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Interaction of onconase with the human ribonuclease inhibitor protein

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ABSTRACT

One of the tightest known protein–protein interactions in biology is that between members of the ribonuclease A superfamily and the ribonuclease inhibitor protein (RI). Some members of this superfamily are able to kill cancer cells, and the ability to evade RI is a major determinant of whether a ribonuclease will be cytotoxic. The archetypal cytotoxic ribonuclease, onconase (ONC), is in late-stage clinical trials for the treatment of malignant mesothelioma. We present here the first measurement of the inhibition of the ribonucleolytic activity of ONC by RI. This inhibition occurs with $K_i = 0.15\,\mu M$ in a solution of low salt concentration.

Protein–protein interactions are prevalent in biological systems. Proper functioning of organisms relies on the formation of complexes, such as those between antibodies and antigens, growth factors and receptors, and enzymes and regulators. These interactions have evolved the necessary affinity for their function, as well as the ability to dissociate within a practical timeframe. The theoretical upper limit for the encounter of two proteins in aqueous solution has been estimated to be $\sim 10^6\,M^{-1}\,s^{-1}$ [1,2]. This rate can be increased via electrostatic steering [3–7], as has been observed with barnase and barstar [8], cytochrome *c* and cytochrome *c* peroxidase [9], and thrombin and hirudin [10].

The association rate for the complex formed between secreted members of the bovine pancreatic ribonuclease (RNase A) superfamily and the cytosolic ribonuclease inhibitor protein (RI) is high ($k_a = \sim 10^8\,M^{-1}\,s^{-1}$ for RNase A and human RI) [11]. The related dissociation rate is low, giving equilibrium dissociation constants in the femtomolar range ($K_d = 0.29$ fM for the complex between RI and human pancreatic ribonuclease (RNase 1); Figs. 1A and B) [7]. This high stability has apparently evolved to protect cells against the adventitious invasion of ribonucleases [12]. Indeed, RNase A and RNase 1 variants that evade RI are cytotoxic [7,13–16].

The interface between RI and ribonucleases contains a higher percentage of charged residues than that typically present in protein–protein complexes [15,17], suggesting a role for electrostatics in complex stability. RI is highly anionic, with an isoelectric point of pI = 4.7 [18], and pancreatic-type ribonucleases are highly cationic. Decreasing the net charge of RNase 1 leads to a decrease in affinity for RI [19]. RI is typically purified by RNase A-affinity chromatography, and eluted from the resin with 3 M NaCl [20]. In addition, the inhibition constant (K_i) for the complex between placental RI and RNase A increases by more than four orders of magnitude from 0.1–1.0 M NaCl [21].

Onconase (ONC) [22,23] is a homologue of RNase A from the Northern leopard frog *Rana pipiens* [24]. Unlike RNase A and RNase 1, ONC is naturally toxic to cancer cells [24] and is currently in Phase IIIb clinical trials for the treatment of malignant mesothelioma [25]. Its cytotoxic activity is ascribed primarily to its low affinity for RI [16,26,27], which is not detectable in assays at physiological salt concentration [28,29] and has been estimated to be $K_i \geqslant 1\,\mu M$ [27]. ONC (104 residues) is markedly smaller than either RNase A (124) or RNase 1 (128). The likely absence of deleterious steric interactions in an RI·ONC complex suggests that ONC should have some intrinsic affinity for RI.

The interaction between RI and ribonucleases is typically measured in solutions of physiological salt concentration, such as PBS [28,29]. In aqueous solutions containing salts, ions interact with charged proteins such as RI and ribonucleases preferentially, with cations primarily interacting with anionic surfaces and anions with cationic surfaces. This phenomenon leads to an unequal distribution of ions in the solution; a higher local concentration of ions is present in the vicinity of the proteins than in the bulk

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Abbreviations: RNase A, bovine pancreatic ribonuclease RI, ribonuclease inhibitor protein PBS, phosphate-buffered saline RNase 1, human pancreatic ribonuclease ONC, onconase.

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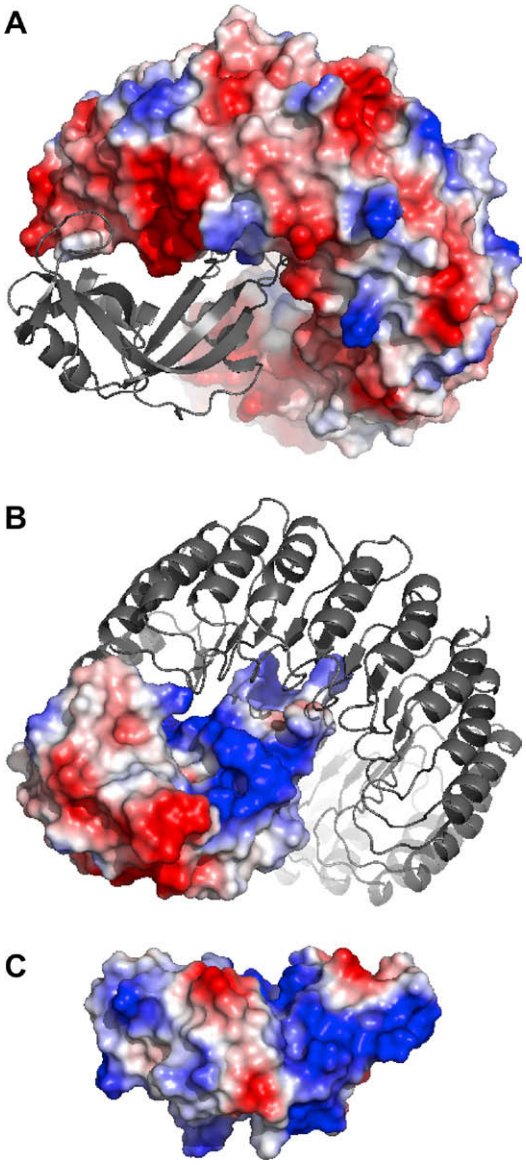

Fig. 1. Crystalline structures of the human RI·RNase 1 complex (Protein Data Bank entry 1z7x [7]) and ONC (entry 1onc [41]). (A) human RI·RNase 1 complex showing electrostatic protein contact potential of human RI and ribbon diagram of RNase 1 (grey). Positively charged surface is shown in blue, negatively charged surface in red, and neutral surface in white. (B) human RI·RNase 1 complex showing electrostatic protein contact potential of RNase 1 and ribbon diagram of human RI. (C) Electrostatic protein contact potential of ONC. Images were generated with the program MacPyMOL (DeLano Scientific, South San Francisco, CA).

solution [30]. When RI binds to ribonucleases, there is a decrease of 2583–3438 Å^2 in the surface area that is exposed to the surrounding solution [16]. This burial results in the release of the ions that were interacting with the surface of the proteins in the interfacial region, and an accompanying increase in the entropy of the system. In solutions of lower salt concentration, the concentration gradient of ions between the bulk water and local water is increased further, increasing the entropy of binding and thus making binding more favorable [30].

Like its homologues, ONC is a highly cationic protein (pI > 9.5, Fig. 1C) [31]. Considering the high charge density of ONC and RI and the large amount of surface area buried in RI·ribonuclease complexes, we reasoned that RI might inhibit ONC in solutions of low salt concentration. Here, we report the first measurement of such an interaction.

Materials and methods

Materials: Escherichia coli BL21(DE3) cells and pET22b(+) plasmid were from Novagen (Madison, WI). Enzymes were obtained from Promega (Madison, WI). Bovine serum albumin (BSA) was obtained as a 20 mg/ml solution (Sigma–Aldrich, St. Louis, MO; product #B8667). 6-Carboxyfluorescein-dArUdGdA-6-carboxytetramethylrhodamine (6-FAM-dArUdGdA-6-TAMRA) [32,33] was obtained from Integrated DNA Technologies (Coralville, IA). Costar 96-well NBS microtiter plates were from Corning Life Sciences (Acton, MA). MES buffer (Sigma–Aldrich) was purified by anion-exchange chromatography to remove oligo(vinylsulfonic acid) (OVS), a potent inhibitor of ribonucleases [34]. All other chemicals used were of commercial grade or better, and were used without further purification.

Instrumentation: Molecular mass was measured by matrix-assisted laser desorption/ionization time-of-flight (MALDI–TOF) mass spectroscopy using a Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems, Foster City, CA) at the University of Wisconsin-Madison Biophysics Instrumentation Facility. The fluorescence intensity in microtiter plates was recorded with a Perkin-Elmer EnVision 2100 plate reader equipped with a FITC filter set (excitation at 485 nm with 14-nm bandwidth; emission at 535 nm with a 25-nm bandwidth; dichroic mirror cutoff at 505 nm) at the W.M. Keck Center for Chemical Genomics.

Purification of ribonuclease inhibitor and ribonucleases: Human RI [7] and RNase A [13] were purified as described previously. ONC was purified as described previously [13], with the following exceptions. ONC was refolded overnight at room temperature after slow dilution (by 10-fold) into 0.10 M Tris–HCl buffer, pH 8.0, containing NaCl (0.10 M), reduced glutathione (3.0 mM), and oxidized glutathione (0.6 mM). After concentration by ultrafiltration, samples were dialyzed overnight against 50 mM sodium acetate buffer, pH 5.0, containing NaCl (0.10 M), and purified by FPLC using Mono S cation-exchange resin (Pharmacia, Uppsala, Sweden). ONC was eluted from the resin with a linear gradient of NaCl (0.15–0.30 M). Protein concentrations were determined by UV spectroscopy using extinction coefficients of $\varepsilon_{280} = 0.88\,(\text{mg/ml})^{-1}\,\text{cm}^{-1}$ for RI [20], $\varepsilon_{278} = 0.72\,(\text{mg/ml})^{-1}\,\text{cm}^{-1}$ for RNase A, and $\varepsilon_{280} = 0.87\,(\text{mg/ml})^{-1}\,\text{cm}^{-1}$ for ONC [13]. The molecular masses of RNase A and ONC were confirmed by MALDI–TOF mass spectrometry.

Determination of K_i value for ONC: A serial dilution (12.5 μM → 96 pM; 2×) of RI in MES–NaOH buffer (20 mM), pH 6.0, containing DTT (5 mM), and BSA (0.1%) was prepared. A 50-μL aliquot of this serial dilution was added to the wells of a 96-well plate. A solution (50-μL) of ONC (100 nM; 2×) in MES–NaOH buffer (20 mM), pH 6.0, containing DTT (5 mM), and BSA (0.1% w/v) was prepared in Eppendorf Protein LoBind Tubes (Fisher Scientific) and added to each well. The negative control contained no RI and the positive control contained excess RNase A (550 μM). A hypersensitive fluorogenic substrate, 6-FAM-dArUdGdA-6-TAMRA (100 nM) [32,33], was added to each well and fluorescence intensity was measured at 25 °C every 30 s over a 5-min period. Data were fitted using nonlinear regression to a dose-response curve using Prism 4 for Macintosh.

Results and discussion

Nonspecific interactions can occur between proteins in solutions of low salt concentration. These interactions can bring together residues that are not at a biologically relevant interface between the proteins. Such weak interactions are screened in solutions of physiological salt concentration [5,35]. To avoid detecting such nonspecific interactions between RI and ONC, we used an activity-based assay to detect binding, measuring the ability of RI to inhibit the ribonucleolytic activity of ONC. These assays were performed

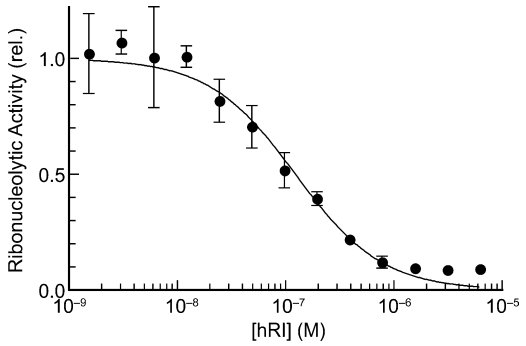

Fig. 2. Inhibition of the ribonucleolytic activity of ONC by human RI. The ribonucleolytic activity of ONC was measured by using a hypersensitive fluorogenic substrate, 6-FAM-dArUdGdA-6-TAMRA (100 nM), in 20 mM MES–NaOH buffer, pH 6.0, containing DTT (5 mM), BSA (0.1% w/v), and ONC (50 nM). The value of $K_i = (0.15 \pm 0.05)\,\mu M$.

at pH 6.0, which is close to the pH-optimum for catalysis by ONC [36]. We found that RI does inhibit the enzymatic activity of ONC under these conditions, and that the value of $K_i = (0.15 \pm 0.05)\,\mu M$ (Fig. 2). We conclude that there is no obstacle that precludes the formation of an RI·ONC complex. Instead, this complex is merely disfavored relative to those of RI and RNase A or RNase 1.

The ribonuclease A superfamily is a vertebrate-specific family that is evolving rapidly [37,38]. Ribonucleases and inhibitors from different classes do not interact [39], and the intraspecies complexes are more stable and form more rapidly than do interspecies complexes [40]. The framework for RI-binding is in place in ONC (i.e., the molecular shape and charge distribution; Figs. 1B and C), but the individual residues that closely interact with RI in other ribonucleases are largely absent in ONC [16,27]. These factors combine to allow ONC to interact with RI in a solution of low salt concentration, but not at physiological salt concentrations. Ongoing work in our laboratory is aimed at isolating an RI homologue from *Rana pipiens*. The measurement of its affinity for amphibian and mammalian ribonucleases will inform our understanding of the evolution of these interesting and important binding partners.

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References

[1] O.G. Berg, P.H. von Hippel, Diffusion-controlled macromolecular interactions, Annu. Rev. Biophys. Biophys. Chem. 14 (1985) 131–160.
[2] S.H. Northrup, H.P. Erickson, Kinetics of protein–protein association explained by Brownian dynamics computer simulation, Proc. Natl. Acad. Sci. USA 89 (1992) 3338–3342.
[3] G. Schreiber, A.R. Fersht, Rapid electrostatically assisted association of proteins, Nat. Struct. Biol. 3 (1996) 427–431.
[4] F.B. Sheinerman, B. Honig, On the role of electrostatic interactions in the design of protein–protein interfaces, J. Mol. Biol. 318 (2002) 161–177.
[5] Y. Shaul, G. Schreiber, Exploring the charge space of protein–protein association: A proteomic study, Proteins 60 (2005) 341–352.
[6] R. Alsallaq, H.X. Zhou, Prediction of protein–protein association rates from a transition-state theory, Structure 15 (2007) 215–224.
[7] R.J. Johnson, J.G. McCoy, C.A. Bingman, G.N. Phillips Jr., R.T. Raines, Inhibition of human pancreatic ribonuclease by the human ribonuclease inhibitor protein, J. Mol. Biol. 367 (2007) 434–449.
[8] G. Schreiber, A.R. Fersht, Interaction of barnase with its polypeptide inhibitor barstar studied by protein engineering, Biochemistry 32 (1993) 5145–5150.
[9] L.B. Vitello, J.E. Erman, Binding of horse heart cytochrome *c* to yeast porphyrin cytochrome c peroxidase: A fluorescence quenching study on the ionic strength dependence of the interaction, Arch. Biochem. Biophys. 258 (1987) 621–629.
[10] S.R. Stone, J. Hofsteenge, Kinetics of the inhibition of thrombin by hirudin, Biochemistry 25 (1986) 4622–4628.
[11] F.S. Lee, D.S. Auld, B.L. Vallee, Tryptophan fluorescence as a probe of placental ribonuclease inhibitor binding to angiogenin, Biochemistry 28 (1989) 219–224.
[12] M.C. Haigis, E.L. Kurten, R.T. Raines, Ribonuclease inhibitor as an intracellular sentry, Nucleic Acids Res. 31 (2003) 1024–1032.
[13] P.A. Leland, L.W. Schultz, B.-M. Kim, R.T. Raines, Ribonuclease A variants with potent cytotoxic activity, Proc. Natl. Acad. Sci. USA 98 (1998) 10407–10412.
[14] P.A. Leland, K.E. Staniszewski, B.-M. Kim, R.T. Raines, Endowing human pancreatic ribonuclease with toxicity for cancer cells, J. Biol. Chem. 276 (2001) 43095–43102.
[15] T.J. Rutkoski, E.L. Kurten, J.C. Mitchell, R.T. Raines, Disruption of shape-complementarity markers to create cytotoxic variants of ribonuclease A, J. Mol. Biol. 354 (2005) 41–54.
[16] T.J. Rutkoski, R.T. Raines, Evasion of ribonuclease inhibitor as a determinant of ribonuclease cytotoxicity, Curr. Pharm. Biotechnol. 9 (2008) 185–199.
[17] B. Kobe, J. Deisenhofer, Mechanism of ribonuclease inhibition by ribonuclease inhibitor protein based on the crystal structure of its complex with ribonuclease A, J. Mol. Biol. 264 (1996) 1028–1043.
[18] K.A. Dickson, M.C. Haigis, R.T. Raines, Ribonuclease inhibitor: Structure and function, Prog. Nucleic Acid Res. Mol. Biol. 80 (2005) 349–374.
[19] R.J. Johnson, T.-Y. Chao, L.D. Lavis, R.T. Raines, Cytotoxic ribonucleases: The dichotomy of Coulombic forces, Biochemistry 46 (2007) 10308–10316.
[20] T.A. Klink, A.M. Vicentini, J. Hofsteenge, R.T. Raines, High-level soluble production and characterization of porcine ribonuclease inhibitor, Protein Exp. Purif. 22 (2001) 174–179.
[21] F.S. Lee, R. Shapiro, B.L. Vallee, Tight-binding inhibition of angiogenin and ribonuclease A by placental ribonuclease inhibitor, Biochemistry 28 (1989) 225–230.
[22] J.E. Lee, R.T. Raines, Ribonucleases as novel chemotherapeutics: The ranpirnase example, BioDrugs 22 (2008) 53–58.
[23] W. Ardelt, K. Shogen, Z. Darzynkiewicz, Onconase and Amphinase, the antitumor ribonucleases from *Rana pipiens* oocytes, Curr. Pharm. Biotechnol. 9 (2008) 215–225.
[24] Z. Darzynkiewicz, S.P. Carter, S.M. Mikulski, W.J. Ardelt, K. Shogen, Cytostatic and cytotoxic effect of Pannon (P-30 Protein), a novel anticancer agent, Cell Tissue Kinet. 21 (1988) 169–182.
[25] N. Pavlakis, N.J. Vogelzang, Ranpirnase—an antitumour ribonuclease: Its potential role in malignant mesothelioma, Expert Opin. Biol. Ther. 6 (2006) 391–399.
[26] Y. Wu, S.M. Mikulski, W. Ardelt, S.M. Rybak, R.J. Youle, A cytotoxic ribonuclease. Study of the mechanism of onconase cytotoxicity, J. Biol. Chem. 268 (1993) 10686–10693.
[27] E. Boix, Y. Wu, V.M. Vasandani, S.K. Saxena, W. Ardelt, J. Ladner, R.J. Youle, Role of the N-terminus in RNase A homologues: Differences in catalytic activity, ribonuclease inhibitor interaction and cytotoxicity, J. Mol. Biol. 257 (1996) 992–1007.
[28] R.L. Abel, M.C. Haigis, C. Park, R.T. Raines, Fluorescence assay for the binding of ribonuclease A to the ribonuclease inhibitor protein, Anal. Biochem. 306 (2002) 100–107.
[29] L.D. Lavis, T.J. Rutkoski, R.T. Raines, Tuning the pK_a of fluorescein to optimize binding assays, Anal. Chem. 79 (2007) 6775–6782.
[30] M.T. Record Jr., W. Zhang, C.F. Anderson, Analysis of effects of salts and uncharged solutes on protein and nucleic acid equilibria and processes: A practical guide to recognizing and interpreting polyelectrolyte effects, Hofmeister effects, and osmotic effects of salts, Adv. Protein Chem. 51 (1998) 281–353.
[31] W. Ardelt, S.M. Mikulski, K. Shogen, Amino acid sequence of an anti-tumor protein from *Rana pipiens* oocytes and early embryos. Homology to pancreatic ribonucleases, J. Biol. Chem. 266 (1991) 245–251.
[32] B.R. Kelemen, T.A. Klink, M.A. Behlke, S.R. Eubanks, P.A. Leland, R.T. Raines, Hypersensitive substrate for ribonucleases, Nucleic Acids Res. 27 (1999) 3696–3701.
[33] J.E. Lee, R.T. Raines, Contribution of active-site residues to the function of onconase, a ribonuclease with antitumoral activity, Biochemistry 42 (2003) 11443–11450.
[34] B.D. Smith, M.B. Soellner, R.T. Raines, Potent inhibition of ribonuclease A by oligo(vinylsulfonic acid), J. Biol. Chem. 278 (2003) 20934–20938.
[35] T. Selzer, G. Schreiber, Predicting the rate enhancement of protein complex formation from the electrostatic energy of interaction, J. Mol. Biol. 287 (1999) 409–419.
[36] J.E. Lee, E. Bae, C.A. Bingman, G.N. Phillips Jr., Structural basis for catalysis by onconase, J. Mol. Biol. 375 (2008) 165–177.
[37] J.J. Beintema, C. Schüller, M. Irie, A. Carsana, Molecular evolution of the ribonuclease superfamily, Prog. Biophys. Mol. Biol. 51 (1988) 165–192.
[38] M.C. Haigis, E.S. Haag, R.T. Raines, Evolution of ribonuclease inhibitor protein by exon duplication, Mol. Biol. Evol. 19 (2002) 960–964.
[39] J.J. Beintema, R.G. Kleineidam, The ribonuclease A superfamily: General discussion, Cell. Mol. Life. Sci. 54 (1998) 825–832.
[40] R.J. Johnson, L.D. Lavis, R.T. Raines, Intraspecies regulation of ribonucleolytic activity, Biochemistry 46 (2007) 13131–13140.
[41] S.C. Mosimann, W. Ardelt, M.N.G. James, Refined 1.7 Å X-ray crystallographic structure of P-30 protein, an amphibian ribonuclease with anti-tumor activity, J. Mol. Biol. 236 (1994) 1141–1153.