structurally characterized and shown to have a substantial population of secondary and tertiary structures, about 40-50% of the native structure by the stopped-flow CD experiment [81]. Using hydrogen deutrium exchange technique with NMR, it was shown that the second helix (residues 24-34), β-sheet region around residues 82-84, and dynamic structure near C-terminal (residues 106-118) β-sheet region are formed in I_ϕ. [82]. Although the regular structure formed in I_ϕ is less stable than that observed in a hydrogen-bonded intermediate, I_1, which is populated in the early stage of U_S^{II} refolding [84], the structural formations occur in the similar regions in both I_ϕ and I_1. Furthermore, the peptide fragment of residues 21-42, including the second α-helix sequence, and the C-terminal hydrophobic core have been suggested as the folding initiation site by structural characterization of the peptide and by thermal unfolding studies, respectively. Thus, ordered structures may be formed in the second α-helix and the C-terminal hydrophobic core regions at the early stage of RNase A folding.

B. Refolding of reduced RNase A

In refolding studies from reduced proteins, the disulfide-bonded intermediates can be chemically trapped, isolated, and structurally characterized. Refolding processes of BPTI [85] and RNase A [86, 87] are extensively studied. It is suggested that fully reduced BPTI still contains extensive ordered structures [88], and most disulfide-bonded intermediates of BPTI are very native-like [89]. Therefore, most of the refolding processes involve disulfide rearrangements rather than the conformational folding. In contrast, RNase A provides a better system to study the folding process through the disulfide-

bond formation, since disulfide formations are coupled to conformation folding.

1. Population of intermediates during the refolding

In the refolding reaction with GSH and GSSG, intermediates were trapped with iodoacetamide (IAA) and monitored by electrospray mass spectrometry (EM-MS) time-course analysis [90, 91]. First, the reduced protein reacts with a single GSH to form a mixed disulfide which then evolves to an intramolecular S-S bond via thiol-disulfide exchange. This reaction produces a single disulfide bond (one-disulfide intermediate). Second, two-disulfide intermediates are accumulated, and more significant than three-disulfide intermediates throughout the refolding. In addition to the first result, the fact that only one disulfide bond forms at a time indicates that the refolding from reduced RNase A proceeds a specific route. In addition, the accumulation of two-disulfide intermediates can be ascribed either to their intrinsic thermodynamic stability compared to other species or to the fast process from three-disulfide intermediates to the native state. From these results, the following equation is suggested.

$$U \rightleftharpoons 1S \rightleftharpoons 2S \overset{\text{very fast}}{\rightleftharpoons} 3S \rightleftharpoons N \qquad (8)$$

2. Isolation of disulfide intermediates

It is difficult to identify the position of disulfide bonds because so many disulfide species are generated during refolding. This is probably caused by formation of mix-disulfide bonds between cysteine residues and glutathione molecules and by rearrangement of disulfide bonds during alkylation with IAA. In contrast, DTT^{ox} molecules form no mixed-disulfide, and AEMTS (aminoethylmethanethiosulfonate), which is at least five orders more reactive towards thiols than IAA. These two reagents make it easy to isolate a disulfide intermediate during refolding [92].

At the early stages of the refolding, the one-disulfide, Cys65-Cys72,

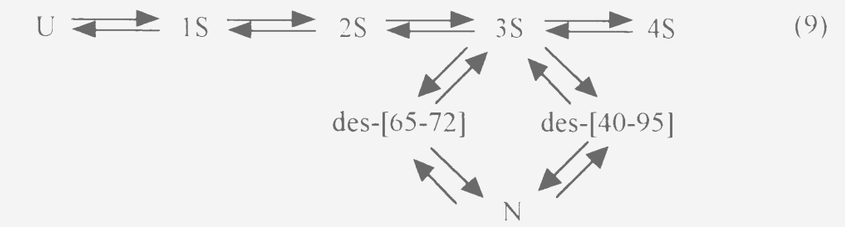
intermediate comprises 40% of the entire one-disulfide species [92]. This means that local interactions within the disulfide loop of residue 65-72, which is suggested as a chain-folding initiation site by structural analysis of the peptide fragment including this sequence [66, 93], force the formation of this disulfide bond.

Although it is still difficult to isolate a specific intermediate possessing two disulfide bonds, the previous result using MS and GSH/GSSG suggests that two-disulfide intermediates accumulate in the long term of the refolding. In addition, two mutants truncated of one disulfide bond, des-[26-84] and des-[58-110] RNase A were found to be thermally unstable compared to the other two mutants, des-[40-95] and des-[65-72] enzymes [94]. This fact indicates that two disulfide bonds, Cys26-Cys84 and Cys58-Cys110, are indispensable to maintain stable, native-like structures. Therefore, the two-disulfide intermediate possessing Cys26-Cys84 and Cys58-Cys110 disulfide bonds is likely accumulated. The putative two-disulfide intermediate, des-[65-72, 40-95] RNase A, was produces by site-directed mutagenesis and characterized [95]. Although all three of the possible two-disulfide pairings appear in this mutant enzyme and their whole structures are predominantly disordered, the native disulfide pairing is formed most preferentially probably due to local interactions. In addition, the NMR analysis indicates formation of local structures in the vicinity of His105, which locates in the bend portion of the native structure.

The three-disulfide intermediates have not been also identified during refolding, while two three-disulfide intermediates, des-[65-72] and des-[40-95], were detected during reductive unfolding by DTT^{red} [87]. After these results, these two intermediates were produced by site-directed mutagenesis [96, 97]. The des-[65-72] and des-[40-95] RNase A are only disturbed around each missed-disulfide bond, and have 22 and 5% hydrolytic activity of the native wild type enzyme, respectively. Thus, they are the intermediates with highly

native-like structures. Twenty and eighty percent of reduced RNase A are regenerated to the native enzyme via des-[65-72] and des-[40-95], respectively [98].

There is a discrepancy that disulfide bond 65-72 formed at first is truncated in the minor three-disulfide intermediate. That is explained by that disufide-rearrangements occur in the final step of refolding since the incorrect disulfide bonds are formed in the process from two-disulfide intermediates to three-disulfide ones. Thus, the regeneration model of reduced RNase A is as follows;



Refolding of reduced RNase A does not occur in native and non-oxidative conditions. This means native-like structures cannot exist without any disulfide bond. On the other hand, local non-covalent interactions force to form specific one disulfide bond, 65-72. Thus, non-covalent interactions, such as hydrogen bonds, hydrophobic interactions, and salt bridges, and disulfide bond formations cooperatively act to proceed folding of RNase A. Two buried disulfide bonds, 26-84 and 58-110, which exist inside hydrophobic cores, are necessary to keep native-like structures in intermediates and the native RNase A. This indicates that hydrophobic interactions in each core are prominent forces to promote folding after the formation of one-disulfide bond, Cy65-Cys72. Furthermore, the putative two-disulfide intermediate has negligible structures around His105 which locates in the bend portion connecting two hydrophobic cores. At this stage, two cores get to interact each other, and the enzyme acquires a native tertiary structure.

Chapter 2. Effect of mutation of Val124 on the properties and regeneration of RNase A

I. Introduction

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 chapter deals the enzymatic and refolding properties of the mutant enzymes in which Val124 is replaced with alanine, tryptophan, lysine, glycine, glutamic acid or leucine residue.

II. Experimental procedures

Materials

Plasmid pETRN coding the RNase A gene was constructed according to the method of delCardayre et al. [99]. 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 Nakalai 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 [100]. 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-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 [101] 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 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 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, 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 x 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 [102] in 0.2 M sodium acetate buffer, pH 5.5, at 25°C by recording the increase in absorbance at 296 nm. The extinction coeffcient was taken as 516.4 M^{-1} cm^{-1}. The substrate concentration was in a range of 0.16 and 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 with 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 with 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 x 50 mm) equilibrated with 0.1 M acetic acid and then 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 [103]. For other experiments, protein

concentrations of unfolded and folded RNase A were determined using the extinction coefficients of 8,500 M^{-1} cm^{-1} at 275 nm [104] and 9,800 M^{-1} cm^{-1} at 277.5 nm [105], 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] [106]. An aliquot of 150 μl removed from the RNase A reoxidation solution of RNase A 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 the extinction coefficient of 13,380 M^{-1} cm^{-1} at 412 nm [107].

III. Results

Expression and Purification of V124 Mutant RNase A

Three to 10 mg of a mutant RNase A were 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. 5) and was used for further experiments.

Properties of V124 Mutant RNase A

The wild type and mutant enzymes showed indistinguishable CD spectra (Fig. 6). The thermal denaturation profiles monitored as to changes in [θ] value at 222 nm were almost identical for the wild type and mutant RNase A (Fig. 7). 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.

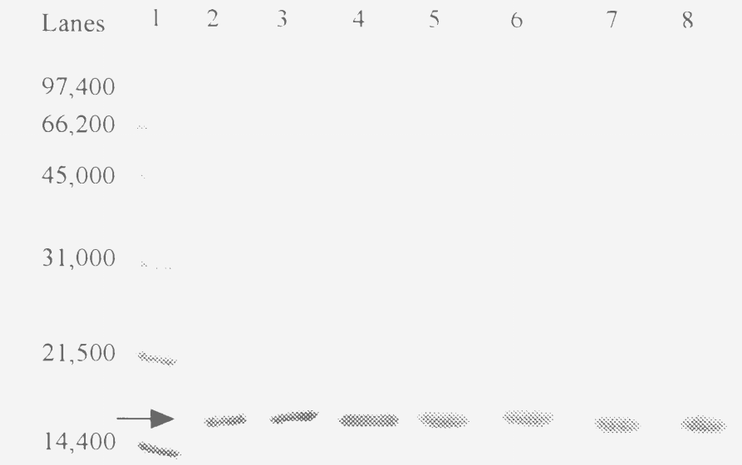


Fig. 5. SDS-PAGE analysis of the purified mutant RNase A. The polyacrylamide concentration of the running gel was 17%. Lane 1, molecular weight standards (phosphorylase *b*, 97,400; serum albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400); Lane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

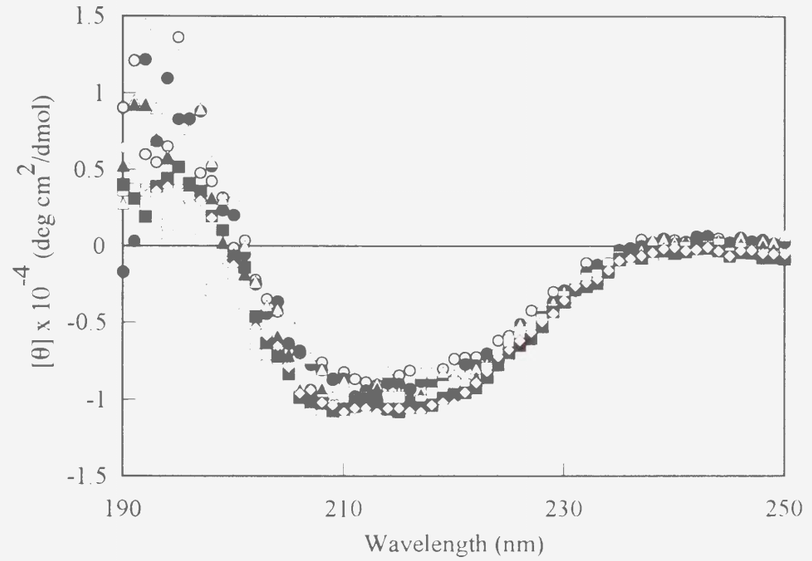


Fig. 6. CD spectra of the wild type enzyme (○), V124A (●), V124W (△), V124K (▲), V124G (□), V124E (■), and V124L (◇).

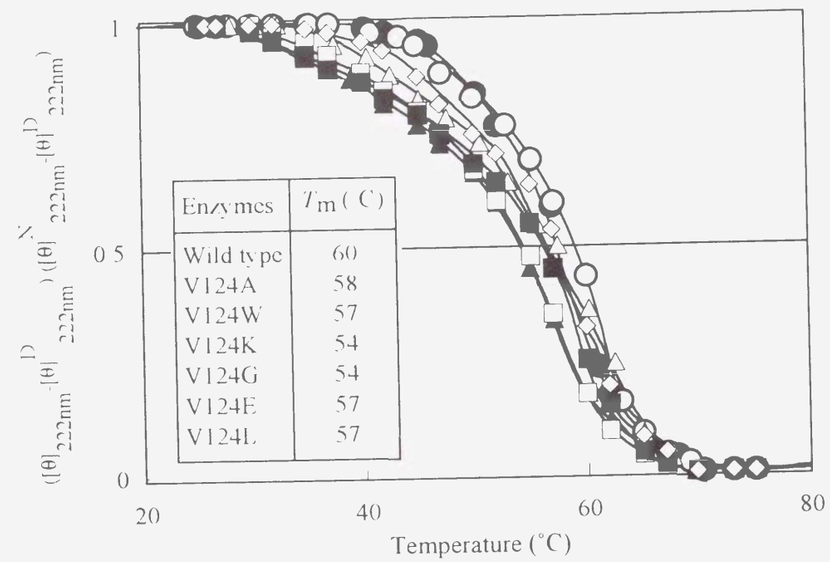


Fig. 7. Thermal denaturation profiles of the wild type enzyme (○), V124A (●), V124W (△), V124K (▲), V124G (□), V124E (■), and V124L (◇).

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 1). 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.

Table 1 Kinetic parameters of the wild type and mutant RNase A for the cleavage of C>p

	K_m	k_{cat}	k_{cat}/K_m
	mM	min^{-1}	$mM^{-1}\,min^{-1}$
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

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. 8). The rate of recovery of the activity at an early period of refolding decreased in 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 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 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).

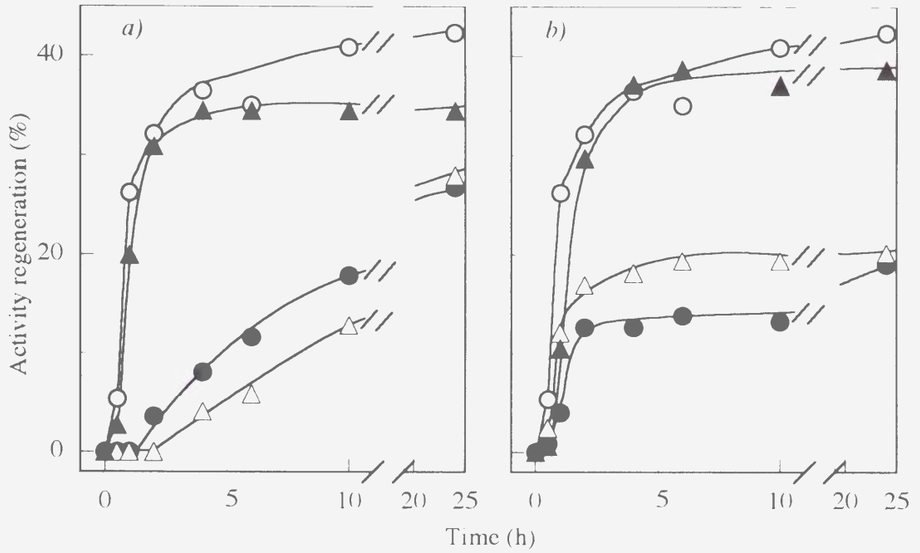


Fig. 8. Recovery of the activity on air oxidation in the absence of glutathione.
Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. Hydrolytic activity toward C>p was measured at various time intervals. The theoretical activity was taken as 100%. (a) Wild type enzyme (○), V124A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

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. 9. The most effective concentrations of glutathiones were 2 mM GSH and 0.2 mM GSSG for the refolding of RNase A [108]. 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 a little lower yield of V124G and V124K mutants.

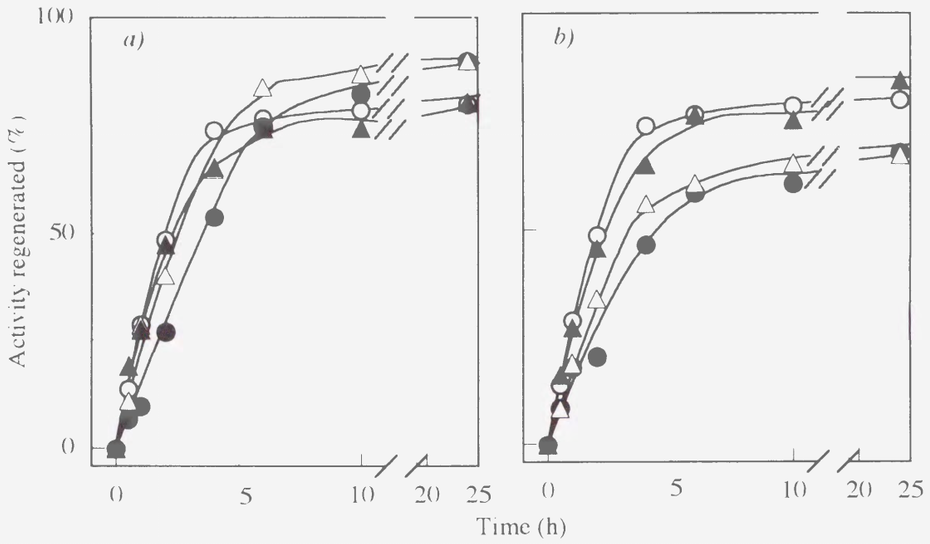


Fig. 9. Recovery of the activity in the presence of GSH and GSSG. Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. (a) Wild type enzyme (○), V124A (●), V124W (△) and V124L (▲). (b) Wild type enzyme (○), V124K (●), V124G (△) and V124E (▲). See Fig. 8 for other conditions.

IV. 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 [109].

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 [109]. 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 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 [109]. It is most likely that the low regeneration rate obtained for V124K is due to the electric repulsion. In contrast, 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) [59, 110, 111], 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 [59]. It is quite likely that the C-terminal region of RNase A is one of the nuclei positions in folding of the enzyme.

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

V. Summary

An important role of C-terminal amino acid residues of bovine pancreatic ribonuclease A (RNase A) in the formation of three-dimensional structure was previously implied. The C-terminal amino acid, Val124, was replaced with amino acid residue 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 recovery rate 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 to 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 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.

Chapter 3. Effects of deletion of the C-terminal region and mutation of Asp121 on the properties and regeneration of RNase A

I. Introduction

The previous chapter showed that the hydrophobic interaction of Val124 with nearby hydrophobic residues, Ile106 and Ile107, is important for efficient packing of the C-terminal region. To investigate the role of the C-terminal Val124, Ser123, Ala122 and Asp121 of RNase A, mutant enzymes of des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A in which these amino acid residue(s) was deleted and D121A, D121E, and D121K RNase A in which Asp121 was replaced with alanine, glutamic acid, or lysine were prepared and subjected to the folding study. 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.

II. Experimental procedures

Materials

Plasmid pETRN coding the RNase A gene was constructed according to the method of delCardayre *et al.* [99]. An *Escherichia coli* strain, *Epicurian Coli XL-1Blue*, was used as host cells for mutagenesis. An *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, Japan). All other reagents were of analytical grade and purchased from Nacalai Tesque (Kyoto, Japan).

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 [112] for the construction of C-terminal deletion and Ala(121-124) mutant enzymes and two kinds of oligonucleotide primers [100]. Mutant plasmid pETRN-V124A was used to make GC content of the primers optimum. The sequences of the oligonucleotide primers designed to construct des-124-RNase A were 5'-C-TTT-GAC-GCC-AGT-TGA-TAA-CTA-GAT-AAG-CTT-GCG-3' and 5'-CGC-AAG-CTT-ATC-TAG-TTA-TCA-ACT-GGC-GTC-AAA-G-3'. The sequences of the oligonucleotide primers designed to construct des-(123-124)-RNase A were 5'-CAC-TTT-GAC-GCC-TGA-GCC-TAA-CTA-GAT-AAG-3' and 5'-CTT-ATC-TAG-TTA-GGC-TCA-GGC-GTC-AAA-GTG-3'. The sequences of the oligonucleotide primers designed to construct des-(122-124)-RNase A were 5'-CT-GTC-CAC-TTT-GAC-TGA-AGT-GCC-TAA-CTA-G-3' and 5'-C-TAG-TTA-GGC-ACT-TCA-GTC-AAA-GTG-GAC-AG-3'. The sequences of the oligonucleotide primers designed to construct des-(121-124)-RNase A were 5'-CCT-GTC-CAC-TTT-TAA-GCC-AGT-GCC-TAA-C-3' and 5'-G-TTA-GGC-ACT-GGC-TTA-AAA-GTG-GAC-AGG-3'. The sequences of the oligonucleotide primers designed to construct Ala(121-124) RNase A were 5'-GTC-CAC-TTT-GCC-GCC-GCC-GCC-TAA-CTA-G-3' and 5'-C-TAG-TTA-GGC-GGC-GGC-GGC-AAA-GTG-GAC-3'. The sequences of the oligonucleotide primers designed to construct D121A RNase A were 5'-CCT-GTC-CAC-TTT-GCC-GCC-AGT-GTT-TAA-C-3' and 5'-G-TTA-AAC-ACT-GGC-GGC-AAA-GTG-GAC-AGG-3'. The sequences of the oligonucleotide primers designed to construct D121E RNase A were 5'-CCT-GTC-CAC-TTT-GAG-GCC-AGT-GTT-TAA-C-3' and 5'-G-TTA-AAC-ACT-GGC-CTC-AAA-GTG-GAC-AGG-3'. The sequences of the oligonucleotide primers designed to construct D121K RNase A were 5'-CCT-GTC-CAC-TTT-AAG-GCC-AGT-GTT-TAA-C-3' and 5'-G-TTA-AAC-ACT-GGC-

CTT-AAA-GTG-GAC-AGG-3'. All the 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).

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Mutant RNase A was expressed by the method of Dodge and Scheraga [101] 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 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, 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 x 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 water.

The purified enzyme was concentrated and kept at 4°C.

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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 with a jacketed cell of 1 cm optical path length. 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, 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 x 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 under the normal condition. The solution was incubated at 25°C.

In the case of the regeneration in the presence of GSH and GSSG, 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 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 [103]. For other experiments, the protein concentrations of unfolded and folded RNase A were determined using the extinction coefficients of 8,500 M^{-1} cm^{-1} at 275 nm [104] and 9,800 M^{-1} cm^{-1} at 277.5 nm [105], 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] [106]. An aliquot of 150 µl removed from the RNase A reoxidation solution of RNase A 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 the extinction coefficient of 13,380 M^{-1} cm^{-1} at 412 nm [107].

Mass Spectrometry Analysis

Mass spectra were analyzed with a Voyager RP Biospectrometry Workstation (PerSeptive Biosystems, Massachusetts, USA). Samples were mixed with the same quantity of a matrix reagent [5 mg/ml α-cyano-4-hydroxy-cinnamic acid and 0.05% trifluoroacetic acid in 50% acetonitrile]. Two microliter of the mixture was applied on a sample tray, dried at room temperature, and used for the analysis.

Cyanogen Bromide Cleavage

Three hundred microliter of the protein solution was mixed with 700 µl of formic acid. One hundred microliter of 10 mg/ml cyanogen bromide was added to the mixture and the solution was incubated at 37°C overnight. The solution was dried with an Automated Environmental SpeedVac System AES1010 (Savant, New York, USA) and dissolved in 50 µl of distilled water. Then, the solution was subjected to the analysis of mass spectrometry to investigate the fragmentation of RNase A.

Amino Acid Analysis

Protein was hydrolyzed with 6 M HCl at 110°C for 22h. After the solution was dried with SpeedVac AES1010, the dried material 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).

III. Results

Expression and Purification of Mutant RNase A

Two to 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 *E. coli* cells (wet weight) collected from 1 liter culture. All of the purified mutant RNase A gave a single-stained band on SDS-PAGE and an expected amino acid composition (Table 2).

Table 2 Amino acid compositions of wild type and mutant RNase A.

	des124-	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
Trp	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
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

Properties and Refolding of C-terminal Deleted Mutant RNase A

The wild type and C-terminal deleted mutant enzymes (des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A) showed a little different CD spectra (Fig. 10). Amount of secondary structures estimated from obtained CD data is shown in Table 3. Helix contents of des-124- and des-(123-124)-RNase A and sheet contents of des-(122-124)- and des-(121-124)-RNase A decreased. Decrease of sheet contents is because of deletion of the C-terminal

amino acid residues which consists of the C-terminal β sheet, but cause of decrease of helix contents is not clear. The thermal denaturation profiles monitored as to changes in [θ] value at 222 nm showed that thermal denaturation of all the mutant enzymes followed a two-state transition (Fig. 11). 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, whereas des-(121-124)-RNase A exhibited much lower value than the wild type enzyme.

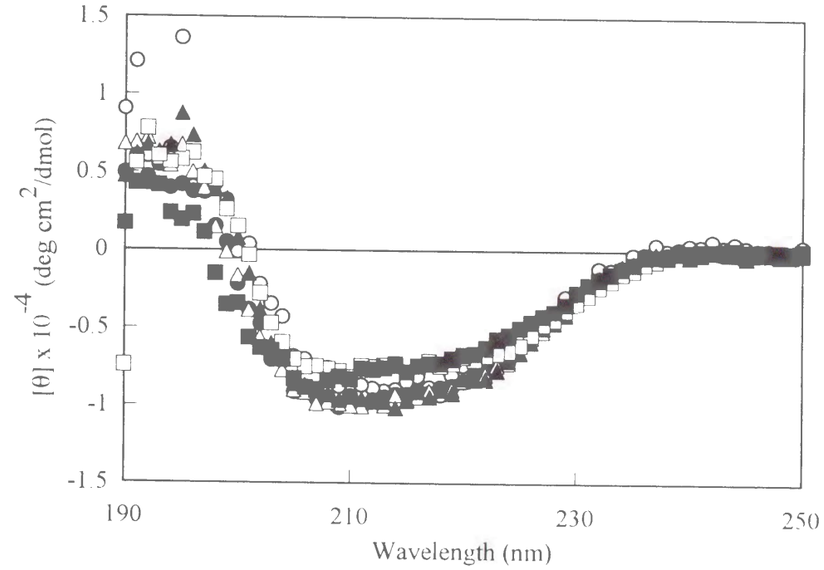

Fig. 10. CD spectra of the wild type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■).

Table 3 Amount of secondary structures of wild type and mutant RNase A

	α-helix (%)	β-sheet (%)
Wild type	21±2	44±4
des-124-	17±2	40±4
des-(123-124)-	17±2	45±4
des-(122-124)-	22±2	36±4
des-(121-124)-	24±2	27±4
Ala(121-124)	12±2	45±4
D121A	15±2	39±4
D121E	15±2	43±4
D121K	16±2	39±4

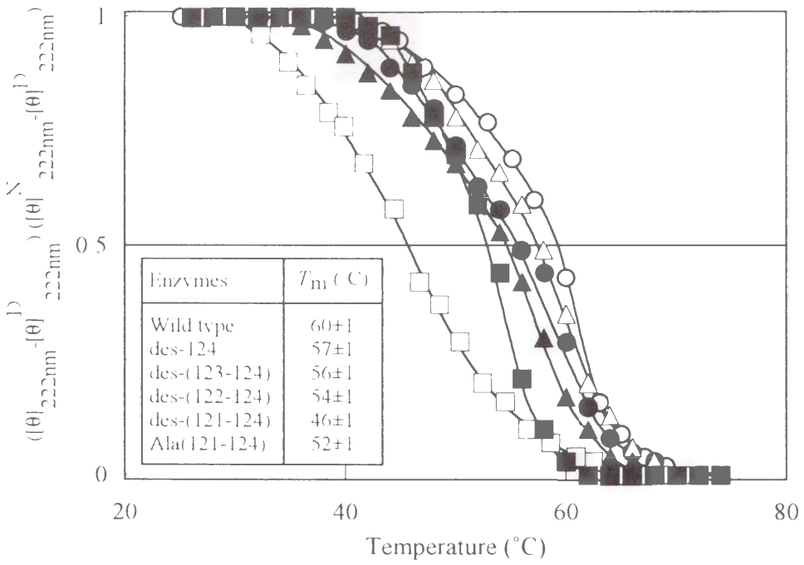

Fig. 11. Thermal denaturation profiles of the wild type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■).

Kinetic parameters of the wild type and C-terminal deleted mutant RNase A for the cleavage of C>p were determined by means of Hanes-Woolf plots (Table 4). K_m of des-124- and des-(123-124)-RNase A was slightly higher and k_{cat} of these mutant enzymes was slightly lower than that of the wild type enzyme; thus k_{cat}/K_m of these mutants was a little 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 construction of the active center. K_m of des-(122-124)- and des-(121-124)-RNase A was higher than that of the wild type and k_{cat} of these mutant enzymes was lower than that of the wild type enzyme; thus k_{cat}/K_m of these mutant RNase A was smaller than that of the wild type enzyme. Ala122 and Asp121 are located near the catalytic residue, His119, so the deletion of these residues affects the hydrolytic activity of RNase A.

Table 4 Kinetic parameters of the wild type and mutant RNase A for the cleavage of C>p

Enzymes	K_m	k_{cat}	k_{cat}/K_m
	mM	min^{-1}	$mM^{-1} \cdot min^{-1}$
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

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. 12). The rate of recovery of the activity at an early period of folding decreased in the order of des-124-, des-(123-124)-, des-(122-124)-, and des-(121-124)-RNase A. The fully recovered activity of des-124-RNase A reached to approximately 90% of that of the wild type enzyme, whereas the recovered activity of other mutant enzymes were as follows; des-(123-124)-RNase A 80% and des-(122-124)-RNase A 70%. Des-(121-124)-RNase A recovered no activity. Duration of the initial lag phase of the regeneration became shorter in the order of des-(122-124)-, des-(123-124)-, and des-124-RNase A. 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 (Fig. 12).

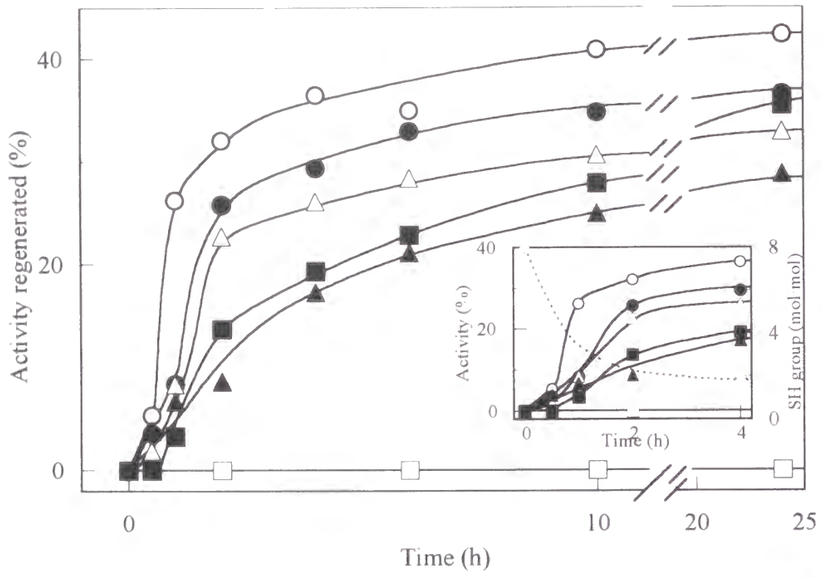

Fig. 12. Activity regeneration of the wild type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■) on air oxidation in the absence of glutathione. Proteins were denatured with 8 M urea and reduced with 2-mercaptoethanol. Hydrolytic activity toward C>p was measured at various time intervals. The activity of each native enzyme was taken as 100%. The insertion shows the activity regeneration and disulfide bond formation in the early period of the regeneration process.

The recovery of the activity in the presence of glutathione for the fully denatured and reduced wild type and mutant RNase A is shown in Fig. 13. The most effective concentrations of glutathiones were 2 mM GSH and 0.2 mM GSSG for the refolding of RNase A as previously reported [108]. The recovery of the activity of both the wild type and all the mutant RNase A significantly increased when glutathione was present. The rates of recovery of the activity at an early period of folding decreased in order of des-(122-124)-, des-124-, des-(123-124)-, and des-(121-124)-RNase A. The recovered activity of the mutant enzymes reached the same level as in the case of the wild type enzyme except des-(121-124)-RNase A. No activity was recovered from denatured and reduced des-(121-124)-RNase A. This is because incubation time for refolding was too short (here, incubation time was 24 h, but incubation for refolding was performed for 72 h in the purification steps).

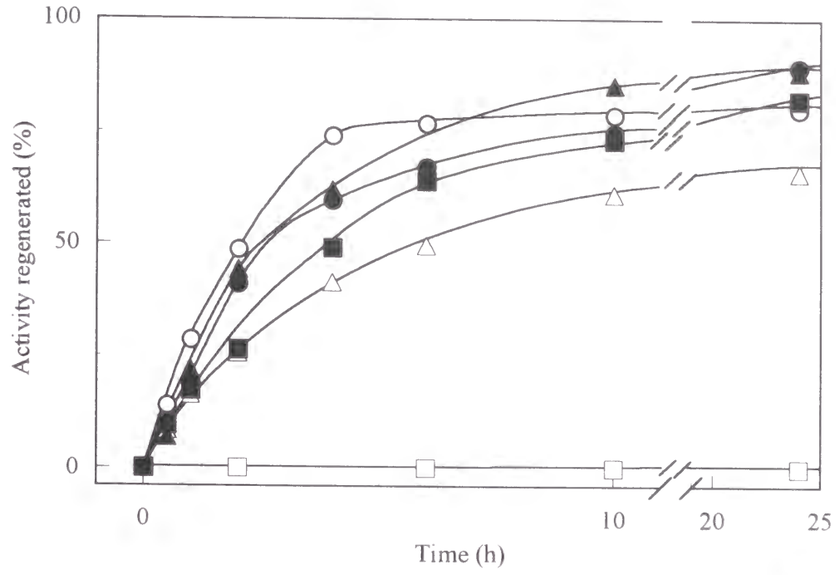

Fig. 13. Activity regeneration of the wild type enzyme (○), des-124- (●), des-(123-124)- (△), des-(122-124)- (▲), des-(121-124)- (□), and Ala(121-124) RNase A (■) in the presence of 2 mM GSH and 0.2 mM GSSG. See Fig. 12 for other details.

After air oxidation for 24 h, C-terminal deletion mutant enzymes were subjected to the cleavage by cyanogen bromide to investigate whether disulfide bonds were correctly paired or not. If disulfide bonds pair correctly, only two peptide fragments, molecular weight of which is about 1,500 and 12,000, are formed by cyanogen bromide cleavage [3]. However, if pairs of disulfide bonds are completely random, other two peptide fragments, molecular weight of which is 5,500 and 6,500, are also formed [3]. Six of 105 sets of four disulfide bonds produce these 5,500 and 6,500 peptide fragments. Among four C-terminal deletion mutant enzymes, only des-(121-124)-RNase A produced peptide fragments of molecular weight 5,500 and 6,500 after air oxidation for 24h. Amount of these fragments was about 6% of that of the fragment of molecular weight 12,000, and this result shows that disulfide bonds of des-(121-124)-RNase A were randomly paired.

Properties and Refolding of Ala(121-124) Mutant RNase A

The wild type and Ala(121-124) mutant enzyme showed indistinguishable CD spectra (Fig. 10). The thermal denaturation profile monitored as to changes in [θ] value at 222 nm showed that thermal denaturation of Ala(121-124) mutant enzyme followed a two-state transition (Fig. 11). The T_m value obtained was nearly the same as that of the wild type enzyme.

Kinetic parameters of the wild type and Ala(121-124) mutant RNase A for the cleavage of C>p were determined by means of Hanes-Woolf plots (Table 4). K_m of Ala(121-124) mutant RNase A became higher than that of the wild type enzyme and its k_{cat} became lower than that of the wild type enzyme; thus k_{cat}/K_m of the mutant RNase A was smaller than that of the wild type enzyme.

Activity regeneration by air oxidation in the absence of glutathione was compared for the fully denatured and reduced forms of the wild type and Ala(121-124) mutant RNase A (Fig. 12). The recovery profile of the activity of Ala(121-124) was almost the same as that of des-(122-124)-RNase A. The fully recovered activity of Ala(121-124) RNase A reached approximately 90% of that of the wild type enzyme. When free sulfhydryl groups were titrated during the refolding process, the sulfhydryl group number of the mutant enzyme decreased at the same rate as for the wild type enzyme, and no lag phase was observed for Ala(121-124) mutant enzyme (Fig. 12).

Activity regeneration in the presence of glutathione from fully denatured and reduced wild type and Ala(121-124) mutant RNase A is shown in Fig. 13. Activity regeneration of both the wild type and the mutant RNase A significantly increased when glutathione was present. The recovered activity of the mutant enzymes reached the same level as in the case of the wild type enzyme.

Properties and Refolding of D121 Mutant RNase A

The wild type and D121 mutant RNase A showed indistinguishable CD spectra (Fig. 14). The thermal denaturation profiles monitored as to changes in [θ] value at 222 nm showed that thermal denaturation of all mutant enzymes followed a two-state transition (Fig. 15). 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 than that of the wild type enzyme.

Kinetic parameters of D121 mutant RNase A for the cleavage of C>p were determined by means of Hanes-Woolf plots (Table 4). K_m of mutant RNase A was almost the same as that of the wild type, but k_{cat} was lower than that of the wild type; thus k_{cat}/K_m of mutant RNase A was smaller than that of the wild type enzyme. This result shows that hydrolytic activity for C>p of the mutant enzymes became lower.

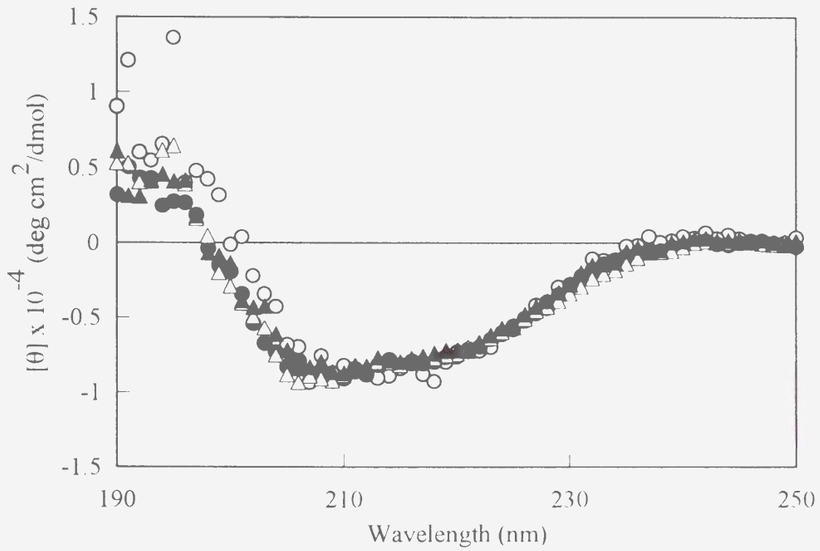

Fig. 14. CD spectra of the wild type enzyme (○), D121A (●), D121E (△), and D121K (▲).

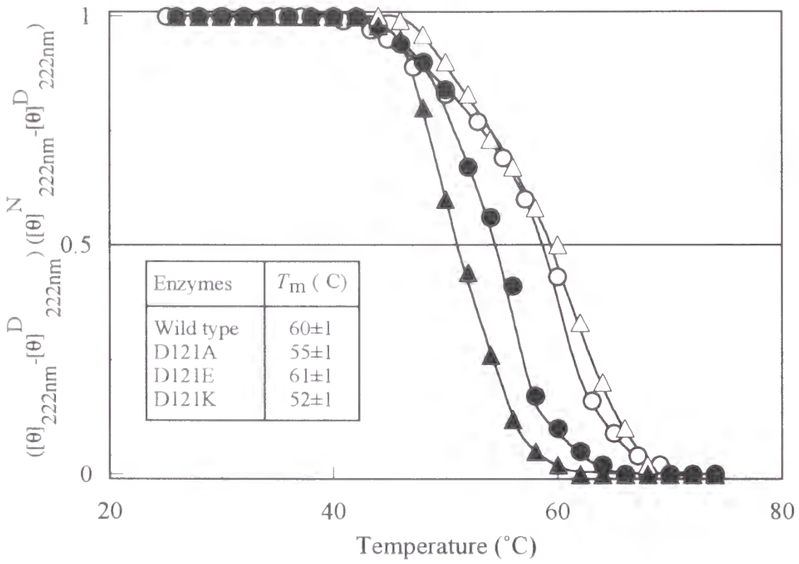

Fig. 15. Thermal denaturation profiles of the wild type enzyme (○), D121A (●), D121E (△), and D121K (▲).

Activity regeneration by air oxidation in the absence of glutathione was compared for the fully denatured and reduced forms of the wild type and D121 mutant RNase A (Fig. 16). The rate of recovery of the activity at an 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 in the case of the wild type enzyme, whereas those of other mutant enzymes were as follows: D121A 91% and D121K 76%. All D121 mutant enzymes showed the initial lag phase of 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. 17. Recovery of the activity of both the wild type and the mutant RNase A significantly increased when glutathione was present. The recovery rates of activity of 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.

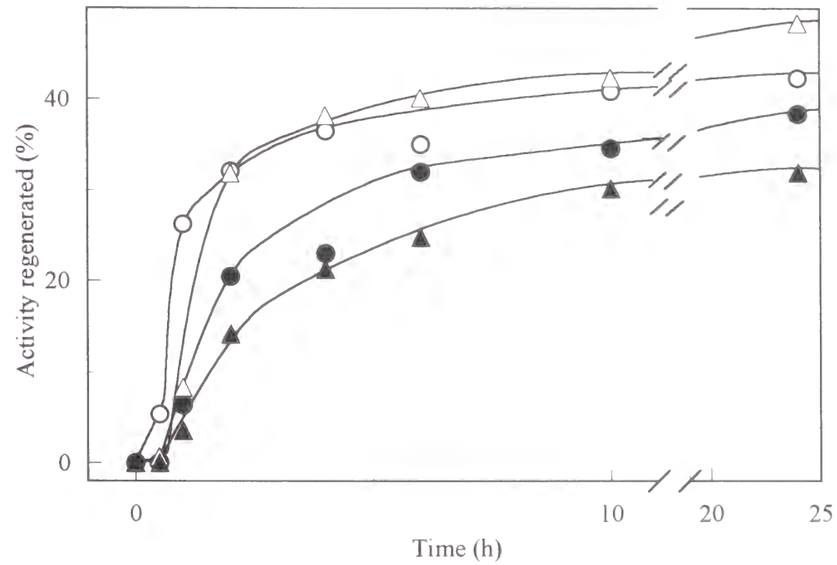

Fig. 16. Activity regeneration of the wild type enzyme (○), D121A (●), D121E (△), and D121K (▲) on air oxidation in the absence of glutathione. See Fig. 12 for other details.

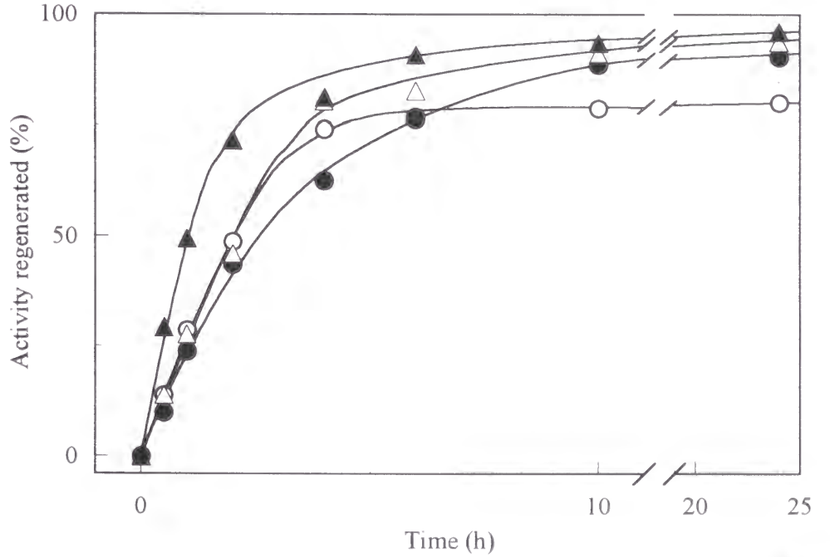

Fig. 17. Activity regeneration of the wild type enzyme (○), D121A (●), D121E (△), and D121K (▲) in the presence of 2 mM GSH and 0.2 mM GSSG. See Fig. 12 for other details.

IV. Discussion

C-terminal four amino acid residues are conserved among many mammalian pancreatic ribonucleases. The C-terminal region of RNase A composes of a β strand [109] and interacts with the surrounding amino acid residues to keep the rigid tertiary structure of RNase A. Deletion or mutation of them produces an enzyme whose secondary structure is almost indistinguishable from that of the wild type enzyme as judged from CD spectra, but it gives adverse effects on the activity and the thermal stability.

Des-124-RNase A is a mutant enzyme lacking the C-terminal amino acid, Val124, of the wild type RNase A. Val124 interacts with nearby hydrophobic residues, Ile106 and Ile107 [109]. When Val124 is deleted, the hydrophobic interaction with isoleucine residues becomes weakened. The lack of this interaction may be a cause for the inability to form compact structure around the C-terminal region, and this may result in slight reduction of 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 [109]. However, only Ser123 has no such an interaction. This may be because hydrolytic activity of des-(123-124)-RNase A was not so reduced from that of the wild type and the activity regeneration rate of this mutant enzyme was not so different from that of des-124-RNase A. Our results show that the formation of these hydrogen bonds is very important element for correct folding of RNase A.

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

Replacement of Asp121 causes slight reduction of the hydrolytic activity for C>p. This result seems to be natural because Asp121 is located near

His119 and has an influence on the activity of RNase A [114-116]. Replacement of this residue also affected on the regeneration of the activity from the fully denatured and reduced forms. However, D121E showed almost the same folding profile as the wild type enzyme. This result implies that a negative charge of Asp121 is an important element for refolding of RNase A to form the compact structure by ionic interaction with surrounding residues during the regeneration.

Des-(121-124)-RNase A recovered no activity. This is because the C-terminal region of this mutant enzyme lacks the side chain interaction and cannot keep the compact structure. Without these amino acid residues, RNase A is not able to form a core structure in the early period of the refolding, and efficiency of the regeneration is reduced because of the formation of random paired disulfide bonds. Our result shows that formation of β sheets around the C-terminus is essential for the construction of the active RNase A structure.

Deletion or mutation of the C-terminal amino acid residues affected on the regeneration of activity from the fully denatured and reduced forms. The regeneration rates of all 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 almost the same when GSH and GSSG were used as oxidizing agents except des-(121-124)-RNase A. A 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, C-terminal deletion mutant enzymes, especially des-(121-124)-RNase A, tend to give randomly formed disulfide bonds; thus, incorrectly folded intermediate(s) may accumulate.

In the regeneration of denatured and reduced 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, there was a significant period of initial lag phase prior to the activity appearance. It was suspected that activity appearance might be directly synchronized to the formation of disulfide bonds. As the disulfide bond-forming rates of the wild type and mutant enzymes were same and there were no lag phase in forming disulfide bonds (Fig. 12), the activity regeneration might be brought about by rearrangement of disulfide bonds. That is, incorrectly paired disulfide bonds are accumulated in the initial regeneration period followed by rearrangement for their correct pairing during the lag phase of the mutant enzymes.

In the previous chapter, it is shown that the formation of compact structure is initiated by hydrophobic interaction by the C-terminal amino acid residue, Val124, and leads efficient packing of the RNase A molecule. Results shown in this chapter suggest that a negative charge of Asp121 is also involved in this process. Therefore, unique characters of the side chains in the C-terminal four residues certainly contribute to the construction of the compact structure, but the length of the protein main chain are also very important factors for refolding of RNase A. This is shown by the present result that Ala(121-124) mutant enzyme, in which all of the C-terminal four amino acid residues replaced with alanine residues, also regenerated the activity from the fully denatured and reduced forms, even though the intrinsic side chain interactions in the C-terminal region must be weakened.

The C-terminal region of RNase A is one of "chain folding initiation sites" (CFIS) [59, 110, 111], in which 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 synthesized C-terminal 20 amino acid peptide of RNase A sequence has a partially ordered structure being stabilized through a hydrophobic interaction [59]. It is quite likely that C-

terminal region of RNase A is one of the nuclei positions in folding of the enzyme.

It is concluded that the C-terminal region of RNase A plays an important role in refolding by providing an environment to form a compact structure in the respect of interaction by side chains of amino acid residues and existence of main chain in the C-terminal region. It may be important that not only the function and structure of the folded mutant protein in which one or some amino acid residues are replaced with other ones but also refolding process is investigated to clear the role of individual amino acid residue in the construction of the protein.

V. Summary

The C-terminal amino acid residues of bovine pancreatic ribonuclease A (RNase A) form a core structure in the initial folding process to lead the construction of the tertiary structure. Roles of the C-terminal four amino acids in structure, function, and refolding were studied with recombinant mutant enzymes in which these residues were deleted or replaced. Mutant enzymes purified were analyzed with secondary structure, thermal stability, and activity regeneration from denatured and reduced state. The C-terminal deleted mutant enzymes showed the lower hydrolytic activity for C>p and almost indistinguishable CD spectra as compared with the wild type enzyme. The rate of recovery of the activity was a significantly different among the C-terminal deleted mutant enzymes when air oxidation was employed in the absence of GSH and GSSG: the rate decreased in the order of des-124-, des-(123-124)-, and des-(122-124) -RNase A. However, the regeneration rates of mutant RNase A in the presence of GSH and GSSG were almost the same. Des-(121-124)-RNase A recovered no activity both in the presence and the absence of glutathione because of random formation of disulfide bonds. Mutant enzyme in which all

of the C-terminal four amino acid residues were replaced with alanines showed the lower hydrolytic activity, indistinguishable CD spectra compared with wild type enzyme, and also recovered its activity by the air oxidation. Hydrolytic activity of the D121 mutant enzymes decreased as compared with the wild type. The D121 mutant enzymes showed indistinguishable CD spectra from that of the wild type. The rates of recovery of activity of D121A and D121K became lower than that of the wild type enzyme, while the rate of recovery of D121E was almost the same as that of the wild type. The C-terminal amino acids significantly influence on the formation of correct disulfide bonds during the refolding process and both interaction of amino acid residues and existence of main chain around the C-terminal are important for an efficient packing of the RNase A molecule.

Conclusion

The rate of recovery of the activity decreased in the order of des-124-, des-(123-124)-, and des-(122-124) -RNase A when air oxidation was performed in the absence of GSH and GSSG. Des-(121-124)-RNase A recovered no activity with air oxidation because of the formation of incorrectly paired disulfide bonds.

The results of the refolding studies using Val124 mutant RNase A imply that the C-terminal amino acid significantly influences the formation of correct disulfide bonds during the refolding process and that the hydrophobic interaction and the size of the Val124 side chain is important for efficient packing of the RNase A molecule.

The results of the refolding studies using Asp121 mutant RNase A imply that a negative charge of Asp121 is an important element for refolding of RNase A to form the compact structure by ionic interaction with surrounding residues during the regeneration.

The results of the study of Ala(121-124) show that not only unique characters of the side chains in the C-terminal four residues but also the length of the protein main chain certainly contribute to the construction of the compact structure of RNase A.

Thus, the C-terminal region of RNase A plays a very important role in the formation of the tertiary structure.

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