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Inhibition of Human Pancreatic Ribonuclease by the Human Ribonuclease Inhibitor Protein

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The ribonuclease inhibitor protein (RI) binds to members of the bovine pancreatic ribonuclease (RNase A) superfamily with an affinity in the femtomolar range. Here, we report on structural and energetic aspects of the interaction between human RI (hRI) and human pancreatic ribonuclease (RNase 1). The structure of the crystalline hRI·RNase 1 complex was determined at a resolution of 1.95 Å, revealing the formation of 19 intermolecular hydrogen bonds involving 13 residues of RNase 1. In contrast, only nine such hydrogen bonds are apparent in the structure of the complex between porcine RI and RNase A. hRI, which is anionic, also appears to use its horseshoe-shaped structure to engender long-range Coulombic interactions with RNase 1, which is cationic. In accordance with the structural data, the hRI·RNase 1 complex was found to be extremely stable ($t_{1/2}$ = 81 days; $K_d = 2.9 \times 10^{-16}$ M). Site-directed mutagenesis experiments enabled the identification of two cationic residues in RNase 1, Arg39 and Arg91, that are especially important for both the formation and stability of the complex, and are thus termed "electrostatic targeting residues". Disturbing the electrostatic attraction between hRI and RNase 1 yielded a variant of RNase 1 that maintained ribonucleolytic activity and conformational stability but had a 2.8×10^3-fold lower association rate for complex formation and 5.9×10^9-fold lower affinity for hRI. This variant of RNase 1, which exhibits the largest decrease in RI affinity of any engineered ribonuclease, is also toxic to human erythroleukemia cells. Together, these results provide new insight into an unusual and important protein–protein interaction, and could expedite the development of human ribonucleases as chemotherapeutic agents.

Keywords: cancer; cytotoxin; protein–protein interaction; tight-binding inhibitor; X-ray crystallography

Introduction

The stability of a protein·protein complex is governed by intermolecular forces that mediate the rates at which the proteins associate and the complex dissociates. The rate of dissociation is affected largely by forces that act over short distances, including hydrophobic forces, hydrogen bonds, and van der Waals interactions. The rate of association, however, depends primarily on diffusion but can be increased by Coulombic forces.[1–7] Although a large energetic penalty is incurred upon desolvation of charged amino acids,[8,9] the rate of association and, consequently, the stability of a complex can be increased by optimizing Coulombic interactions.[5,6,10]

Abbreviations used: BS-RNase, bovine seminal ribonuclease; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol; EDN, eosinophil-derived neurotoxin; FADE, fast atomic density evaluator; 6-FAM, 6-carboxyfluorescein; hRI, human ribonuclease inhibitor; MALDI–TOF, matrix-assisted laser desorption/ionization–time-of-flight; ONC, Onconase® (a registered trademark of Alfacell, Inc.); PBS, phosphate-buffered-saline; PDB, Protein Data Bank; pRI, porcine ribonuclease inhibitor; RI, ribonuclease inhibitor; RNase A, bovine pancreatic ribonuclease; RNase 1, human pancreatic ribonuclease; rmsd, root-mean-square deviation; 6-TAMRA, 6-carboxytetramethylrhodamine; TB, terrific broth; TCEP, Tris(2-carboxyethyl)phosphine.

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The ribonuclease inhibitor protein forms a tight ($K_d = k_d/k_a \approx 10^{-15}$ M) complex with multiple members of the bovine pancreatic ribonuclease (RNase A[11]; EC 3.1.27.5) superfamily, as shown in equation (1) for the human inhibitor (hRI) and enzyme (RNase 1).[12,13]

$$\text{hRI} + \text{RNase 1} \underset{k_d}{\overset{k_a}{\rightleftharpoons}} \text{hRI·RNase 1} \qquad (1)$$

RI achieves its high affinity for ribonucleases through the burial of a large surface area (2908 Å^2 for the hRI·angiogenin complex[14]), along with one of the largest known electrostatic energies of interaction ($\Delta U = -12.3$ kcal/mol for the hRI·angiogenin complex[15]). Indeed, among 68 heterodimeric protein·protein complexes, the hRI·angiogenin complex ranked behind only the karyopherin β2–Ran complex in the relative contribution of electrostatic energy to complex formation.[15]

The evasion of RI by ribonucleases has medicinal implications, as variants of RNase A that evade RI are toxic to cancer cells.[16,17] By using the structure of the complex between porcine RI (pRI) and RNase A,[18] we designed variants of RNase A that are more toxic to human leukemic cells *in vitro* than is Onconase® (ONC), a naturally cytotoxic homologue from *Rana pipiens* that is now in Phase III clinical trials as a cancer chemotherapeutic agent.[17] Disruption of the pRI·RNase A interface was accomplished by designing RNase A variants with amino acid substitutions that disturbed regions of high shape-complementarity.[17] These substitutions targeted short-range pRI·RNase A interactions by instilling steric hindrance or excising hydrogen bonds. We also applied this strategy to bovine seminal ribonuclease (BS-RNase, 87% sequence similarity), another homologue of RNase A that is dimeric in its native state.[19,20] A BS-RNase variant with substitutions in the same high shape-complementarity regions was also more cytotoxic than ONC *in vitro*.[20]

Designing proteins that have diminished affinity for a cognate protein could be accomplished by targeting either component of the equilibrium dissociation constant: k_d or k_a. Previous studies of the RI·ribonuclease interface have focused on short-range intermolecular contacts between the proteins, thereby raising the dissociation rate.[21,22,17] Diminishing the affinity of RNase 1, the human homologue of RNase A, by modulating short-range interactions has, however, proven to be difficult.[23–25] Although RNase 1 and RNase A share 70% sequence identity, mutagenesis studies have indicated substantial variation in how each is recognized by RI.[23–25] We sought to elaborate how RNase 1 is recognized by hRI.

Here, we report the atomic structure of the crystalline hRI·RNase 1 complex. We use this structure to design RNase 1 variants that reveal the contribution of specific residues to the affinity for hRI and to design a variant that has micromolar (rather than femtomolar) affinity for hRI. This variant is toxic to human erythroleukemia cells. Our findings cause us to re-evaluate the stability of the wild-type hRI·RNase 1 complex, which we find to have a K_d value that is nearly 10^3-fold lower than any reported previously. Overall, this work highlights the structural basis for intraspecies regulation of ribonucleolytic activity as well as facilitates the development of chemotherapeutic agents based on human ribonucleases.

Results

Important interactions between hRI and RNase 1

The three-dimensional crystal structure of the hRI·RNase 1 complex was refined to an R_{cryst} value of 0.175 ($R_{free} = 0.236$) at a resolution of 1.95 Å (Table 1). The asymmetric unit of the crystal of the hRI·RNase 1 complex resembles that of the hRI·angiogenin complex in its containing two

Table 1. Crystallographic, data processing, and refinement statistics

Data collection statistics	
Space group	$P2_12_12_1$
Unit cell parameters	a=71.338, b=107.546, c=155.036
Alpha beta gamma	90.00 90.00 90.00
X-ray energy (keV)	12.399
X-ray wavelength (Å)	0.99997
Overall resolution range (Å)	47.17–1.95 (2.00–1.95)
Number of reflections	Measured 573,939, unique 84,446
Completeness (%)	97.0 (72.6)
R_{merge}[a]	0.078 (0.424)
Redundancy	6.8 (3.6)
Mean I/σ (I)	16.96 (2.94)
Phasing	
MR correlation coefficient (MOLREP)[d]	0.223
MR model[d]	1DFJ
Refinement and model statistics from REFMAC 5.2.0005	
Number of reflections (total)	80,141
R_{cryst}[b] (R_{free}[c])	0.175 (0.236)
rmsd bonds (Å)	0.016
rmsd angles (°)	1.515
ESU based on R_{free} (Å)[d]	0.166
Average B factor (Å^2)	28.04
Number of water molecules	854
Ramachandran plot	
Residues in most favorable region (%)	86.8
Residues in additional allowed region (%)	12.8
Residues in generously allowed region (%)	0.4
Residues in disallowed region (%)	0.0

Values in parentheses refer to the highest resolution shell.

[a] $R_{merge} = \sum_h \sum_i | I_i(h) - \langle I(h) \rangle | / \sum_h \sum_i I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

[b] $R_{cryst} = \sum_h | F_{obs} - F_{calc} | / \sum_h F_{obs}$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

[c] R_{free} was calculated as R_{cryst} using 5.0% of the randomly selected unique reflections that were omitted from structure refinement.

[d] Abbreviations used: MR, molecular replacement; ESU, estimated standard uncertainty.