A synapomorphic disulfide bond is critical for the conformational stability and cytotoxicity of an amphibian ribonuclease

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Abstract Onconase® (ONC) is a homolog of ribonuclease A (RNase A) that has unusually high conformational stability and is toxic to human cancer cells in vitro and in vivo. ONC and its amphibian homologs have a C-terminal disulfide bond, which is absent in RNase A. Replacing this cystine with a pair of alanine residues greatly decreases the conformational stability of ONC. In addition, the C87A/C104A variant is 10-fold less toxic to human leukemia cells. These data indicate that the synapomorphic disulfide bond of ONC is an important determinant of its cytotoxicity.

Key words: Synapomorphic disulfide bond; Ribonuclease A; Onconase; Leukemia

1. Introduction

Onconase® (ONC; registered trademark of Alfacell Corp., Bloomfield, NJ, USA) is a ribonuclease that is present in the oocytes and early embryos of the Northern leopard frog (*Rana pipiens*) [1,2]. ONC was discovered based on its potent anti-cancer activity [3]. ONC is now in Phase III human clinical trails for the treatment of malignant mesothelioma, an asbestos-related lung cancer.

The amino acid sequence of ONC is 30% identical to that of bovine pancreatic ribonuclease A (RNase A [4]; EC 3.1.27.5) [5], the defining member of the pancreatic-type ribonuclease superfamily [6]. The catalytic triad of residues characteristic of pancreatic-type ribonucleases (His12, Lys41 and His119 in RNase A) is conserved in ONC as His10, Lys31 and His97. ONC also retains three of the four disulfide bonds common to the pancreatic-type ribonucleases (Fig. 1) [7,8]. In addition, ONC has a synapomorphic disulfide bond between residues 87 and 104. The Cys87–Cys104 disulfide bond in ONC tethers its C-terminal residue to a central β-strand and appears to be unique to the amphibian homologs of RNase A [2,6].

ONC has unusually high conformational stability [9]. Here, we determine the contribution of the Cys87–Cys104 disulfide bond to the conformational stability of ONC. Specifically, we replace the half-cystines at residues 87 and 104 with alanine residues. We show that this variant of ONC (C87A/C104A ONC) has substantially less conformational stability than does the wild-type enzyme. In addition, we show that removal of the Cys87–Cys104 disulfide bond results in a significant decrease in cytotoxic activity.

2. Materials and methods

2.1. Materials

K-562 cells, which are from a continuous human chronic myelogenous leukemia line, were from the American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* strain BL21(DE3) was from Novagen (Madison, WI, USA).

Enzymes used for DNA manipulation were from Promega (Madison, WI, USA) or New England Biolabs (Beverly, MA, USA). Ribonuclease inhibitor protein (RI) was from Promega. Oligonucleotides used for site-directed mutagenesis were from Integrated DNA Technologies (Coralville, IA, USA). The ribonuclease substrate 6-carboxyfluorescein ~ dArUdAdA ~ 6-carboxytetramethylrhodamine [6-FAM ~ dArU(dA)$_2$ ~ 6-TAMRA [10]] was from Integrated DNA Technologies. [*methyl*-^{3}H]Thymidine was from DuPont/NEN (Boston, MA, USA). 2-(*N*-Morpholino)ethanesulfonic acid (MES) was from Sigma (St. Louis, MO, USA). All other chemicals were of commercial reagent grade or better and were used without further purification.

2.2. Production of ribonucleases

Plasmid pONC directs the production of wild-type ONC in *E. coli* [9]. Oligonucleotide-mediated site-directed mutagenesis of plasmid pONC was used to replace Cys87 and Cys104 with a pair of alanine residues. The integrity of the C87A/C104A ONC cDNA was confirmed by dye-terminator cycle sequencing using a BigDye cycle sequencing kit from Perkin Elmer (Norwalk, CT, USA) and an ABI 377XL Automated DNA Sequencer at the University of Wisconsin Biotechnology Center.

Wild-type ONC was produced, folded and purified as described previously [9]. The concentration of ONC was determined by ultraviolet spectroscopy using an extinction coefficient of $\varepsilon_{280} = 0.87$ ml mg^{-1} cm^{-1}, which was calculated with the method of Pace et al. [11]. The C87A/C104A variant of ONC was produced, folded and purified by using methods identical to those described for wild-type ONC [9]. Removal of a disulfide bond in ONC is expected to change the extinction coefficient by less than 1%, compared to that of the wild-type protein [11]. Hence, the concentration of C87A/C104A ONC was also determined using an extinction coefficient of $\varepsilon_{280} = 0.87$ ml mg^{-1} cm^{-1}. Matrix-assisted laser desorption-ionization (MALDI) mass spectrometry was performed on a Bruker Biflex III instrument at the University of Wisconsin Biotechnology Center.

2.3. Circular dichroism (CD) spectroscopy

CD spectra of ONC and C87A/C104A ONC (0.2 mg/ml in phosphate-buffered saline; PBS) were collected at 25°C on a Model 202 SF CD spectrometer from Aviv (Lakewood, NJ, USA) equipped with an Aviv temperature controller. Raw data were converted to molar ellipticity ([Θ]) by using a mean residue mass of 110 Da. PBS contained (in 1 l) KCl (0.20 g), KH_2PO_4 (0.20 g), NaCl (8.0 g) and $Na_2HPO_4{\cdot}7H_2O$ (2.16 g).

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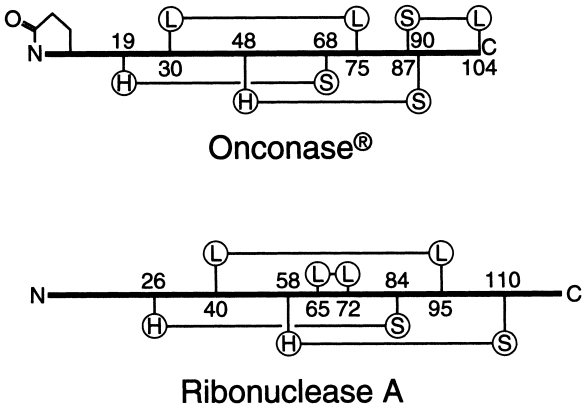

Fig. 1. Connectivity of the disulfide bonds in Onconase® (104 residues) and ribonuclease A (124 residues). The secondary structural context of each half-cystine residue is indicated by H (α-helix), S (β-sheet) or L (loop). The N-terminal residue of Onconase® is a pyroglutamate.

2.4. Determination of conformational stability

The conformational stability of ONC was determined previously by using CD spectroscopy [9]. The conformational stability of C87A/C104A ONC was likewise determined by using CD spectroscopy to monitor the change in molar ellipticity at 204 nm ($[\Theta_{204}]$) with increasing temperature. The temperature of a solution of C87A/C104A ONC (0.2 mg/ml in PBS) was increased from 35 to 85°C in 2°C-increments. The decrease in ellipticity at 204 nm (Θ_{204}) was recorded after a 3 min equilibration at each temperature. CD data were fitted to a two-state model for denaturation [12]. The melting temperature (T_m) is the temperature at the midpoint of the thermal denaturation curve.

2.5. Assay for ribonucleolytic activity

Ribonucleolytic activity was determined with a hypersensitive assay based on the cessation of fluorescence quenching [10]. Assays were performed at (23 ± 2)°C in 2.00 ml of 0.10 M MES–NaOH buffer (pH 6.0) containing NaCl (0.10 M), 6-FAM ~ dArU(dA)$_2$ ~ 6-TAMRA (60 nM), enzyme (0.10–0.34 μM) and RI (0.1–1.6 nM). The increase in fluorescence upon substrate cleavage was measured with a QuantaMaster 1 photon-counting fluorescence spectrometer from Photon Technology International (South Brunswick, NJ, USA) using excitation and emission wavelengths of 495 and 515 nm, respectively. Values of k_{cat}/K_M were determined by a linear least-squares regression analysis of initial velocity data using:

$$k_{cat}/K_M = \frac{(\Delta F/\Delta t)}{F_{max} - F_0} \cdot \frac{1}{[E]} \quad (1)$$

In Eq. 1, $\Delta F/\Delta t$ is the slope from the linear regression, F_{max} is the maximal fluorescence intensity, F_0 is the initial fluorescence intensity and [E] is the enzyme concentration. F_{max} was determined by adding RNase A (~0.1 μM final concentration) to the reaction after acquiring enough initial velocity data for the least-squares regression analysis.

2.6. Determination of cytotoxic activity

The effect of ONC and C87A/C104A ONC on the proliferation of K-562 cells was measured as described previously [9]. Data represent the average of quadruplicate samples within an individual assay. Results from two independent cytotoxicity assays did not deviate significantly.

3. Results

3.1. Production and characterization of ribonucleases

Wild-type ONC and C87A/C104A ONC were produced in *E. coli* with isolated yields of 25 and 15 mg l^{-1} of culture, respectively. Our yield of wild-type ONC is comparable to that from other methods for the production and purification of the enzyme [13]. Both proteins migrate as a single band of appropriate M_r during sodium dodecyl sulfate–polyacrylamide gel electrophoresis (data not shown). Analysis of an equimolar mixture of wild-type and C87A/C104A ONC with MALDI mass spectrometry gave two peaks with a difference of 61 Da, which is close to the change in molecular mass expected for the C87A/C104A substitution (data not shown). The CD spectra of wild-type and C87A/C104A ONC do, however, differ (Fig. 2A). To facilitate the comparison, CD spectra were normalized by using the molar ellipticity at 210 nm (Fig. 2A, inset). Notably, the molar ellipticity of C87A/C104A ONC becomes positive at a lower wavelength than does the signal of wild-type ONC. This change is consistent with the accumulation of random secondary structures, potentially at the expense of the β-sheet, in the C87A/C104A variant of ONC [14].

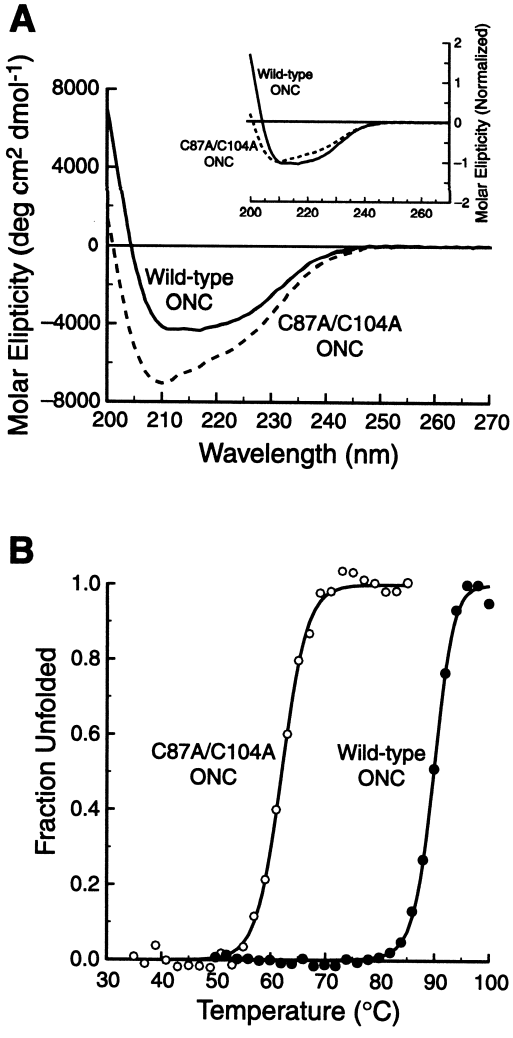

Fig. 2. Conformation and conformational stability of wild-type Onconase® and the C87A/C104A variant in PBS. A: CD spectrum of wild-type Onconase® (solid line) and C87A/C104A Onconase® (broken line) at 25°C. Inset: spectra normalized using the molar ellipticity at 210 nm as a reference. B: Thermal unfolding of wild-type Onconase® (●) and the C87A/C104A variant (○) monitored by CD spectroscopy at 204 nm. Data for wild-type Onconase® are from [9].

Table 1
Biophysical and biochemical parameters of Onconase® and its C87A/C104A variant

Onconase®	T_m [a] (°C)	k_{cat}/K_M [b] (10^2 M^{-1} s^{-1})	IC_{50} [c] (μM)
Wild-type	90 ± 2	2.2 ± 0.1	0.2
C87A/C104A	62 ± 2	1.1 ± 0.1	2.0

[a]Values of T_m (±S.E.M.) were determined by CD spectroscopy (Fig. 2B).
[b]Values of k_{cat}/K_M (±S.E.M.) are for catalysis of 6-FAM~dArU(dA)$_2$~6-TAMRA cleavage at pH 6.0 and 23°C in the presence of RI (0.1–1.6 nM), which inhibits any contaminating pancreatic-type ribonucleases.
[c]Values of IC_{50} are for toxicity to human leukemia cell line K-562 (Fig. 3).

3.2. Contribution of the Cys87–Cys104 disulfide bond to conformational stability

ONC has remarkable conformational stability, its T_m is 90°C. Previously, we suggested that the high conformational stability of ONC, compared to that of RNase A (T_m of 62°C), is due to a disulfide bond between ONC residues 87 and 104 [9]. Cytotoxic ribonucleases from the oocytes of *Rana japonica* (Japanese rice paddy frog) and *Rana catesbeiana* (bullfrog) each retain a disulfide bond analogous to the Cys87–Cys104 pairing in ONC [15–17]. Significantly, the *R. catesbeiana* and *R. japonica* ribonucleases also have high conformational stability ($T_m > 75$°C) [18].

The T_m of C87A/C104A ONC was measured by using CD spectroscopy to record the change in molar ellipticity at 204 nm with increasing temperature. As shown in Fig. 2B and listed in Table 1, the Cys87–Cys104 disulfide bond does contribute significantly to the conformational stability of ONC. Indeed, the T_m of the C87A/C104A variant of ONC is 62°C, which is almost 30°C lower than that of the wild-type enzyme.

3.3. Contribution of the Cys87–Cys104 disulfide bond to ribonucleolytic activity

The ribonucleolytic activity of ONC is less than that of RNase A. Nevertheless, ribonucleolytic activity is necessary for the cytotoxic activity of ONC [19]. The contribution of the Cys87–Cys104 disulfide bond to ribonucleolytic activity was determined in MES–NaOH buffer (pH 6.0) containing NaCl and RI. Because RI binds to mammalian pancreatic-type ribonucleases with extraordinarily high affinity ($K_d = 4.4\times10^{-14}$ M for the RI–RNase A complex [20]) but is a weak inhibitor of ONC (estimated $K_i \geq 10^{-6}$ M [21]), the presence of a small amount of RI ($\leq 3\times10^{-10}$ M) in the assay mixture eliminates any trace of contaminating ribonucleolytic activity from mammalian pancreatic-type ribonucleases without inhibiting the catalytic activity of ONC. Likewise, the addition of RI to a rabbit reticulocyte lysate has not previously been shown to have diminished the ability of ONC to inhibit protein synthesis [22].

As reported in Table 1, ONC cleaves a tetranucleotide substrate with a k_{cat}/K_M value of 2.2×10^2 M^{-1} s^{-1}. This value is slightly lower than that reported previously [23]. The difference could be due to contaminating ribonucleolytic activity in previous assays, which is eliminated by the addition of RI to the assays described herein. This method of eliminating contaminating activity yields a valid value of k_{cat}/K_m only if C87A/C104A ONC is not inhibited significantly by RI in the assay mixture. Indeed, the value of k_{cat}/K_m for C87A/C104A ONC determined in the presence of 0.55 nM RI did not differ significantly from that determined in the presence of 55 nM RI (data not shown), as expected if $K_i \geq 10^{-6}$ M [21]. At 1.1×10^2 M^{-1} s^{-1}, the value of k_{cat}/K_M for C87A/C104A ONC is within two-fold of that for the wild-type enzyme (Table 1).

3.4. Contribution of the Cys87–Cys104 disulfide bond to cytotoxicity

The effect of ONC and the C87A/C104A variant on the viability of a human leukemic cell line was tested by measuring the incorporation of a ^{3}H-labeled nucleotide into DNA after a 44 h incubation with the ribonucleases. As shown in Fig. 3 and listed in Table 1, ONC inhibited the proliferation of K-562 cells with an IC_{50} of 0.2 μM. Deletion of the Cys87–Cys104 disulfide bond reduced substantially the cytotoxic activity of ONC. At 2 μM, the IC_{50} value for C87A/C104A ONC was 10-fold higher than that of the wild-type enzyme.

4. Discussion

Wild-type ONC is 10-fold more cytotoxic than is its C87A/C104A variant. ONC kills cells by cleaving RNA [19]. Changes that compromise ribonucleolytic activity should also compromise cytotoxic activity. For example, alkylation of the active-site histidine residues yields an ONC derivative that lacks catalytic activity and is ineffective as a cytotoxin [19]. Similarly, the Met(-1) variant of ONC is an inefficient catalyst and a weak cytotoxin [21,22]. We find that the value of k_{cat}/K_M for C87A/C104A ONC is within two-fold of that of the wild-type enzyme (Table 1). Removing the Cys87–Cys104 disulfide bond is therefore likely to decrease cytotoxicity by a mechanism unrelated to a decrease in ribonucleolytic activity.

ONC likely encounters proteases while in route to the cytosol, or within the cytosol itself. The high conformational stability of ONC (T_m of 90°C) may limit susceptibility to cellular proteases and thus allow for the accumulation of ribonucleolytic activity in the cytosol. CD spectra indicate that the structure of C87A/C104A ONC is more random than that of wild-type ONC (Fig. 2A), a difference that is consistent with the loss of a covalent crosslink. In addition, although both enzymes are >99% folded at physiological temperature, the conformational stability of the C87A/C104A variant is significantly lower than that of the wild-type enzyme (Fig. 2B and Table 1). These changes may render the variant more susceptible to degradation by proteases. Indeed, C87S/des104 ONC has a T_m that is 19°C less than wild-type ONC and is more vulnerable to degradation by pepsin [24]. Accordingly, a higher concentration of enzyme would be necessary to deliver enough ribonucleolytic activity to kill a cell.

Restricting the terminus of a polymer causes a maximal decrease in the conformational entropy of its unfolded state and thereby results in a maximal increase in the conformational stability of its native state [25]. We had shown previously that the two terminal disulfide bonds in RNase A (that is, Cys26–Cys84 and Cys58–Cys110; Fig. 1) contribute more to the stability of that enzyme than do the two embedded ones [26]. Moreover, removing the four C-terminal residues of RNase A (Asp121 through Val124) decreases its T_m by almost 20°C [27]. Yet, the identity of the C-terminal residue is not critical, as replacing Val124 of RNase A has little impact on

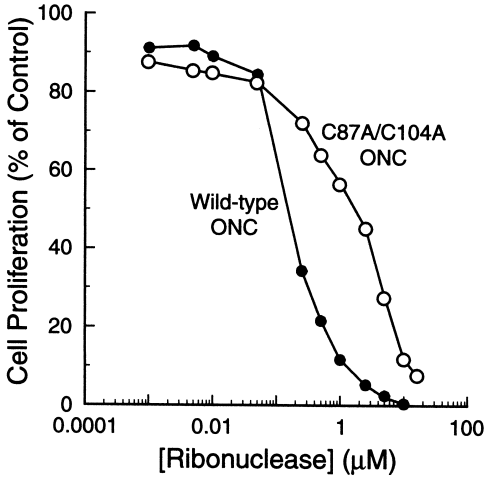

Fig. 3. Proliferation of human leukemia cell line K-562 in the presence of wild-type Onconase® (●) and the C87A/C104A variant (○). Cell proliferation was measured by incorporation of [*methyl*-^{3}H]-thymidine into cellular DNA after a 44 h incubation with the ribonucleases. Values are the mean of four cultures and are expressed as the percentage of control cultures lacking an exogenous ribonuclease. The standard error of each point is $<11\%$.

conformational stability [28]. The C-terminal residues in native RNase A form a β-hairpin, with His105 and Val124 forming a mainchain–mainchain hydrogen bond in a crystal [29] and exhibiting a strong NH–NH NOE in solution [30]. Apparently, ONC uses the Cys87–Cys104 disulfide bond to fortify its native state beyond that attainable with non-covalent interactions alone. Interestingly, the T_m of C87A/C104A ONC (62°C; Table 1) is almost identical to that of RNase A (63°C [9]).

In addition to defining secondary structure and enhancing conformational stability, the C-terminal disulfide bond of ONC may protect ONC from degradation by carboxypeptidases. In this respect, it is intriguing that the N-terminal residue in ONC, as well as that in the *R. catesbeiana* and *R. japonica* ribonucleases, is a pyroglutamate (which forms from the N-terminal glutamine residue [31,32]). This cyclic residue could confer resistance to exopeptidases that would otherwise act at the N-terminus of wild-type ONC and the C87A/C104A variant. The C-terminus of the variant is, however, unobstructed and could be a substrate for carboxypeptidases.

Recently, we used a cytotoxic RNase A variant to probe the relationship between conformational stability and cytotoxic activity [33]. By removing a native disulfide bond, or by incorporating a non-native disulfide bond in a cytotoxic variant of RNase A, we created a family of cytotoxins with differing conformational stabilities. Significantly, as the conformational stability of a variant increases, so does its cytotoxicity. Moreover, increased conformational stability correlates with reduced susceptibility to proteolysis. A variant with high conformational stability exists in an unfolded state to a lesser extent than does one with low conformational stability, decreasing proteolytic susceptibility [34]. Thus, the ribonucleolytic activity and, consequently, the cytotoxicity of ribonucleases appear to be dependent on proteolytic susceptibility. Apparently, ribonucleases with higher conformational stability retain catalytic activity within the cytosol for a longer time and, therefore, are more effective cytotoxins.

5. Conclusions

We find that the Cys87–Cys104 disulfide bond makes a substantial contribution to the conformational stability of ONC. A variant lacking this disulfide bond is 10-fold less toxic for human leukemia cells. We conclude that the Cys87–Cys104 disulfide bond is an important determinant of the cytotoxicity of ONC.

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