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Conformational Stability Is a Determinant of Ribonuclease A Cytotoxicity*

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Onconase™, a homolog of bovine pancreatic ribonuclease A (RNase A) with high conformational stability, is cytotoxic and has efficacy as a cancer chemotherapeutic agent. Unlike wild-type RNase A, the G88R variant is toxic to cancer cells. Here, variants in which disulfide bonds were removed from or added to G88R RNase A were used to probe the relationship between conformational stability and cytotoxicity in a methodical manner. The conformational stability of the C40A/G88R/C95A and C65A/C72A/G88R variants is less than that of G88R RNase A. In contrast, a new disulfide bond that links the N and C termini (residues 4 and 118) increases the conformational stability of G88R RNase A and C65A/C72A/G88R RNase A. These changes have little effect on the ribonucleolytic activity of the enzyme or on its ability to evade the cytosolic ribonuclease inhibitor protein. The changes do, however, have a substantial effect on toxicity toward human erythroleukemia cells. Specifically, conformational stability correlates directly with cytotoxicity as well as with resistance to proteolysis. These data indicate that conformational stability is a key determinant of RNase A cytotoxicity and suggest that cytotoxicity relies on avoiding proteolysis. This finding suggests a means to produce new cancer chemotherapeutic agents based on mammalian ribonucleases.

The free energy difference between the native and unfolded states of a protein is small—typically 5–15 kcal/mol (1, 2). In their native state, many proteins are less susceptible to proteolytic degradation than when unfolded (3). In the unfolded state, the steric protection of peptide bonds provided by the compact native state is lost (4). For example, bovine pancreatic ribonuclease A (RNase A)[1] (EC 3.1.27.5 (5, 6)) is degraded more readily by proteases in the presence of denaturants or elevated temperatures (7–10) and less readily when glycosylated (11).

The rates of intracellular protein turnover vary by 10^3-fold (12). Apparently, some proteins are better able to thwart the proteolytic machinery (13, 14). These proteins remain intact and retain activity longer within the cell. A correlation between the conformational stability of a protein and its catabolism was reported over 20 years ago (15). Since then, studies using unrelated proteins have either supported (16) or contradicted (14, 17) this correlation. Drawing conclusions from these studies is problematic because the proteins were divergent in characteristics that have been implicated in metabolic turnover. In contrast, variants of a single protein, the N-terminal domain of the repressor protein from bacteriophage λ, have been used to demonstrate a definite link between conformational stability and metabolic turnover (18). More recently, the conformational stability of bovine pancreatic trypsin inhibitor variants was shown to correlate with the yield of intact protein produced in a heterologous system (19).

RNase A homologs elicit diverse biological activities, including specific toxicity to cancer cells (20, 21). Onconase™, which is a homolog of RNase A in the Northern leopard frog, is now in Phase III clinical trials for the treatment of malignant mesothelioma. It has been shown that the ribonucleolytic activity of ribonucleases is essential to their cytotoxicity (49). Nonetheless, ribonucleolytic activity is not the only requirement for a ribonuclease to be a cytotoxin. For example, relative to Onconase, RNase A is a 500-fold more effective catalyst of RNA cleavage (51), yet RNase A is not toxic to cancer cells. Still, the high ribonucleolytic activity of RNase A can engender a potent cytotoxin. Indeed, substitutions at Gly^{88} enable RNase A to mimic the ability of Onconase to evade the endogenous ribonuclease inhibitor (RI) protein and become cytotoxic (22). Still, no RNase A homolog is as cytotoxic as Onconase.

Onconase and RNase A differ substantially in another property—conformational stability. The value of T_m (which is the temperature at the midpoint of the thermal transition) for Onconase (T_m = 90 °C) is much greater than that of RNase A (T_m = 63 °C) (22). Is conformational stability a determinant of ribonuclease cytotoxicity? Answering this question requires a means to increase or decrease conformational stability without altering other important properties of the enzyme. We suspected that adding or removing disulfide bonds would provide such a subtle means.

RNase A contains four disulfide bonds (Cys^{26}–Cys^{84}, Cys^{40}–Cys^{95}, Cys^{58}–Cys^{110}, and Cys^{65}–Cys^{72}; Fig. 1). Previously, we dissected the contribution of each disulfide bond to conformational stability by replacing each cystine with a pair of alanine residues (23). Of the four disulfide bonds, the Cys^{40}–Cys^{95} and Cys^{65}–Cys^{72} cross-links are the least important to conformational stability (23, 24). Removing these disulfide bonds leads to RNase A variants that have T_m values below that of the wild-type enzyme but above physiological temperature.

Here, we investigate the relationship between the conformational stability and cytotoxicity of RNase A. Specifically, we construct G88R RNase A variants that are missing the Cys^{40}–Cys^{95} or Cys^{65}–Cys^{72} disulfide bonds. We also introduce a new disulfide bond into G88R RNase A and a variant missing the Cys^{65}–Cys^{72} disulfide bond. We find that the T_m values for G88R RNase A and its four disulfide variants vary by nearly

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[1] The abbreviations used are: RNase A, bovine pancreatic ribonuclease A; 6-FAM, 6-carboxyfluorescein; 6-TAMRA, 6-carboxytetramethylrhodamine; MES, 2-(*N*-morpholino)ethanesulfonic acid; RI, ribonuclease inhibitor.

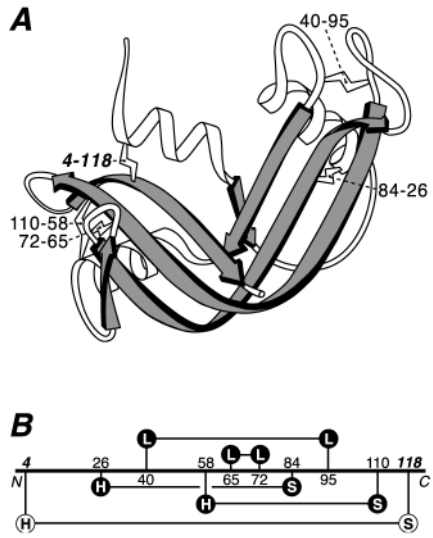

FIG. 1. **Structural representations of ribonuclease A.** *A,* ribbon diagram with inscriptions referring to the location of native (Cys^{26}–Cys^{84}, Cys^{40}–Cys^{95}, Cys^{58}–Cys^{110}, and Cys^{65}–Cys^{72}) and nonnative (Cys^{4}–Cys^{118}) disulfide bonds. *B,* scheme showing the connectivity of the five native and nonnative disulfide bonds. The secondary structural context of the half-cystines is indicated as follows: *H,* α-helix; *L,* surface loop; *S,* β-sheet.

30 °C. We show that conformational stability is linked to protease susceptibility *in vitro*. Finally, we demonstrate that each variant is toxic to cancer cells and, most significantly, that cytotoxicity is correlated with conformational stability.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strain BL21(DE3) was from Novagen (Madison, WI). *E. coli* strain DH5α, RPMI medium, fetal bovine serum, proteinase K, penicillin, and streptomycin were from Life Technologies, Inc. *E. coli* strain CJ236 and helper phage M13K07 were from Bio-Rad (Richmond, CA). A plasmid encoding G88R RNase A was a generous gift of P. A. Leland (22). All enzymes for the manipulation of recombinant DNA were from Promega (Madison, WI) or New England Biolabs (Beverly, MA). RI was from Promega. [*methyl*-^{3}H]Thymidine (6.7 Ci/mmol) was from NEN Life Science Products (Boston, MA). K-562 human erythroleukemia cells were from the American Type Culture Collection (Manassas, VA).

Purified oligodeoxyribonucleotides and the fluorogenic substrate 6-FAM~(dA)rU(dA)$_2$~6-TAMRA were from Integrated DNA Technologies Inc. (Coralville, IA). Poly(cytidylic acid) was from Midland Certified Reagents (Midland, TX) and was precipitated from ethanol and washed with aqueous ethanol (70% v/v) before use. DNA was sequenced with an ABI 373XL automated sequencer using an Perkin-Elmer Big Dye kit, FS (Foster City, CA), and an MJ Research PTC-100 programmable thermal controller (Watertown, MA) at the University of Wisconsin Biotechnology Center (Madison, WI).

Isopropyl-1-thio-β-D-galactopyranoside was from Gold Biotechnology (St. Louis, MO). Bacto-yeast extract, Bacto-tryptone, Bacto-peptone, and Bacto-agar were from Difco (Detroit, MI). LB medium contained (in 1.00 liter) Bacto-tryptone (10 g), Bacto-yeast extract (5 g), and sodium chloride (10 g). TB medium contained (in 1.00 liter) Bacto-tryptone (12 g), Bacto-yeast extract (24 g), glycerol (4 ml), KH_2PO_4 (2.31 g), and K_2HPO_4 (12.54 g). Phosphate-buffered saline (pH 7.3) contained (in 1.00 liter) KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g), and Na_2HPO_4:$7H_2O$ (2.16 g). *E. coli* cell lysis was performed using a French pressure cell from SLM Aminco (Urbana, IL). FPLC Hiload 26/60 Superdex 75 gel filtration column and mono-S HR10/10 cation-exchange column were from Amersham Pharmacia Biotech. All media were prepared in distilled, deionized water and autoclaved. All other chemical reagents were of commercial reagent grade or better and were used without further purification.

Instruments—Ultraviolet and visible absorbance measurements were made with a Cary 3 double beam spectrophotometer equipped with a Cary temperature controller from Varian (Sugar Land, TX). Calorimetry experiments were performed with an MCS differential scanning calorimeter from MicroCal (Northampton, MA). Fluorescence measurements were performed on a QuantaMaster1 photon counting fluorometer from Photon Technology International (South Brunswick, NJ) equipped with sample stirring. The concentration of 6-FAM~(dA)rU(dA)$_2$~6-TAMRA was determined using ϵ = 102,400 M^{-1} cm^{-1} at 260 nm (25).

Design of a New Disulfide Bond—The C-terminal residue of Onconase, which has greater conformational stability than does any other known homolog of RNase A, is a heterologous half-cystine. To attempt to enhance the conformational stability of RNase A, a new disulfide bond was designed to cross-link the N and C termini. Such a cross-link would greatly restrict the number of conformations of the polypeptide chain, thereby destabilizing the unfolded state relative to the native state (26). The program SYBYL (Tripos, St. Louis, MO) and atomic coordinates derived from crystalline RNase A (PDB entry 7RSA (27)) were used to determine the placement of the new disulfide bond. According to our molecular modeling, replacing Ala^4 and Val^{118} with two half-cystine residues would cross-link the N and C termini without perturbing the three-dimensional structure of RNase A (Fig. 1). Replacing Arg^4 and Val^{118} in the human homolog of RNase A with two half-cystine residues has been reported to make that enzyme more resistant to proteolysis by trypsin.[2]

Preparation of Ribonuclease A Variants—Oligonucleotide-mediated site-directed mutagenesis was used to create the RNase A variants. Plasmids encoding the G88R (22), C40A/C95A, and C65A/C72A (23) variants were derived from plasmid pBXR, which directs the expression of RNase A in *E. coli* (29). Mutagenesis was performed on plasmids encoding the G88R, C40A/C95A, and C65A/C72A variants replicated in *E. coli* strain CJ236 (30). To produce plasmids encoding the C40A/G88R/C95A and C65A/C72A/G88R variants, the GGC codon for Gly^{88} was replaced in DNA encoding the C40A/C95A and C65A/C72A variants using the oligodeoxyribonucleotide TTGGAGCT**ACG**CGTCTCACG (reverse complement in boldface). To produce plasmids encoding the A4C/G88R/V118C and A4C/C65A/C72A/G88R/V118C variants, the GCA codon for Ala^4 was replaced in DNA encoding the G88R and C65A/C72A/G88R variants using the oligodeoxyribonucleotide CTTGGCTGC**ACA**AGTTTCCTTGC (reverse complement in boldface), and the GTC codon for Val^{118} was replaced using the oligodeoxyribonucleotide GCATCAAAGTG**ACA**TGGCACATACGGGTTTCC (reverse complement in boldface).

Wild-type RNase A and its variants were produced and purified by methods described previously (29, 31) except for minor modifications in the conditions for oxidative folding (22, 23). The purity of each protein was assessed by SDS-polyacrylamide gel electrophoresis. Adding or removing a disulfide bond is expected to alter the extinction coefficient of RNase A by <1% (32, 33). Hence, the concentrations of wild-type RNase A and the disulfide variants were determined by using ϵ = 0.72 ml mg^{-1} cm^{-1} at 277.5 nm (34).

Assays of Conformational Stability—Differential scanning calorimetry was used to determine the conformational stability of each RNase A variant. Differential scanning calorimetry experiments were performed as described (23), with the following modifications. Protein solutions (0.99–5.6 mg/ml) were dialyzed exhaustively against phosphate-buffered saline and then centrifuged at 15,300 × *g* for 30 min to remove particulate matter. Data were collected with the program ORIGIN (MicroCal Software, Northampton, MA). The unfolding of wild-type RNase A and each of the variants was >90% reversible, as demonstrated by reheating of the protein samples (data not shown).

Assays of Cytotoxicity—The cytotoxicity of the RNase A variants was assessed using the continuous human erythroleukemia cell line K-562 as described (22). Briefly, K-562 cells were grown at 37 °C in RPMI medium supplemented with fetal bovine serum (10% v/v) with penicillin (100 units/ml) and streptomycin (100 μg/ml) in the presence of wild-type RNase A, a variant, or a phosphate-buffered saline control for 44 h, followed by a 4-h pulse with [*methyl*-^{3}H]thymidine (0.20 μCi per well). Each experiment is the average of triplicate determinations for each ribonuclease concentration. The standard error of the values from each protein concentration is ≤20%. The IC_{50} value is the concentration of an RNase A variant that kills 50% of the K-562 cells.

Assays of Ribonucleolytic Activity—A fluorogenic substrate, 6-FAM~(dA)rU(dA)$_2$~6-TAMRA, was used to determine the values of k_{cat}/K_m for the RNase A variants (25). A large increase in fluorescence occurs upon cleavage of the P-O$^{5'}$ bond on the 3′ side of the single ribonucleotide residue embedded within this substrate (25). Assays were performed with stirring in 2.0 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), 6-FAM~(dA)rU(dA)$_2$~6-TAMRA (60 nM), and enzyme (5 pM to 0.50 nM). Fluorescence was monitored at 515 nm

[2] J. Futami, M. Seno, and H. Yamada, personal communication.

TABLE I
Conformational stability, cytotoxicity, ribonucleolytic activity, inhibition by ribonuclease inhibitor, and protease susceptibility of wild-type ribonuclease A and disulfide variants

Ribonuclease A	T_m[a]	IC_{50}[b]	k_{cat}/K_m[c]	K_i[d]	$t_{1/2}^{proteinase\ K}$[e]
	°C	μM	$10^5\ M^{-1}s^{-1}$	nM	h^{-1}
A4C/G88R/V118C	68.8 ± 0.1	3	49 ± 6	0.65 ± 0.11	6.9
G88R	64.0 ± 0.1	9	150 ± 30	0.24 ± 0.05	5.3
Wild-type	63.2 ± 0.1	>100	360 ± 40[f]	ND	ND
A4C/C65A/C72A/G88R/V118C	50.4 ± 0.1	17	4.0 ± 0.7	3.9 ± 0.3	5.3
C65A/C72A/G88R	45.9 ± 0.1	26	36 ± 2	0.78 ± 0.16	4.1
C40A/G88R/C95A	40.4 ± 0.2	25	76 ± 1	0.35 ± 0.05	2.4

[a] Values (± S.E.) from differential scanning calorimetry are for triplicate experiments performed in protein-buffered saline. Determinate errors for T_m values are approximately 1%.
[b] Proliferation was measured by incorporation of [^{3}H]thymidine into cellular DNA after incubation with a ribonuclease at 37 °C for 44 h. Values are from triplicate measurements for each ribonuclease concentration from each of three independent experiments.
[c] Values (± S.E.) for cleavage of 6-FAM~(dA)rU(dA)$_2$~6-TAMRA are for triplicate experiments at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M).
[d] Values (± S.E.) for RI affinity are for triplicate experiments at 25 °C in 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M) and dithiothreitol (8.0 mM).
[e] Values for inactivation by proteinase K are from triplicate experiments at 25 °C in 50 mM Tris-HCl buffer (pH 8.0) containing $CaCl_2$ (1.0 mM).
[f] Value from Ref. 25.

with excitation at 495 nm. Values of k_{cat}/K_m were determined using the following equation,

$$I = I_0 + (I_f - I_0)(k_{cat}/K_m)[E]_t \quad \text{(Eq. 1)}$$

where I is the fluorescence intensity in photon counts per second, I_0 is the intensity of the substrate prior to addition of enzyme, I_f is the final intensity when all the substrate is cleaved, and $[E]_t$ is the total enzyme concentration. Values of k_{cat}/K_m were determined by linear least squares regression analysis of initial velocity data, assuming that assays were performed at substrate concentrations below the K_m, which is near 22 μM (25).

Assays of Inhibition by Ribonuclease Inhibitor—RI is a competitive inhibitor of RNase A that binds with a 1:1 stoichiometry (35–37). We had shown previously that as RI affinity decreases, RNase A cytotoxicity increases (22). Commercial RI is stored in aqueous glycerol (50% v/v). To eliminate errors caused by pipetting viscous solutions, a buffer exchange to remove glycerol was performed, and the RI activity in the resulting low-viscosity solutions was determined as described (38).

To determine values of K_i, we monitored the cleavage of 6-FAM~(dA)rU(dA)$_2$~6-TAMRA by the RNase A variants as a function of RI concentration. Assays were performed with stirring in 2.00 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M), dithiothreitol (8.0 mM), 6-FAM~(dA)rU(dA)$_2$~6-TAMRA (60 nM), and enzyme (5 pM to 0.50 nM). Fluorescence was monitored at 515 nm, with excitation at 495 nm. The value of k_{cat}/K_m was determined using Equation 1. After 5 min, an aliquot (0.5 μl) of RI (11.5 nM) was added, and the rate of substrate turnover was determined again. Additional aliquots of RI were added at 5-min intervals until the fluorescence change decreased to less than 10% of its original value. Values of K_i were determined by nonlinear least-squares regression analysis of data fitted to the following equation (25),

$$\Delta I/\Delta t = (\Delta I/\Delta/t)_0(K_i/(K_i + [I])) \quad \text{(Eq. 2)}$$

where $(\Delta I/\Delta t)_0$ is the turnover rate prior to addition of RI.

Assays of Proteolytic Susceptibility—Proteinase K is a nonspecific protease that catalyzes the hydrolysis of accessible peptide bonds in proteins. A discontinuous assay was employed to determine the susceptibility of each RNase A variant to proteinase K. An RNase A variant (6.1 μM) was incubated in triplicate at 25 °C with proteinase K (5 μg/ml) in 50 mM Tris-HCl buffer (pH 8.0) containing $CaCl_2$ (1.0 mM). Controls were incubated in buffer without proteinase K. At known times, aliquots were removed and diluted with 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M). The turnover of 6-FAM~(dA)rU(dA)$_2$~6-TAMRA (60 nM) was monitored in 2.00 ml of 0.10 M MES-NaOH buffer (pH 6.0) containing NaCl (0.10 M) and enzyme (5–600 pM). Fluorescence was monitored at 515 nm with excitation at 495 nm. Values of k_{cat}/K_m were determined using Equation 1. A plot of relative ribonucleolytic activity as a function of time was fitted to a first-order rate equation to determine the pseudo first-order rate constant (k) for inactivation by proteinase K digestion. Values of $t_{1/2}^{proteinase\ K}$ were determined from the rate constant using the equation $t_{1/2}^{proteinase\ K} = \ln 2/k$.

RESULTS

Conformational Stability—The reversible thermal transitions of wild-type RNase A and the G88R, A4C/G88R/V118C, C40A/G88R/C95A, C65A/C72A/G88R, and A4C/C65A/C72A/G88R/V118C variants were fitted to equations describing a two-state model for unfolding: N ⇄ U, where N is the native state and U is the unfolded state. Removing a native disulfide bond in RNase A decreases the stability significantly (24, 23). The C65A/C72A/G88R and C40A/G88R/C95A variants are approximately 90 and 60% folded at 37 °C, respectively (data not shown). Compared with G88R RNase A, the T_m values for the C65A/C72A/G88R and C40A/G88R/C95A variants are decreased by 18.1 and 23.6 °C, respectively. In contrast to these variants, wild-type RNase A and the G88R, A4C/G88R/V118C, and A4C/C65A/C72A/G88R/V118C variants are >99% folded at 37 °C. The T_m values of wild-type RNase A and G88R RNase A are 63.2 and 64.0 °C, respectively (Table I). Most interestingly, the new Cys^4–Cys^{118} disulfide bond in A4C/G88R/V118C RNase A increases the T_m by 4.8 °C relative to that of G88R RNase A. A similar increase in T_m occurs in the A4C/C65A/C72A/G88R/V118C variant relative to that of C65A/C72A/G88R RNase A.

Cytotoxicity—The cytotoxicity of wild-type RNase A and the G88R, A4C/G88R/V118C, C40A/G88R/C95A, C65A/C72A/G88R, and A4C/C65A/C72A/G88R/V118C variants was assessed by measuring the incorporation of [*methyl*-^{3}H]thymidine into cellular DNA after a 44-h incubation with K-562 cells. Wild-type RNase A is not cytotoxic to K-562 cells at the concentrations used in this assay (Table I). The IC_{50} value of 9 μM for G88R RNase A agrees closely with those reported previously (22, 38). Most significantly, adding the disulfide bond between residues 4 and 118 decreases the IC_{50} value for A4C/G88R/V118C RNase A by 3-fold (IC_{50} = 3 μM). Although still cytotoxic, each of the remaining variants is less cytotoxic than is G88R RNase A. The IC_{50} values for the C40A/G88R/C95A, C65A/C72A/G88R, and A4C/C65A/C72A/G88R/V118C variants are 25, 26, and 17 μM, respectively.

Ribonucleolytic Activity—A highly sensitive fluorometric assay was used to assess ribonucleolytic activity. The values of k_{cat}/K_m for wild-type RNase A and variants are the average of three independent assays. Each variant is a potent catalyst of RNA cleavage. At 25 °C, the values of k_{cat}/K_m for the variants are 2.4–90-fold lower than that of the wild-type enzyme (Table I).

Inhibition by Ribonuclease Inhibitor—Inhibition of the ribonuclease-catalyzed cleavage of 6-FAM~(dA)rU(dA)$_2$~6-TAMRA by

RI was assessed in a continuous assay. The effect of RI concentration on the ribonucleolytic activity of each variant is reported as K_i (Table I). The value of K_i = 0.24 nM for G88R RNase A agrees closely with those reported previously (22, 38). Removing or adding a disulfide bond to G88R RNase A affects only slightly its affinity for RI. The K_i values for the A4C/G88R/V118C, C40A/G88R/C95A, C65A/C72A/G88R, and A4C/C65A/C72A/G88R/V118C variants are 0.65, 0.35, 0.78, and 3.9 nM, respectively.

Protease Susceptibility—The proteolytic susceptibility of each of the RNase A variants was assessed by monitoring ribonucleolytic activity after exposure to proteinase K. The C40A/G88R/C95A and C65A/C72A/G88R variants are the most susceptible to proteinase K digestion. Furthermore, the addition of the Cys^4–Cys^{118} disulfide bond decreased the proteinase K susceptibility of A4C/G88R/V118C RNase A and A4C/C65A/C72A/G88R/V118C RNase A relative to the G88R and C65A/C72A/G88R variants. The values of $t_{1/2}^{\text{proteinase K}}$ for the A4C/G88R/V118C, G88R, A4C/C65A/C72A/G88R/V118C, C65A/C72A/G88R, and C40A/G88R/C95A variants are 6.9, 5.3, 5.3, 4.1, and 2.4 h^{-1}, respectively (Table I).

DISCUSSION

Disulfide bonds contribute to protein stability principally by limiting the flexibility of the unfolded polypeptide chain and thereby destabilizing the unfolded state relative to the native state (26). The loss of entropy in the unfolded state is not, however, the only effect of disulfide bonds on conformational stability. A disulfide bond can enhance (39–41) or diminish (42–45) interactions in the folded state that contribute to conformational stability. Thus, predicting the precise contribution of a particular native or nonnative disulfide bond to conformational stability is difficult. Still, we find that replacing a cystine with a pair of alanine residues is a superb means of altering conformational stability without affecting severely the other attributes of a protein. Moreover, the judicious addition of a new cystine to a protein expands the range of accessible stabilities.

Conformational Stability—RNase A contains four disulfide bonds that are important to its conformational stability (23). Substitutions at Gly^{88} have little effect on conformational stability but endow RNase A with cytotoxic activity (22). We constructed four disulfide variants of G88R RNase A that are mostly folded at physiological temperature, which is the temperature of the cytotoxicity assays. The T_m values of the C65A/C72A/G88R and C40A/G88R/C95A variants are 18.1 and 23.6 °C lower, respectively, than that of G88R RNase A (Table I). These decreases in conformational stability are similar to those suffered by the analogous disulfide variants of wild-type RNase A (23). The other two variants have a new disulfide bond. A4C/G88R/V118C RNase A is the only known disulfide variant of RNase A with greater conformational stability than the wild-type enzyme. Moreover, conformational stability lost by removing the native Cys^{65}–Cys^{72} disulfide bond is recovered by adding the nonnative Cys^4–Cys^{118} disulfide bond. The T_m values of the A4C/G88R/V118C and A4C/C65A/C72A/G88R/V118C variants are 4.8 and 4.5 °C higher, respectively, than that of the G88R and C65A/C72A/G88R variants (Table I). Thus, the T_m values of the RNase A variants studied herein range from 40.4 to 68.8 °C.

Within the G88R RNase A variants, cytotoxicity correlates well with conformational stability (Fig. 2). For example, A4C/G88R/V118C RNase A has the highest T_m value of the five enzymes and is the most potent cytotoxin. In contrast, the C40A/G88R/C95A and C65A/C72A/G88R variants have the lowest T_m values and the highest IC_{50} values. The G88R and A4C/C65A/C72A/G88R/V118C variants have intermediate T_m values and intermediate IC_{50} values. These data are consistent with a model in which conformational stability is a determinant of cytotoxicity.

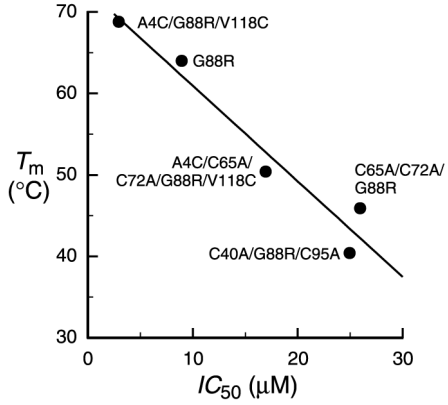

FIG. 2. **T_m values *versus* IC_{50} values for variants of ribonuclease A.** Data are from Table I.

This model ignores the low thermodynamic stability of disulfide bonds in the reducing environment of the cytosol (46). The four disulfide bonds in wild-type RNase A are virtually inaccessible to solvent (23) and have considerable kinetic stability in a highly reducing environment (47). Indeed, the $t_{1/2}$ of RNase A in the cytosol is >44 h (48), which is the incubation time of our cytotoxicity assays. Hence, we suspect that the disulfide bonds of RNase A are not susceptible to reduction in the cytosol within the time course of our assays.

Ribonucleolytic Activity—Ribonucleases must retain ribonucleolytic activity to be toxic to cells (49). We determined the values of k_{cat}/K_m for the cleavage of 6-FAM~(dA)rU$(dA)_2$~6-TAMRA to determine whether differences in cytotoxicity were caused by differences in ribonucleolytic activity. Each of the disulfide variants is a somewhat less efficient catalyst of RNA cleavage than is G88R RNase A (Table I). Ribonucleolytic activity does not, however, correlate with cytotoxicity. For example, G88R RNase A has a 3-fold larger k_{cat}/K_m value than does A4C/G88R/V118C RNase A but is less cytotoxic. Furthermore, the C65A/C72A/G88R and C40A/G88R/C95A variants are 9- and 19-fold more efficient catalysts of RNA cleavage but are less cytotoxic than is A4C/C65A/C72A/G88R/V118C RNase A.

Inhibition by Ribonuclease Inhibitor—Cytosolic ribonucleolytic activity is controlled by the presence of RI (37). RI is a 50-kDa scavenger of pancreatic-type ribonucleases and forms a tight noncovalent complex with wild-type RNase A ($K_d = 4 \times 10^{-14}$ M (36)). Hence, wild-type RNase A has low cytotoxicity. Still, large quantities of RNase A in the cytosol can overwhelm the sentry. For example, microinjection of RNase A greatly increases its cytotoxicity (50).

Ribonucleases that are capable of evading cytosolic RI are cytotoxic at much lower concentrations than is RNase A (51, 52). Indeed, Onconase has $>10^8$-fold lower affinity for RI than does RNase A (53). Variations at Gly^{88}, which is an important contact point within the RI·RNase A complex, allows RNase A to evade RI binding more effectively and increases its cytotoxicity (22, 54).

Each of the G88R disulfide variants has slightly less affinity for RI than does G88R RNase A (Table I). The K_i values of the disulfide variants are between 1.5- and 16-fold lower than that of G88R RNase A. Nonetheless, the affinity of RI for the G88R disulfide variants does not correlate with their cytotoxicity (Table I). For example, A4C/C65A/C72A/G88R/V118C RNase A has a 16-fold lower affinity for RI than does G88R RNase A but is less cytotoxic.

Proteinase K Susceptibility—Proteolytic susceptibility is greater in misfolded or unfolded proteins, relative to folded

proteins (3, 10). Steric hindrance of the peptide bonds in a folded protein blocks possible protease cleavage sites. Indeed, simple glycosylation can slow proteolysis (11). A protein with low conformational stability will exist in an unfolded state to a greater extent than a protein with high conformational stability, increasing proteolytic susceptibility (18).

Is the conformational stability of a ribonuclease linked to its cytotoxicity via its proteolytic susceptibility? C40A/G88R/C95A RNase A and C65A/C72A/G88R RNase A have the lowest conformational stability and the highest IC_{50} values and are also the most susceptible to inactivation by proteinase K digestion (Table I). In contrast, A4C/G88R/V118C RNase A has the highest T_m value, is the most cytotoxic, and is the least susceptible to inactivation by proteinase K digestion. The addition of the Cys^4–Cys^{118} disulfide bond increases the T_m value and decreases both the IC_{50} value and the $t_{1/2}^{\text{proteinase K}}$ value of two different enzymes. Thus, high conformational stability is linked to both high cytotoxicity and low proteolytic susceptibility.

Implications for the Mechanism of Cytotoxicity—The cytotoxicity of ribonucleases is manifested in the cytosol. To gain access to the cytosol, an extracellular ribonuclease must cross a cellular membrane. Protein traffic through membranes is thought to require unfolding prior to translocation (55, 56). For example, the translocation of barnase variants across the mitochondrial membrane decreases with increasing disulfide cross-links and conformational stability (62). Our data are in apparent conflict with this model, as the disulfide variants with greater conformational stability are more cytotoxic (Table I). An explanation of this discrepancy is that the translocation of RNase A and its variants could be relatively rapid and another step, such as cytosolic proteolysis, could limit cytotoxicity. Indeed, protease inhibitors are known to increase the cytotoxicity of other protein cytotoxins (57). Similarly, the addition of proteolytic degradation signals can decrease protein cytotoxicity (58). Finally, it is noteworthy that protein toxins such as diphtheria toxin, enterotoxin, and abrin II have T_m values near that of RNase A (59–61). Increasing the conformational stability of these protein toxins could increase their cytotoxicity, as we have shown with G88R RNase A.

Relevance to Cancer Chemotherapy—Onconase, which is from an amphibian, is on the verge of approval as a human cancer chemotherapeutic agent. We find that enhanced conformational stability can increase the toxicity of a mammalian homolog of Onconase toward cancer cells. This finding suggests a means to produce new cancer chemotherapeutic agents based on mammalian ribonucleases.

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