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Stabilization of Human RNase 1 by Introduction of a Disulfide Bond between Residues 4 and 118[1]

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In order to stabilize human RNase 1 by introduction of an intramolecular cross-link, a mutant protein (4-118CL RNase 1), in which Arg4 and Val118 are replaced with cysteine residues and linked by a disulfide bond, was designed and expressed in *Escherichia coli* as inclusion bodies. The 4-118CL RNase 1 that refolded under redox conditions was a monomer without free SH groups and retained 11% of the activity of the wild-type recombinant RNase 1, indicating that the mutant enzyme was correctly folded with the formation of an additional disulfide bond between Cys4 and Cys118. From guanidium chloride denaturation experiments based on the assumption of a two-state transition for unfolding, it was demonstrated that the introduction of the present cross-link increased the thermodynamic stability of RNase 1 by 2.0 kcal/mol. This value was lower than that, 5.4 kcal/mol, theoretically calculated from the reduction of chain entropy of the unfolded state due to the introduction of the cross-link. These results suggest that the present cross-link also destabilized the folded state of RNase 1 by 3.4 kcal/mol. Along with the increase in the thermodynamic stability, the stability of RNase 1 against trypsin digestion was also significantly increased by the introduction of this cross-link. It is likely, although not proven, that stabilized human RNases are favorable for clinical use, because human RNase-based immunotoxins should have long half-lives as to proteolytic degradation after endocytosis.

Key words: chain entropy, disulfide, proteolysis, RNase, stabilization.

Human pancreatic RNase (RNase 1) is a secretory small globular protein possessing chemotherapeutic potential due to its ability to degrade RNA molecules when it enters the cytosol (*1–3*). Fusion proteins of human RNases with growth factors (*1*, *2*) or with an antibody (*4*) have been shown to be cytotoxic for targeted cells expressing the corresponding ligand receptors or cell surface antigen (*5*). However, the cytotoxicity of RNase 1 was not so high (*1*), probably because of its high affinity to endogenous RNase inhibitor and of its low stability as to proteolytic degradation. On the other hand, onconase from frog oocytes, a cytotoxic RNase, is extremely stable compared with most nontoxic members of the pancreatic-type RNase family (*6*). Furthermore, most bacterial and plant toxins employed so far as toxic domains for immunotoxins are unusually stable and resistant to proteolysis (*7*). After internalization of an immunotoxin through a specific cell surface receptor or antigen, the endosome will fuse to lysosome containing lysosomal proteases such as cathepsins. Therefore, the stability against protease would determine the half-life of a protein after endocytosis, and would be a key characteristic of the toxic domain. In this regard, stabilization of RNase 1 may be useful for its application to immunotoxins.

A globular protein is usually in equilibrium between the folded (N) and unfolded (D) states (N $\rightleftarrows$ D, K_D = [D]/[N], K_D is the equilibrium constant for reversible denaturation). Under physiological conditions, the equilibrium greatly favors the folded state. As discussed by Imoto *et al.* (*8*) and Yamada *et al.* (*9*), the proteolytic degradation of a globular protein usually does not directly proceed *via* the folded state (N) but *via* the unfolded state (D), and the rate of proteolysis of the protein is proportional to the concentration of D ([D] or K_D), unless the concentration of protease is extremely high. Therefore, in order to stabilize a protein against proteolysis, we should increase the thermodynamic stability of the protein to shift the unfolding equilibrium in the direction of N. Theoretically, the introduction of an intramolecular cross-link decreases the chain entropy of an unfolded protein (*10*), thereby destabilizing the unfolded state and, as a result, stabilizing the protein thermodynamically. In fact a number of proteins, such as chicken lysozyme (*11*, *12*), bovine pancreatic RNase (RNase A) (*13*), subtilisin (*14*), and T4 lysozyme (*15*, *16*), have been successfully stabilized by means of chemically or genetically introduced intramolecular cross-links. However, the introduction of an intramolecular cross-link often induces strain in the folded state of a protein and unfavorable cross-links sometimes completely compensate for the stabilization effects mentioned above (*9*, *16*). These results suggest that the introduction of an engineered disulfide is an effective strat-

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egy for stabilizing RNase 1, as long as mutation sites are properly selected.

Here, we selected a pair of residues, 4 and 118, in RNase 1 based on molecular modeling and converted them to cysteine residues by means of site-directed mutagenesis. The engineered RNase 1, possessing a Cys-4/Cys-118 bond (4-118CL RNase 1), was found to be thermodynamically stabler by about 2 kcal/mol and also kinetically stabler against trypsin digestion than wild-type RNase 1.

MATERIALS AND METHODS

Materials—RNase A was obtained from Sigma (Type XII-A). RNase 1 was a recombinant protein expressed in *Escherichia coli* and purified as described previously (*17*). *E. coli* strain MM294(DE3)/pLysS was that described previously (*18*). L-1-(*p*-Tosylamido)-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. All other chemicals were of the highest purity commercially available.

Expression and Purification of 4-118CL RNase 1—The Arg4→Cys and Val118→Cys mutations were introduced by means of a Quick Chang Site-directed Mutagenesis Kit (Stratagene) into a RNase 1 expression plasmid, pBO26 (*17*), to give a plasmid, pBO244, for the expression of 4-118CL RNase 1. The transformed cells, MM294(DE3)/pLysS/pBO244, were used for protein expression. The procedures for expression, isolation of inclusion bodies, and solubilization of the protein were the same as described previously (*1*). The optimized folding reaction was performed as described previously (*19*). Briefly, inclusion bodies of 4-118CL RNase 1 were solubilized in 6 M guanidium chloride (GdmCl) in the presence of 2-mercaptoethanol, diluted to a final protein concentration of 100 μg/ml with a redox buffer [10 mM Tris-HCl, pH 8.5, containing 0.5 mM oxidized glutathione (Kojin, Tokyo), 2 mM reduced glutathione/2-mercaptoethanol, 30% (v/v) glycerol, and 0.4 M GdmCl], and then incubated for 36 h at 4°C. After removal of insoluble materials by centrifugation, the pH was adjusted to 5.0 with acetic acid, and the supernatant was diluted four times with water. The refolded protein was concentrated by adsorption to a column of 10 ml of CM-Toyopearl 650C (Tosoh, Tokyo), followed by elution with 0.4 M NaCl, pH 5.0. The eluted protein was further purified by ion-exchange chromatography on an open column of CM-Toyopearl 650M (Tosoh; 13 × 680 mm), which was eluted with a linear gradient from 1 liter of 50 mM sodium acetate buffer, pH 5.0, to 1 liter of the same buffer containing 0.8 M NaCl. The fractions containing pure 4-118CL RNase 1 were pooled, precipitated with saturated ammonium sulfate, dissolved in a small amount of water, exhaustively dialyzed against water for 4 days, and then lyophilized to give 4-118CL RNase 1.

GdmCl-Induced Unfolding of 4-118CL RNase 1—In order to compare the thermodynamic stabilities of 4-118CL RNase 1, RNase 1, and RNase A, denaturation experiments were carried out in the presence of various concentrations of GdmCl (Katayama Chemical, Okayama) in 50 mM sodium acetate buffer at a protein concentration of 20 μM, pH 5.4, and 32°C. GdmCl-induced unfolding of proteins was monitored by measuring the change in absorbance at 287 nm using a 1-cm light path quartz cell.

Stability of 4-118CL RNase 1 against Trypsin Digestion—The trypsin digestion of each RNase was carried out under physiological conditions. Five micrograms of RNase was dissolved in 15 μl of 75 mM Tris-HCl buffer, pH 8.0, in the presence or absence of trypsin, and then incubated at 37°C for 40 min. The reactions were stopped by the addition of 15 μl of ×2 sample buffer consisting of 2% SDS, 2% 2-mercaptoethanol, 40% glycerol and 50 mM Tris-HCl, pH 6.8. The mixture was boiled for 3 min and then analyzed by SDS-PAGE on a 15% polyacrylamide gel.

Stability of Cys-4/Cys-118 Bond against Reduction—The stability of the Cys-4/Cys-118 bond in 4-118CL RNase 1 against reduction was examined under various redox conditions. That is, 4-118CL RNase 1 (250 μg/ml, 17 μM) was equilibrated in 50 mM Tris-HCl buffer, pH 8.0, containing a total 10 mM glutathiones comprising various ratios of reduced (GSH) and oxidized (GSSG) forms at 37°C for 40 min. All remaining sulfhydryl groups were alkylated with 25 mM iodoacetamide. For comparison, RNase 1 was also treated with 10 mM oxidized glutathione (GSH/GSSG = 0) or with 10 mM reduced glutathione (GSH/GSSG = ∞) at pH 8.0 and 37°C for 40 min, followed by the addition of 25 mM iodoacetamide. Completely reduced and *S*-carboxamidomethylated RNase 1 and 4-118CL RNase 1 samples were prepared by reduction with 2.5 mM dithiothreitol (DTT) at pH 8.0 and 50°C for 40 min, followed by *S*-alkylation with 25 mM iodoacetamide. The protein samples thus prepared were then analyzed by SDS-PAGE on a 15% polyacrylamide gel under non-reducing conditions.

Assay for RNase Activity—The RNase activities of RNases at pH 7.5 and 25°C were measured using yeast RNA as a substrate as described elsewhere (*17*).

RESULTS

Forecast of Increase in Stability of 4-118CL RNase 1—The double mutation (R4C and V118C) was introduced with the aim of stabilizing RNase 1 through the introduction of a disulfide bridge between residues 4 and 118, because the introduction of an intramolecular cross-linkage decreases the chain entropy of an unfolded protein, resulting in the destabilization of the denatured state and therefore in the net stabilization of the protein. Although the three-dimensional structure of RNase 1 has not been determined, its structure is considered to be similar to that of RNase A, because the overall structures of RNases belonging to a pancreatic-type RNase family, of which the three-dimensional structures have been determined, closely resemble each other (*20–26*). Therefore, the distance information on RNase A (PDB entry 9RTA) (*20*) was used to select the best pair of mutations. For this purpose, pairs of C_βs were searched for so as to minimize possible strain energy induced by the introduction of an additional disulfide bridge into the folded conformation, and the Arg4 and V118 pair was found. Namely, the distances between C_βs in four disulfide bridges of RNase A are 3.8–3.9 Å, while the distance between C_βs of the corresponding Ala4 and Val118 for RNase A (9RTA) is 4.18 Å.

According to the method of Lin *et al.* (*13*), the change in chain entropy of the unfolded state of RNase 1 due to the cross-linking between residues 4 and 118 (4-118CL) can be calculated to be −17.75 eu, which corresponds to an increase in the stability of RNase 1 of 5.4 kcal/mol at 32°C. Therefore, it is expected that 4-118CL RNase 1 is stabler

than RNase 1 by 5.4 kcal/mol at 32°C unless the folded state of RNase 1 is affected by the cross-linking.

Expression and Purification of 4-118CL RNase 1—The mutant protein, 4-118CL RNase 1, was expressed in *E. coli* as inclusion bodies. The refolding and purification of 4-118CL RNase 1 were carried out as described under "MATERIALS AND METHODS," and 8 mg of pure 4-118CL RNase 1 was obtained from 1 liter of culture. The 4-118CL RNase 1 thus purified gave a single sharp peak on cation-exchange HPLC and reverse-phase HPLC, respectively, and no free SH groups were detected on analysis with dithiobisdinitrobenzoate (data not shown). Amino acid analysis of 4-118CL RNase 1 confirmed the consistency with the design. SDS-PAGE analysis under non-reducing conditions indicated that 4-118CL RNase 1 migrated faster than wild-type RNase 1 (Fig. 1, lanes 3 and 4). However, under reducing conditions, 4-118CL RNase 1 showed the same mobility as the wild-type protein (Fig. 1, lanes 1 and 2). These results indicate that 4-118CL RNase 1 is a monomer with all five disulfide bonds, and has a smaller hydrodynamic volume than wild-type RNase 1 with four disulfide bonds under denaturing and non-reducing conditions. This smaller hydrodynamic volume is a clear indication of the reduced chain entropy of the unfolded 4-118CL RNase 1. Although Val118 is close to the catalytic residue (His119) and highly conserved in the RNase family, 4-118CL RNase 1 retained 11.2% of the activity of RNase 1 against yeast RNA as a substrate (*19*) (Table I). Thus, we concluded that all five disulfide bonds were correctly formed on the refolding of 4-118CL RNase 1 from the reduced form to give the active enzyme.

GdmCl-Induced Unfolding and Thermodynamic Stability of 4-118CL RNase 1—As shown in Fig. 2A and Table I, RNase 1 ($C_{1/2}$ = 1.81 M) was much less stable than RNase A ($C_{1/2}$ = 2.66 M) against GdmCl at pH 5.4 and 32°C. However, 4-118CL RNase 1 ($C_{1/2}$ = 2.45 M) was only slightly less stable than RNase A. By assuming a two-state transition for unfolding, the equilibrium constant between the folded (N) and unfolded (D) states, K_D = D/N, and the free energy of unfolding, $\Delta G_D = -RT \ln K_D$, at a given concentration of GdmCl, were calculated from a GdmCl-induced unfolding transition curve (Fig. 2A). All results fitted the following equation,

$$\Delta G_D = \Delta G_D(H_2O) - m[\text{GdmCl}]$$

where $\Delta G_D(H_2O)$ is the ΔG_D value in the absence of GdmCl (Fig. 2B and Table I). The results indicate that the introduced disulfide bond stabilizes RNase 1 by 1.6 kcal/mol, which is lower than the theoretical value of 5.4 kcal/mol. Since a small error in m results in a large deviation in $\Delta G_D(H_2O)$, and since the concentration of GdmCl at the midpoint of the denaturation, $C_{1/2}$, could be accurately determined, the $\Delta G_D(H_2O)$ values for 4-118CL RNase 1 and RNase 1 were re-calculated using an average m value (3.05) and the equation, $\Delta G_D(H_2O) = mC_{1/2}$ (Table I). The stabilization energy $[\Delta\Delta G_D(H_2O)]$ of 2.0 kcal/mol thus obtained was not much different from the value obtained above. Thus, the present results are explained as follows. The introduction of a disulfide bridge between residues 4 and 118 destabilizes the denatured state by 5.4 kcal/mol due to the loss of the chain entropy, and also destabilizes the folded state by about 3.4 kcal/mol due to the strain induced by the cross-linkage. As a net effect, 4-118CL RNase 1 becomes thermodynamically stabler than intact RNase 1 by about 2 kcal/mol (Fig. 3).

Stability of 4-118CL RNase 1 against Trypsin Digestion—As shown in Fig. 4, RNase A was not appreciably

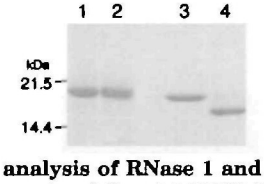

Fig. 1. **SDS-PAGE analysis of RNase 1 and 4-118CL RNase 1.** Purified RNase 1 (lanes 1 and 3) and 4-118CL RNase 1 (lanes 2 and 4) were loaded onto an SDS–15% polyacrylamide gel and then electrophoresed under reducing conditions (lanes 1 and 2) or non-reducing conditions (lanes 3 and 4). The positions of some molecular protein size markers are shown by bars on the left.

TABLE I. **Thermodynamic parameters for GdmCl-induced unfolding, and enzymatic activities of RNase A, RNase 1, and 4-118CL RNase 1.**

Molecule	Thermodynamic parameters[a]			Relative activity against yeast RNA[b] (%)
	$C_{1/2}$ (M)	m (kcal/mol/M)	$\Delta G_D(H_2O)$ (kcal/mol)	
RNase A	2.66	3.17	8.42	267
RNase 1	1.81	3.14	5.67 (5.51)[c]	100
4-118CL RNase 1	2.45	2.96	7.24 (7.46)[c]	11.2

[a]GdmCl-induced unfolding at pH 5.4 and 32°C. [b]RNase activity at pH 7.5 and 25°C. [c]Values in parentheses are those calculated using the average m value (3.05). Details are given in the text.

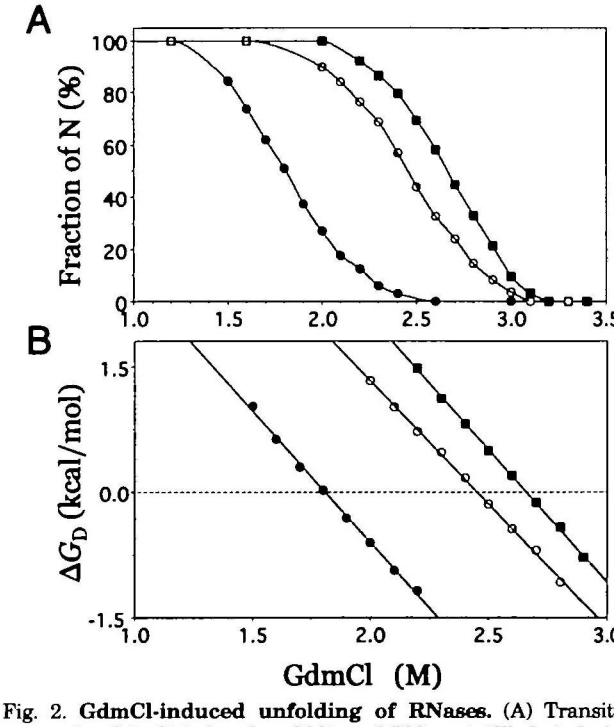

Fig. 2. **GdmCl-induced unfolding of RNases.** (A) Transition curves for GdmCl-induced unfolding of RNase 1 (filled circles), 4-118CL RNase 1 (open circles), and RNase A (squares) at pH 5.4 and 32°C. Transitions were monitored by measuring the decrease in absorbance at 287 nm. (B) ΔGs for the unfolding of RNases as a function of the GdmCl concentration.

digested by trypsin while RNase 1 was. The trypsin digestion of RNase 1 appeared to proceed in an all-or-nothing manner, because no accumulation of intermediate products was detected, suggesting the trypsin digestion of RNase 1 proceeded *via* the unfolded state. On the other hand, 4-118CL RNase 1 seemed to be resistant to trypsin under the conditions employed. The decreased susceptibility of 4-118CL RNase 1 to trypsin well agreed with its increased thermodynamic stability (Fig. 2).

Stability of 4-118CL RNase 1 against Reduction—As mentioned above (Fig. 1), disulfide (SS)-intact 4-118CL RNase 1 (with 5 SS bonds) migrated faster than SS-intact RNase 1 (with 4 SS bonds) on SDS-PAGE, while the two completely reduced RNases (without SS bonds) co-migrated but more slowly than the SS-intact ones. These observations indicate that the degree of migration is sensitive to the reduction of the Cys-4/Cys-118 bond. Therefore, the stability of the Cys-4/Cys-118 bond in 4-118CL RNase 1 at pH 8.0 and 37°C under various redox conditions with a total 10 mM GSH/GSSG was investigated by means of SDS-PAGE analysis, which was carried out after quenching the reaction with iodoacetamide (Fig. 5). As the ratio of GSH/GSSG increased, SS-intact 4-118CL RNase 1 (5-SS) gradually decreased, an intermediate with the same mobility as SS-intact RNase 1 (4-SS) transiently accumulated, and then a completely reduced species (0-SS) became predominant. To a lesser extent, another intermediate showing faster mobility than RNase 1 also transiently accumulated along with the above intermediate. When 4-118CL RNase 1 was partially reduced with DTT and quenched by iodoacetamide, the latter intermediate was not detected while the former one was obtained (data not shown). On the other hand, RNase 1 was scarcely reduced even under the high reducing conditions at GSH/GSSG = ∞, indicating that the native sets of disulfide bonds inaccessible to the solvent in

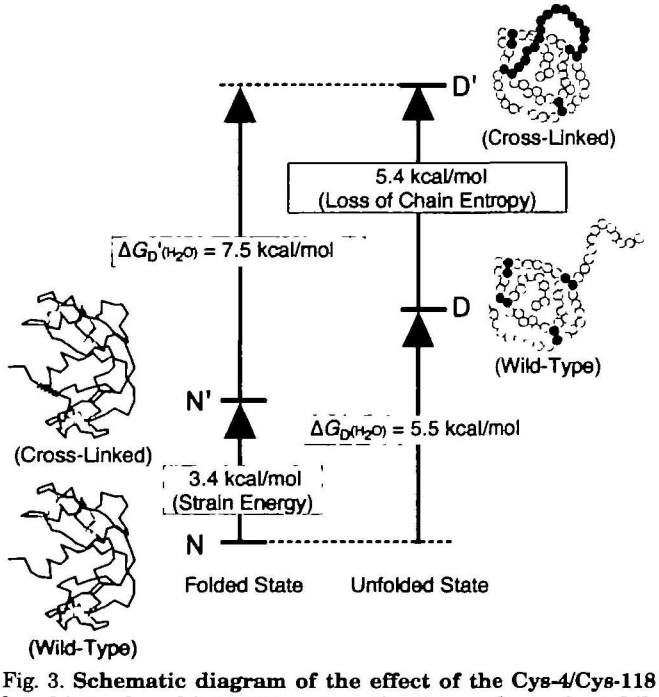

Fig. 3. **Schematic diagram of the effect of the Cys-4/Cys-118 bond introduced into RNase 1 on the thermodynamic stability at pH 5.4 and 32°C.** N and D indicate the folded and unfolded states of wild type RNase 1, and N' and D' those of 4-118CL RNase 1, respectively. $\Delta G_D(H_2O)$ and $\Delta G_D'(H_2O)$ indicate the free energy changes for the unfolding of RNase 1 and 4-118CL RNase 1 in the absence of GdmCl, respectively. The predicted folded structures of RNase 1 and 4-118CL RNase 1 are shown on the left side assuming they are essentially the same as that of RNase A. The position of the engineered disulfide bond is indicated by a dashed line. Schematic unfolded polypeptide structures are shown on the right side. One circle, except solid ones, represents about two amino acid residues. The pairs of solid circles are cystines and the shadowed circles are polypeptide chains in which the degree of freedom is restricted by the cross-linking between residues 4 and 118.

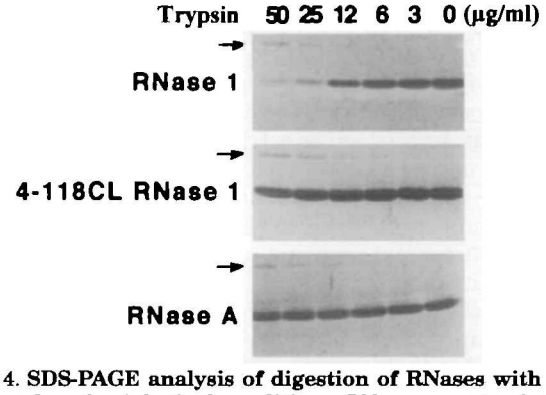

Fig. 4. **SDS-PAGE analysis of digestion of RNases with trypsin under physiological conditions.** RNases were incubated in the presence of various concentrations of trypsin at pH 8.0 and 37°C for 40 min, and then subjected to SDS-PAGE analysis under reducing conditions. The concentrations of trypsin are indicated above the lanes, respectively. Higher molecular mass bands (indicated by arrows) are those of trypsin.

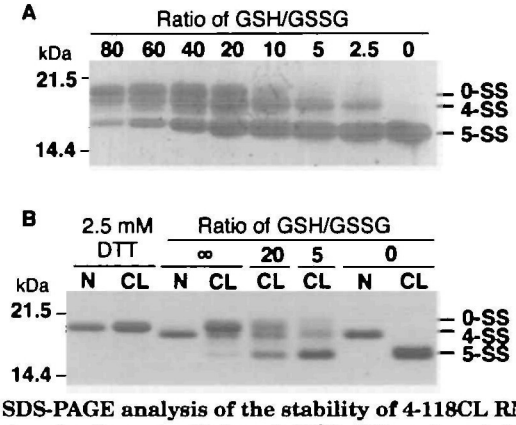

Fig. 5. **SDS-PAGE analysis of the stability of 4-118CL RNase 1 against reduction at pH 8 and 37°C.** RNase 1 and 4-118CL RNase 1 were exposed to various reducing conditions with total 10 mM mixture of reduced and oxidized glutathiones at pH 8 and 37°C for 40 min and then quenched with iodoacetamide. Samples were then analyzed by non-reducing SDS-PAGE. The GSH/GSSG ratios are indicated above the lanes, respectively. The bars, 0-SS, 4-SS, and 5-SS, on the right indicate the positions of completely reduced and *S*-carboxamidomethylated RNase 1 or 4-118CL RNase 1, SS-intact RNase 1, and SS-intact 4-118CL RNase 1, respectively. The positions of some molecular protein size markers are shown by bars on the left. (A) 4-118CL RNase 1. (B) RNase 1 (N) and 4-118CL RNase 1 (CL). The completely reduced and *S*-carboxamidomethylated RNase 1 and 4-118CL RNase 1 samples were prepared by reduction with 2.5 mM DTT at 50°C for 40 min, followed by *S*-alkylation with iodoacetamide. These are indicated by "2.5 mM DTT" above the lane in (B). See "MATERIALS AND METHODS" for details.

the folded conformation would only be reduced through the unfolded conformation. These results strongly suggest that the major intermediate with the same mobility as RNase 1 is a 4-SS intermediate in which the Cys-4/Cys-118 bond is reduced and that the minor one is the same 4-SS intermediate except that either Cys-4 or Cys-118 forms a mixed disulfide with glutathione.

The important thing is that, under the reducing conditions with the ratio of GSH/GSSG = 10, only about half of the Cys-4/Cys-118 bond in 4-118CL RNase 1 was reduced. Furthermore, SS-intact 4-118CL RNase 1 existed in a fairly large amount at GSH/GSSG = 80, and was still detectable even at GSH/GSSG = ∞. All of these results indicate that the Cys-4/Cys-118 bond is fairly stable against reduction, that is, 4-118CL RNase 1 is considerably stable against reduction.

DISCUSSION

In order to introduce an additional disulfide bond into RNase 1, we expressed a mutant protein, in which Arg4 and Val118 were both replaced with cysteine residues, in *E. coli* as inclusion bodies. The introduced Cys-4 and Cys-118 were spontaneously bridged in a disulfide bond with the formation of native sets of disulfide bonds to give an active enzyme (4-118CL RNase 1) during the *in vitro* folding from a crude extract of inclusion bodies according to the methods developed previously (*19*). Since these two residues were selected based on the distance information on RNase A without knowing the three-dimensional structure of RNase 1, these results strongly suggest that the three-dimensional structure of RNase 1 is very similar to that of RNase A.

In the present study, drastic stabilization of human RNase 1 was achieved by means of a *de novo* designed engineered disulfide bridge between residues 4 and 118. Along with the increase in the thermodynamic stability, the stability against trypsin digestion was also increased in 4-118CL RNase 1. Furthermore, 4-118CL RNase 1 was fairly resistant to reduction. These results suggest that the stability of 4-118CL RNase 1 against proteolysis in the reducing environment of the cytosol also increases. Thus, the introduction of a designed disulfide bond is concluded to be an effective strategy for increasing the stability of RNase 1 against protease digestion.

The Cys-4/Cys-118 disulfide bond in 4-118CL RNase 1 was found to contribute by about 2.0 kcal/mol to the increase in ΔG_D for unfolding of RNase 1 at pH 5.4 and 32°C. This value is smaller than the theoretical value (5.4 kcal/mol). Therefore, it is suggested that the Cys-4/Cys-118 bond not only destabilizes the unfolded state by 5.4 kcal/mol due to the loss of the chain entropy, but also destabilizes the folded state by about 3.4 kcal/mol (Fig. 3). We suppose that the introduction of the present mutations and/or the formation of the extra disulfide bond induces some strain in the folded conformation of RNase 1.

From a structural view, the Cys-4/Cys-118 bond in 4-118CL RNase 1 is considered to be located in a molecular surface area just beside the active site. On the other hand, the other four native disulfide bonds would be buried and inaccessible to the solvent in the folded conformation. In fact, RNase 1 was hardly reduced even under high reducing conditions, while the Cys-4/Cys-118 bond in 4-118CL RNase 1 was more easily reduced, leading to the transient accumulation of 4-SS intermediates having the native sets of disulfide bonds (Fig. 5). Thus, the Cys-4/Cys-118 bond in 4-118CL RNase 1 may be directly reduced in the folded conformation. Since a considerable fraction of 4-118CL RNase 1 was completely reduced under high reducing conditions, the Cys-4/Cys-118 bond-cleaved species seemed no longer to be stabler than RNase 1.

We are aiming to improve the human RNase-based immunotoxin. Therefore, RNase should have several properties as an efficient cytotoxic domain. These are, first, RNase must be an active catalyst (*27*), second, RNase should evade endogenous RNase inhibitor (*28*), and third, RNase should be stable against proteolysis. Onconase showing high cytotoxicity has almost all the properties described above. The RNase activity of onconase is very low (about 0.02% of the activity of RNase A), but it exhibits unusual thermodynamic stability (T_m = 90°C) compared to bovine RNase A (T_m = 63°C) (*6*). These results imply that the stability of RNase strongly contributes to the cytotoxicity. Although 4-118CL RNase 1 has somewhat decreased activity (11% of the activity of RNase 1), its increased stability should be much more favorable for the utilization of a human RNase as a toxic domain for immunotoxins because 4-118CL RNase 1 is resistant to proteolysis under physiological conditions (Fig. 4). However, the ΔG_D value for unfolding of 4-118CL RNase 1 is still smaller by 1.2 kcal/mol than that of RNase A (Fig. 3). Further stabilizing mutation may be required to obtain a more efficient toxic domain for the human RNase-based immunotoxin.

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