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FOR THE RECORD

Variants of ribonuclease inhibitor that resist oxidation

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Abstract: Human ribonuclease inhibitor (hRI) is a cytosolic protein that protects cells from the adventitious invasion of pancreatic-type ribonucleases. hRI has 32 cysteine residues. The oxidation of these cysteine residues to form disulfide bonds is a rapid, cooperative process that inactivates hRI. The most proximal cysteine residues in native hRI are two pairs that are adjacent in sequence: Cys94 and Cys95, and Cys328 and Cys329. A cystine formed from such adjacent cysteine residues would likely contain a perturbing *cis* peptide bond within its eight-membered ring, which would disrupt the structure of hRI and could facilitate further oxidation. We find that replacing Cys328 and Cys329 with alanine residues has little effect on the affinity of hRI for bovine pancreatic ribonuclease A (RNase A), but increases its resistance to oxidation by 10- to 15-fold. Similar effects are observed for the single variants, C328A hRI and C329A hRI, suggesting that oxidation resistance arises from the inability to form a Cys328–Cys329 disulfide bond. Replacing Cys94 and Cys95 with alanine residues increases oxidation resistance to a lesser extent, and decreases the affinity of hRI for RNase A. The C328A, C329A, and C328A/C329A variants are likely to be more useful than wild-type hRI for inhibiting pancreatic-type ribonucleases in vitro and in vivo. We conclude that replacing adjacent cysteine residues can confer oxidation resistance in a protein.

Keywords: angiogenin; *cis* peptide bond; cysteine; cystine; disulfide bond; leucine-rich repeat; protein stability; ribonuclease A

Mammalian cells contain a ribonuclease inhibitor [RI (Lee & Vallee, 1993; Hofsteenge, 1997)]. RI is a 50-kDa protein that constitutes ≤0.01% of the protein in the cytosol of a typical mammalian cell (Blackburn & Moore, 1982). The complexes between RI and pancreatic-type ribonucleases are extremely tight. For example, RI binds to bovine pancreatic ribonuclease A (RNase A) to form a complex with K_d near $10^{-13.3}$ M (Lee et al., 1989; Vicentini et al., 1990). This complex has no detectable ribonucleolytic activity. RI may, therefore, serve to protect the RNA in the cytosol of mammalian cells from the adventitious invasion of ribonucleases (Leland et al., 1998). And, as the most effective known inhibitor of pancreatic-type ribonucleases (Raines, 1998), RI is used widely for protecting RNA during laboratory experiments.

In 1993, the three-dimensional structure of crystalline porcine RI (pRI) was determined by X-ray diffraction analysis (Kobe & Deisenhofer, 1993). pRI has an unusual nonglobular shape that resembles a horseshoe. This shape derives from 16 homologous leucine-rich repeats (Kobe & Deisenhofer, 1994). Crystalline structures have also been determined for complexes of pRI and RNase A (Kobe & Deisenhofer, 1995) and of human RI (hRI) and human angiogenin (Fig. 1) (Papageorgiou et al., 1997). The orientation of the proteins in these two homologous complexes is similar.

The sensitivity of RI to oxidation lessens its utility as a reagent. hRI has 32 cysteine residues. In the native protein, none of these cysteine residues participates in a disulfide bond. The oxidation of pRI, which has 30 cysteine residues, is known to be a highly cooperative process (Fominaya & Hofsteenge, 1992). After the initial oxidation of a small number of cysteine residues, a conformational change occurs that causes an increase in reactivity of the remaining thiols. The result is the formation of 15 disulfide bonds and inactive pRI. In the cytosol, oxidized pRI suffers proteolysis (Blázuez et al., 1996). Oxidation could, thus, trigger the physiological inactivation of RI (Aoki & Natori, 1981; Fominaya et al., 1988). Bound RNase A protects pRI from oxidation, but not completely (Ferreras et al., 1995).

We have analyzed the three-dimensional structure of hRI with the goal of creating RI variants that still bind tightly to RNase A, but that are resistant to oxidation. The most proximal pairs of cysteine residues in the native structure are those arising from cysteine residues that are adjacent in the amino acid sequence. We find that replacing these cysteine residues with alanine endows hRI with oxidation resistance.

Results and discussion: *Design of oxidation-resistant variants of ribonuclease inhibitor:* Our goal was to hinder the cataclysmic oxidation of hRI. We reasoned that those pairs of cysteine residues that were closest in space would have the greatest tendency to form disulfide bonds (Raines, 1997). To identify such residues, we surveyed the three-dimensional structure of hRI (Fig. 1). The most

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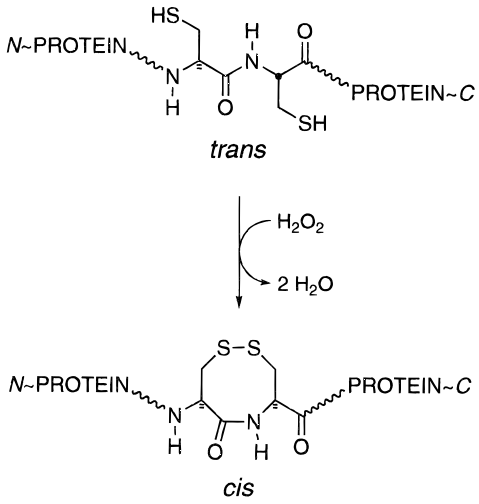
Abbreviations: DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethanesulfonic acid); hRI, human ribonuclease inhibitor; poly(C), poly(cytidylic acid); pRI, porcine ribonuclease inhibitor; RI, ribonuclease inhibitor; RNase A, bovine pancreatic ribonuclease A.

proximal cysteine residues in native hRI are those that are adjacent in the amino acid sequence (Lee et al., 1988): Cys94 and Cys95 (which are in a loop), and Cys328 and Cys329 (which are in an α-helix). None of these four cysteine residues contact angiogenin in the hRI·angiogenin complex (Papageorgiou et al., 1997). pRI has one pair of adjacent cysteine residues, which are homologous to Cys328 and Cys329 (Hofsteenge, 1997). In contrast, RI from rat has no pairs of adjacent cysteine residues. The oxidative stability of the rat protein, as well as its three-dimensional structure, is unknown.

When adjacent cysteine residues form a disulfide bond, the resulting cystine residue defines an eight-membered ring that includes a peptide bond. Normally, a *trans* (i.e., *Z*) peptide bond is more favorable by $\Delta G^\circ = 3$ kcal/mol than is a *cis* (i.e., *E*) peptide bond (Scherer et al., 1998). A *trans* bond is, however, especially unstable in an eight-membered ring. Cyclooctene, like a CysCys cystine residue, has two sp^2 centers in an eight-membered ring. Over 40 years ago, Turner and Meador (1957) showed that *trans*-cyclooctene is $\Delta G^\circ = 9.2$ kcal/mol less stable than is *cis*-cyclooctene. These conformational energetics suggest that a peptide bond within a cystine formed from adjacent cysteine residues will be in the *cis* (rather than *trans*) conformation:



The strain of the *cis* peptide bond is overcome by the strength of the covalent S–S bond, which has a dissociation energy of $D^\circ_{298} = 65$ kcal/mol in $CH_3S–SCH_3$ (Nicovich et al., 1992). Indeed, such cystine residues with *cis* peptide bonds have been found in crystalline methanol dehydrogenase (Blake et al., 1994) and crystalline peptides (Capasso et al., 1977; Mez, 1993). Further, the stability of an intramolecular disulfide bond in Cys–$(Ala)_n$–Cys peptides is greater for $n = 0$ than for $n = 2$, 4, or 5 (Zhang & Snyder, 1989). In solution, the peptide bond within CysCys cystine residues appear to be in a conformational equilibrium, with either the *trans* conformation (Sukumaran et al., 1991; García-Echeverría & Rich, 1994) or the *cis* conformation (Gehrmann et al., 1998) predominating.

The formation of a disulfide bond between adjacent cysteine residues has structural consequences. In particular, a *cis* peptide bond is not tolerated in an α-helix or β-sheet. Replacing a *trans* peptide bond with a *cis* peptide bond is, therefore, likely to distort the structure of the native protein. In hRI, this distortion could congregate other pairs of cysteine residues, leading to further oxidation. Hence, we chose to replace the two pairs of adjacent cysteine residues with alanine.

Fig. 1. Ribbon diagram of the three-dimensional structure of human ribonuclease inhibitor. The structure was determined by X-ray diffraction analysis of the human ribonuclease inhibitor·angiogenin complex (Papageorgiou et al., 1997). The side chains of the 32 cysteine residues are shown, and the four cysteine residues replaced herein are highlighted. Angiogenin is not shown.

Inhibition of ribonuclease A: Cys94, Cys95, Cys328, and Cys329 do not contact angiogenin in the hRI·angiogenin complex (Papageorgiou et al., 1997). So, we did not anticipate that replacing any of these cysteine residues with alanine would impair significantly the ability of hRI to bind to RNase A. As shown in Figure 2, each variant is indeed an effective inhibitor of ribonucleolytic activity. Still, C94A/C95A hRI and C94A/C95A/C328A/C329A hRI are less effective inhibitors than is the C328A/C329A variant (Fig. 2A). The affinity of the two single variants, C328A hRI and C329A hRI, for RNase A is between that of wild-type hRI and the C328A/C329A variant (Fig. 2B).

Oxidation resistance: Replacing adjacent cysteine residues with alanine makes hRI oxidation resistant. As our oxidant, we chose H_2O_2, which oxidizes thiols to disulfides (Trost, 1991) but is easier to dispense than O_2(g) and does not form mixed disulfides (as does oxidized glutathione). As shown in Figure 3, H_2O_2 has a greater effect on C94A/C95A hRI than on the C328A/C329A variant. In our assays, wild-type hRI loses 50% of its activity at 0.007% v/v H_2O_2. In contrast, C328A/C329A hRI retains 50% of its activity at 0.09% v/v H_2O_2. By this measure, the C328/C329A variant is 10- to 15-fold more resistant to oxidative damage than is wild-type hRI.

The enhanced oxidation resistance of C328A/C329A hRI appears to result from the preclusion of a disulfide bond between Cys328 and Cys329. As shown in Figure 3B, the individual C328A and C329A variants of hRI are as resistant to oxidation by H_2O_2 as is C328A/C329A hRI. The simplest explanation of this result is that oxidation of the wild-type protein results in a Cys328–Cys329 disulfide bond, which cannot form in the C328A, C329A, or C328A/C329A variant.

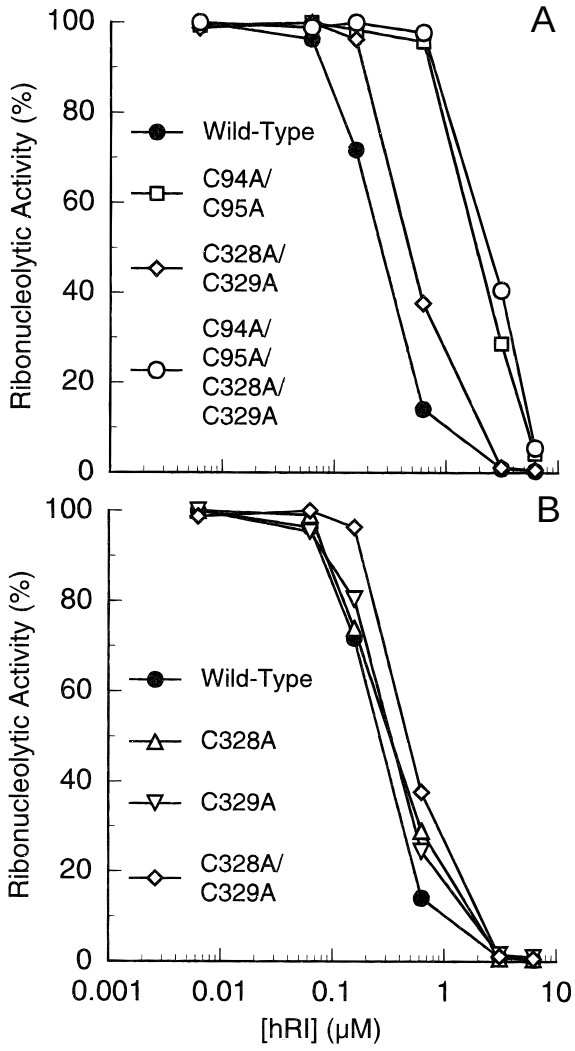


Fig. 2. Inhibition of ribonuclease A by human ribonuclease inhibitor and five variants. Wild-type (filled circle), C94A/C95A (open square), C328A (open up triangle), C329A (open down triangle), C328A/C329A (open diamond), and C94A/C95A/C328A/C329A (open circle). Inhibition experiments were performed in 20 mM HEPES-HCl buffer, pH 7.6, containing RNase A (40 nM), hRI (none; 5 nM to 5 μM), and KCl (50 mM). Ribonucleolytic activity was assessed by monitoring poly(C) cleavage. Data for wild-type hRI and the C328A/C329A variant are shown in both **A** and **B** to ease comparison. Results from duplicate experiments did not differ significantly.

High levels of H_2O_2 (such as 0.09% v/v = 0.04 M) inactivate all five variants of hRI (Fig. 3). At least two explanations are possible for this result. Disulfide bonds could form between thiols of nonadjacent cysteine residues. Alternatively, thiols (RSH) of hRI that contact RNase A in the hRI·RNase A complex could be oxidized to sulfonates (RSO_3^-). Such overoxidation is more likely with H_2O_2 than with O_2(g) (Trost, 1991).

Prospectus: Commercial hRI (e.g., RNasin® from Promega Corp., Madison, Wisconsin) is distributed in solutions containing millimolar levels of dithiothreitol (DTT). The presence of this reducing agent is necessary to maintain hRI in a reduced, and hence active, form. Such reducing agents are incompatible with some laboratory protocols. Moreover, reducing agents are oxidized, and thus rendered ineffective, by the ubiquitous oxidant O_2(g) and by transition metal ions. We find that replacing only 1 (i.e., Cys328 or Cys329) of the 32 cysteine residues of hRI with alanine increases substantially its resistance to oxidation without compromising its affinity for RNase A. We anticipate that variants lacking Cys328 or Cys329 (or both) will be more useful than wild-type hRI in many laboratory protocols.

Oxidation-resistant variants of hRI could serve another purpose. Angiogenin, like RNase A, is bound tightly by hRI. As its name implies, angiogenin promotes neovascularization—the formation of new blood vessels. hRI has been shown to be effective in inhibiting angiogenin-mediated neovascularization (Shapiro & Vallee, 1987; Polakowski et al., 1993). In such physiological experiments, hRI is exposed to an oxidative environment, which could compromise its ability to inhibit angiogenin. We, therefore, suspect that

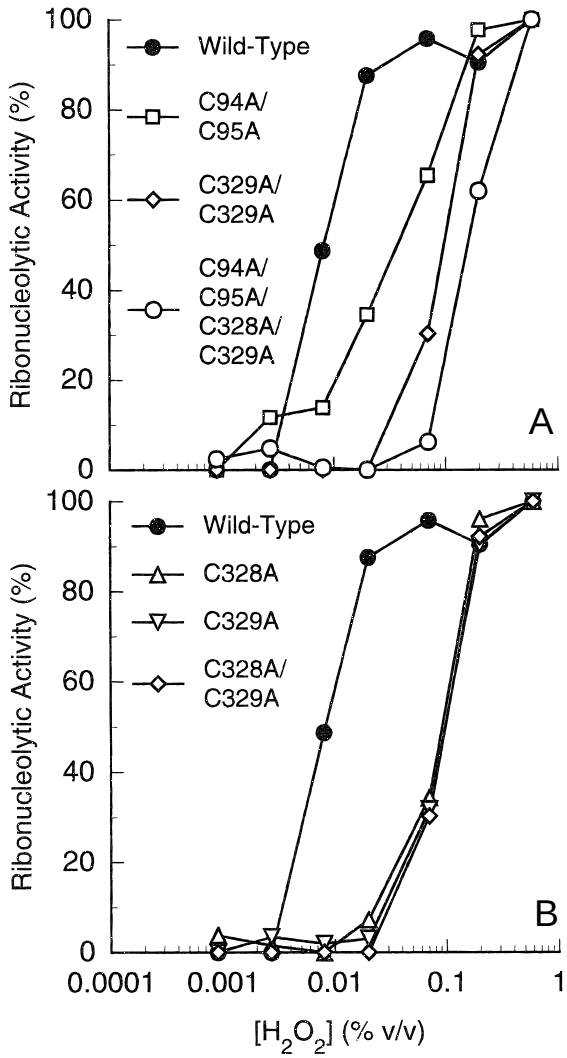


Fig. 3. Resistance to oxidation of human ribonuclease inhibitor and five variants. Wild-type (filled circle), C94A/C95A (open square), C328A (open up triangle), C329A (open down triangle), C328A/C329A (open diamond), and C94A/C95A/C328A/C329A (open circle). Oxidation experiments were performed in 20 mM HEPES-HCl buffer, pH 7.6, containing hRI (none; 5 μM), H_2O_2 (0.002–1% v/v), and KCl (50 mM). Inhibition was assessed as described in Figure 2. Data for wild-type hRI and the C328A/C329A variant are shown in both **A** and **B** to ease comparison. Results from duplicate experiments did not differ significantly.

the oxidation-resistant variants described herein could be more effective than wild-type hRI at inhibiting angiogenin-mediated angiogenesis.

Conclusion: The five variants described herein are all more oxidation resistant than is wild-type hRI. The C94A/C95A variant gains the least resistance (Fig. 3A) and loses affinity for RNase A (Fig. 2A). The quadruple variant, C94A/C95A/C328A/C329A hRI, also loses affinity for RNase A. The variant at Cys328 or Cys329 (or both) is superior at retaining affinity for RNase A and acquiring resistance to oxidation. Thus, the formation of a disulfide bond between Cys328 and Cys329 could trigger the cataclysmic inactivation of hRI. Variants lacking one or both of these cysteine residues are likely to be more useful than wild-type hRI for many purposes. Moreover, we suspect that our strategy is general—replacing adjacent cysteine residues would enhance the conformational stabilities of other proteins in an oxidative environment.

Materials and methods: *Proteins:* RNase A was produced in *Escherichia coli* with a recombinant DNA expression system, as described (delCardayré et al., 1995). Wild-type hRI and its variants were produced in *E. coli* by using plasmid pET–RI, which directs the expression of hRI (Leland et al., 1998). To produce hRI variants, the cDNA that codes for hRI was mutated by the method of Kunkel et al. (1987). The oligonucleotides used were BMK14 (C94A/C95A; *Hin*dIII): GGCCCCCGTCAG**CGCCGC**GTTCTG GAGGCTAAGCTTCTG; BMK16 (C328A/C329A; *Nhe*I): GCT GAAGTGGCT**AGCGGC**GGCGGCTGTGAA; BMK17 (C328A; *Sph*I): GCTGAAGTGGGAGCA**TGC**GGCGGCTGTGAA; and BMK18 (C329A; *Nhe*I): GCTGAAGTGGCT**AGC**GCAGGCGG CTGTGAA. In these sequences, the reverse complement of new alanine codons is in bold type, and new restriction endonuclease sites are underlined. cDNA sequences of mutated plasmids were determined with an ABI 373 Automated Sequencer.

Wild-type hRI and the variants were produced and purified essentially as described (Leland et al., 1998). The key step in the purification protocol is affinity chromatography on RNase A–Sepharose 4B resin. Briefly, *E. coli* lysate in 50 mM potassium phosphate buffer, pH 7.5, containing glycerol (15% v/v), DTT (5 mM), and EDTA (1 mM) was loaded onto the resin. Only active molecules of hRI are bound by the immobilized RNase A. The loaded resin was washed with 50 mM potassium phosphate buffer, pH 7.5, containing NaCl (0.5 M) and DTT (8 mM), and eluted with 0.10 M sodium acetate buffer, pH 5.0, containing glycerol (15% v/v), NaCl (3.0 M), and DTT (8 mM).

The presence of 8 mM DTT would interfere with assays of oxidation resistance. To prepare hRI for the assays described below, the concentration of DTT was reduced by 10^3-fold (to 8 μM) by concentration/dilution. Briefly, hRI was concentrated 10-fold by ultrafiltration using a Microcon 10 microconcentrator from Amicon (Beverly, Massachusetts). The resulting solution was diluted 10-fold with degassed 20 mM HEPES–HCl buffer, pH 7.6, containing glycerol (50% v/v) and KCl (50 mM). This treatment was repeated three times. hRI thus treated retains full activity, provided that its exposure to air is minimal.

General methods: Concentrations of RNase A were determined by assuming that $A = 0.72$ at 277.5 nm for a 1.00 mg/mL solution. Concentrations of hRI were determined by assuming that $A = 0.88$ at 280 nm for a 1.00 mg/mL solution (Ferreras et al., 1995). Concentrations of poly(cytidylic acid) [poly(C)] were determined by assuming that $\epsilon = 6{,}200\ M^{-1}\ cm^{-1}$ per nucleotide at 268 nm (Yakovlev et al., 1992).

Inhibition of ribonuclease A: The ability of each hRI to inhibit the ribonucleolytic activity of RNase A was assayed qualitatively as follows. Serial dilutions were made to produce six solutions (10 μL each) of 20 mM HEPES–HCl buffer, pH 7.6, containing KCl (50 mM) and hRI (10 nM to 10 μM). A solution (10 μL) of 20 mM HEPES–HCl buffer, pH 7.6, containing KCl (50 mM) and RNase A (80 nM) was added to each of the hRI solutions. The resulting mixtures were incubated at 37 °C for 5 min. The ribonucleolytic activity in each mixture was then assessed by using a spectrophotometric assay for poly(C) cleavage, as described (delCardayré et al., 1995), with [poly(C)] = 37 μM. This experiment was performed at least twice with wild-type hRI and each variant.

Oxidation resistance: The resistance of each hRI to oxidation was assayed qualitatively as follows. Serial dilutions were made to produce seven solutions (5 μL each) of 20 mM HEPES–HCl buffer, pH 7.6, containing KCl (50 mM) and H_2O_2 (0.004–2% v/v, which is 2 mM–0.9 M). A solution (5 μL) of 20 mM HEPES–HCl buffer, pH 7.6, containing KCl (50 mM) and hRI (10 μM) was added to each of the H_2O_2 solutions. The resulting mixtures were incubated at 37 °C for 30 min. A solution (10 μL) of 20 mM HEPES–HCl buffer, pH 7.6, containing KCl (50 mM) and RNase A (80 nM) was then added to each H_2O_2 plus hRI solution. The resulting mixtures were incubated at 37 °C for 5 min. The ribonucleolytic activity in each mixture was then assessed by using a spectrophotometric assay for poly(C) cleavage as described (delCardayré et al., 1995), with [poly(C)] = 37 ρM. This experiment was performed at least twice with wild-type hRI and each variant.

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