Supporting Information

Protein Prosthesis: A Semisynthetic Enzyme with a β-Peptide Reverse Turn

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Materials. Chemicals were of commercial reagent grade or better, and were used without further purification.

Instruments. Peptides were synthesized with an Applied Biosystems Pioneer synthesizer at the University of Wisconsin Biotechnology Center. Mass spectra were obtained with Perkin Elmer Sciex API 365 electrospray ionization (ESI) and Bruker Biflex III matrix-assisted laser desorption/ionization (MALDI) instruments at the University of Wisconsin Biotechnology Center. Ultraviolet and visible absorbance was recorded with a Cary Model 3 UV/VIS spectrophotometer from Varian (Palo Alto, CA) equipped with a Varian temperature controller.

Plasmid Construction. Plasmid pTXB1 was from New England Biolabs (Beverly, MA). This plasmid directs the expression of the *mxe*-intein fused to a chitin-binding domain. Plasmid pBXR encodes the cDNA for wild-type RNase A.[1] DNA sequences encoding both full-length RNase A and its 1–94 fragment were inserted into pTXB1 by standard techniques using the PCR. The resulting plasmids direct the expression of wild-type RNase A or RNase A(1–94) fused to the *mxe*-intein. It is noteworthy that both proteins produced by this method possess an additional methionine residue at their N-terminus: Met(−1).

Production of Fusion Proteins. Luria–Bertani medium [which contained (in 1 liter) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g)] containing ampicillin (100 μg/ml) was inoculated with a fresh culture of *Escherichia coli* ER2566 cells (New England Biolabs) that had been transformed with a plasmid that directs the expression of wild-type RNase A or RNase A(1–94) fused to the *mxe*-intein. Cultures were grown with shaking at 37°C until O.D. = 0.5 at 600 nm. Gene expression was then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; to 0.5 mM), and the cultures were grown for an additional 3–4 h at 25°C. The lower temperature prevents the formation of inclusion bodies. The yield of harvested cells was ~2.3 g per liter of bacterial culture. Cells were stored at −20°C until used.

Production of Thioester-Tagged Wild-Type RNase A and RNase A(1–94). After thawing, cells were suspended in lysis and column buffer, which was 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH buffer (pH 6.8) containing NaCl (0.50 M), ethylenediaminetetraacetic acid (EDTA; 0.1 mM), and Triton X-100 (0.1% v/v). Cells were lysed by sonication, and the lysate was clarified by centrifugation at 15,000*g* for 30 min. The supernatant was applied slowly at 4°C to an equilibrated column of chitin resin (New England Biolabs). The loaded resin was washed thoroughly with column buffer. Intein cleavage was induced by incubating the resin at 4°C for 12 h with cleavage buffer, which was 50 mM MOPS–NaOH buffer (pH 6.8) containing NaCl (0.50 M), 2-mercaptoethanesulfonic acid (MESNA; 50 mM), and EDTA (0.1 mM). MESNA effects the transthioesterification of the fusion protein to form a thioester-tagged protein. The cleavage product was eluted from the resin at 4°C with 2 column-volumes of cleavage buffer. The intact wild-type RNase A was subjected

to folding directly (*vide infra*). The RNase A(1–94) thioester was precipitated by the addition of an aqueous solution of sodium deoxycholate (NaDOC; to 0.24 mM) and trichloroacetic acid (TCA; to 30 mM).[2] This precipitate was washed with acetone, dissolved in 2 M guanidine–HCl to a concentration of approximately 5 mg/ml, and used in a ligation reaction.

A potential obstacle arose from the presence of an asparagine residue at position 94 of the RNase A sequence. In natural intein-containing proteins, intein splicing involves cyclization of a C-terminal asparagine residue.[3-5] Asn94 could cyclize similarly, and thereby destroy the thioester that is necessary for ligation. After cleavage of the fusion protein by transthioesterification with MESNA, mass spectrometry revealed the presence of three RNase A(1–94) species, each with a distinct C-terminus: thioester, succinimide (from cyclization), and free acid (from hydrolysis). Thus, ligation of the RNase A(1–94) cleavage product to the synthetic peptide resembling residues 95–124 of the RNase A sequence was successful, despite the presence of a C-terminal asparagine residue in the cleavage product. The ligation yields were, however, low (*vide infra*).

Synthesis of Reverse-Turn Mimic. The β-peptide reverse-turn mimic, *R*-nipecotic acid–*S*-nipecotic acid (*R*-Nip–*S*-Nip), and its diastereomer, *R*-nipecotic acid–*R*-nipecotic acid (*R*-Nip–*R*-Nip), were synthesized with Fmoc protection as described previously.[6-8]

Synthesis of Peptides. A methylbenzhydrylamine polystryrene resin that had been functionalized with a 4-hydroxymethylphenoxy acid labile linker and loaded with valine (which is the C-terminal residue of RNase A) was used for all peptide syntheses. Monomers were coupled by using cycles of *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium

hexafluorophosphate/diisopropylethyl amine activation of the carboxylic acid group, followed by piperidine deprotection of the Fmoc group. Syntheses were done on a 50- to 100-μmol scale with a 4-fold molar excess of each amino acid monomer or β-peptide reverse-turn mimic. The sequence of the wild-type peptide was CAYKTTQANKHIIVACEGNPYVPVHFDASV (which corresponds to residues 95–124), and the sequence of the variant peptide was CAYKTTQANKHIIVACEG-mimic-YVPVHFDASV, (where "mimic" refers to *R*-Nip–*S*-Nip or *R*-Nip–*R*-Nip). The monoisotopic mass of each synthetic peptide is listed in Table S1.

Ligation of Thioester-Tagged Protein and Peptide. Ligation reactions were performed in 0.20 M Tris–HCl buffer (pH 8.0) containing guanidine–HCl (2.0 M), the thioester-tagged protein (5 mg/ml), and the peptide (12.5 mg/ml; 40-fold molar excess). The pH was adjusted to ~8 with 1 M NaOH, and the ligation reaction was allowed to proceed at room temperature for at least 16 h. Although changing the pH between 7.0 and 9.0 or the identity of residues 113 and 114 had little effect on the yield of the ligation reaction, a high molar excess of peptide was critical for obtaining a high yield (Figure S1).

Protein Folding. Prior to folding the semisynthetic proteins, gel filtration chromatography was used to remove excess guanidine–HCl and unligated peptide from the ligation reaction mixture. Specifically, a column of Superdex75 HiLoad™ 26/60 gel filtration resin (Amersham Biosciences; Piscataway, NJ) was equilibrated with 0.10 M sodium acetate buffer (pH 5) containing NaCl (0.10 M). The ligation reaction mixture was applied to the column, and fractions containing the ligation product were collected. These fractions were pooled, and the pooled fractions were diluted with water to <0.1 mg of protein per milliliter. Reduced glutathione

(GSH) and oxidized glutathione (GSSG) were added with stirring at 4°C to final concentrations of 1.0 and 0.2 mM, respectively. After adjusting the pH to ~8 with 1 M NaOH, the solution was transferred to room temperature and protein folding was allowed to proceed for at least 16 h.

Protein Purification. Prior to application of the folding reaction to columns, any precipitate formed during folding was removed by centrifugation at 15,000*g* for 30 min. The first purification step employed either affinity chromatography or hydrophobic interaction chromatography. The resulting protein was purified by cation-exchange chromatography. Samples from throughout the purification procedure were analyzed by SDS–PAGE through a 15% (w/v) acrylamide gel.[9]

Affinity Chromatography. The folding reaction mixture was diluted with water to contain less than 30 mM NaCl, and the pH was adjusted to 5.2 with 1 M acetic acid. The resulting solution was applied slowly to a column of guanosine 5′-diphosphate~agarose resin (Sigma Chemical; 5 ml) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.2). The loaded resin was washed thoroughly with equilibration buffer. The protein was eluted with the same buffer containing NaCl (0.25 M). Fractions (2 ml) were collected, and absorbance at 278 nm was used to locate the protein.

Hydrophobic Interaction Chromatography. Solid ammonium sulfate was added slowly and with stirring to the folding reaction mixture to a final concentration of 1.7 M. This sample was then applied to a column of Phenyl Sepharose™ 6 Fast Flow resin (Amersham Biosciences; 6 ml), that had been equilibrated with 0.10 M sodium phosphate buffer (pH 7.0) containing ammonium sulfate (1.7 M). The loaded resin was washed thoroughly with equilibration buffer. The protein was eluted with a linear reverse gradient (30 + 30 ml) of ammonium sulfate

(1.7–0.0 M). Fractions (2 ml) were collected, and absorbance at 278 nm was used to locate the protein.

Cation-Exchange Chromatography. Fractions that contained ribonucleolytic activity (*vide infra*) were pooled, and the pooled fractions were diluted with water to contain <0.2 M NaCl and <0.1 M ammonium sulfate. The pH was adjusted to ~4.5 with 1 M acetic acid. The resulting solution was loaded on to a column of HiTrap SP Sepharose™ cation-exchange resin (Amersham Biosciences; 5 ml) that had been equilibrated with 20 mM sodium acetate buffer (pH 5.0). The loaded resin was washed with 10 ml of equilibration buffer, 10 ml of H_2O, and 10 ml of 5 mM sodium phosphate buffer (pH 8.0). Semisynthetic RNase A variants were eluted with 10 ml of 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (25 mM). Fractions (2 ml) were collected, and absorbance at 278 nm was used to locate the protein. If necessary, samples were concentrated by partial lyophilization. It is noteworthy that the semisynthetic proteins behaved in an identical manner during their purification by chromatography, which is consistent with their being of similar composition and structure.

After purification, 30 mg of peptide yielded 1 mg of a semisynthetic RNase A with Asn–Pro as residues 113 and 114 (wild-type RNase A) or *R*-Nip–*S*-Nip replacing residues 113 and 114 (β*RS* RNase A) (40% yield based on thioester-tagged protein; 1% yield based on peptide). The semisynthetic RNase A with *R*-Nip–*R*-Nip replacing residues 113 and 114 (β*RR* RNase A) did not fold properly and could not be purified after ligation. The monoisotopic mass of each synthetic peptide is listed in Table S1. A mass spectrum of each semisynthetic protein produced by intein-mediated ligation is shown in Figure S2, and the mass of each variant and wild-type protein is listed in Table S2. The mass spectrum of β*RR* RNase A is of the unpurified ligation mixture, and shows the presence of the unligated fragment corresponding to residues 1–94. The

mass of the unligated fragment (observed *m*/*z* 10,557) suggests that the MESNA thioester had undergone hydrolysis (expected *m*/*z* 10,557 for MH^+ with 3 disulfide bonds).

Wild-Type Ribonuclease A. The analysis of semisynthetic enzyme with a prosthetic group requires a meaningful comparison to an enzyme lacking the prosthetic group. As listed in Tables 1 and S2, wild-type RNase A was produced in three distinct ways. "Semisynthetic" RNase A was produced by the ligation of RNase A(1–94) with a peptide containing Asn–Pro as residues 113 and 114. RNase A from "*E. coli*" was produced by the spontaneous hydrolysis of thioester-tagged wild-type RNase A(1–124). These two wild-type enzymes contained an N-terminal methionine residue [Met(–1)]. RNase A from "bovine pancreas" was from Sigma Chemical (St. Louis, MO). This enzyme did not contain an N-terminal methionine residue.

Thermal Denaturation. UV spectroscopy was used to determine the effect on the thermal stability of RNase A of replacing Asn113 and Pro114 with an artificial reverse-turn mimic. As RNase A is unfolded, its six tyrosine residues become exposed to the solvent and its molar absorptivity at 287 nm decreases significantly.[10] The thermal stabilities of RNase A and its semisynthetic variants were determined by monitoring the change in absorbance at 287 nm with temperature.[11] Protein samples (0.2 mg/ml) were in 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (25 mM). The thermal transition upon heating at 0.15°C/min was monitored at 287 nm.

Ribonucleolytic Activity. Values of k_{cat}/K_M for the enzymatic cleavage of a fluorogenic substrate, 6-carboxyfluorescein~dArU$(dA)_2$~6-carboxytetramethylrhodamine, were determined as described previously.[12]

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Table S1. Monoisotopic Mass of Synthetic Peptides for the Semisynthesis of Ribonuclease A

Residues 113–114	MALDI (MH^+)	expected (MH^+)
R-Nip–*S*-Nip (**2**)	3286.2	3286.6
R-Nip–*R*-Nip	3287.0	3286.6
Asn–Pro (**1**)	3275.2	3275.6

Table S2. Mass of Ribonuclease A Variants

residues 113–114	origin	mass		
		ESI	MALDI	expected
R-Nip–*S*-Nip (**2**)	semisynthesis	13,827	13,830	13,824
R-Nip–*R*-Nip	semisynthesis	13,828	13,826	13,824
Asn–Pro (**1**)	semisynthesis	13,813	13,809	13,813
Asn–Pro (**1**)	*E. coli*	13,818	13,815	13,813
Asn–Pro (**1**)	bovine pancreas	ND[a]	ND	13,683[b]

[a] ND, not determined. [b] This protein lacks an N-terminal methionine residue.

FIGURE LEGENDS

Figure S1. Polyacrylamide gel (15% w/v) after electrophoresis of samples from the ligation reaction of thioester-tagged RNase A(1–94) and peptide. Lanes refer to ligation reactions performed at pH 7.0 or 9.0, Asn–Pro (wild-type) or *R*-Nip–*S*-Nip (mimic) as residues 113 and 114, and a protein:peptide molar ratio of 1:4 or 1:40.

Figure S2. MALDI mass spectra of three semisynthetic proteins created by intein-mediated ligation: β*RS* ribonuclease A, β*RR* ribonuclease A, and wild-type ribonuclease A (Table S2). Each protein has a methionine residue at its N-terminus [Met(–1)]. The mass spectrum of β*RR* ribonuclease A is of the unpurified ligation mixture, and shows the presence of the unligated fragment corresponding to residues 1–94.

Figure S3. Representative thermal denaturation curve of β*RS* ribonuclease A. Top, raw data. Bottom, transformed data. Protein samples (0.2 mg/ml) were in 50 mM sodium phosphate buffer (pH 8.0) containing NaCl (25 mM). The thermal transition upon heating at 0.15 °C/min was monitored at 287 nm.

Figure S1

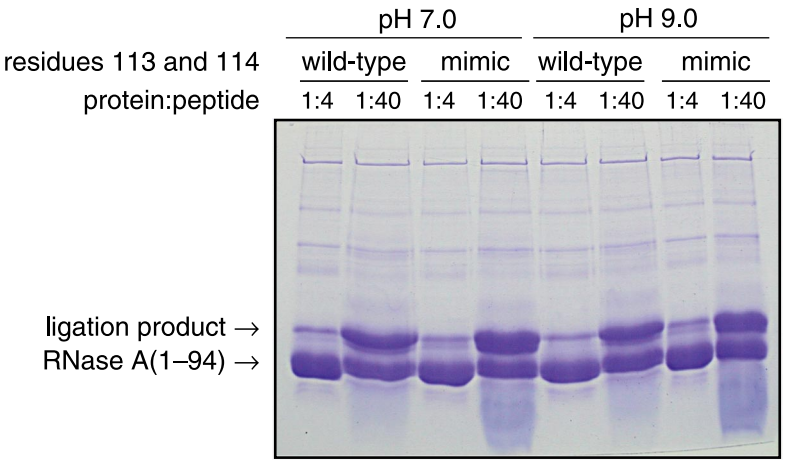
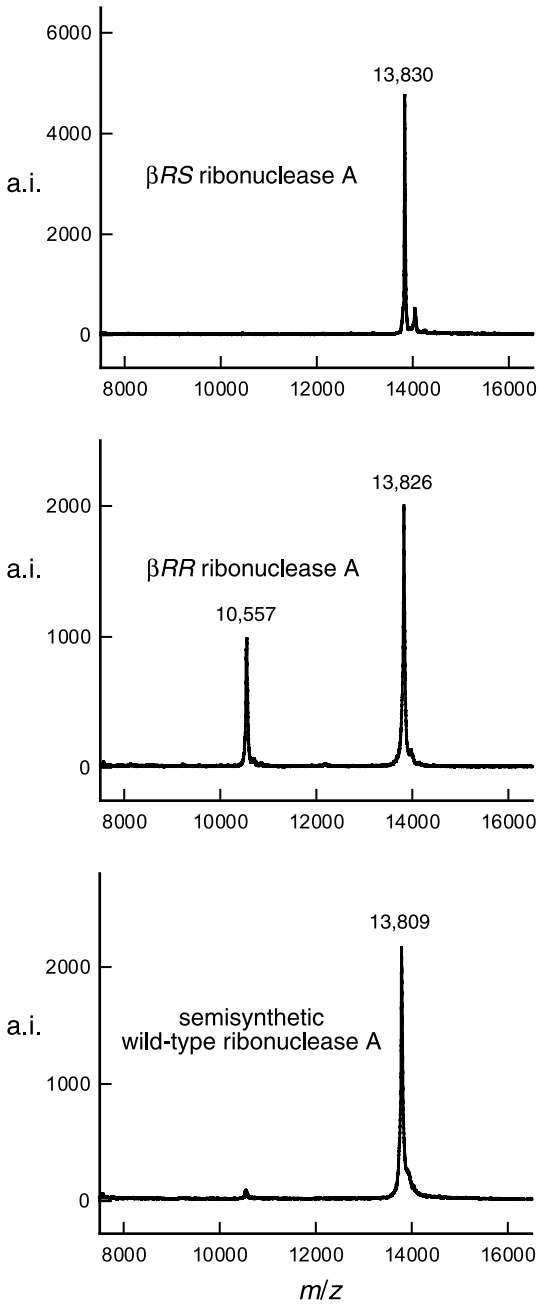

pH 7.0
pH 9.0
residues 113 and 114
wild-type
mimic
wild-type
mimic
protein:peptide
1:4
1:40
1:4
1:40
1:4
1:40
1:4
1:40
ligation product →
RNase A(1–94) →

Figure S2


6000
4000
2000
0
a.i.
13,830
βRS ribonuclease A
8000
10000
12000
14000
16000
2000
1000
0
a.i.
13,826
βRR ribonuclease A
10,557
8000
10000
12000
14000
16000
2000
1000
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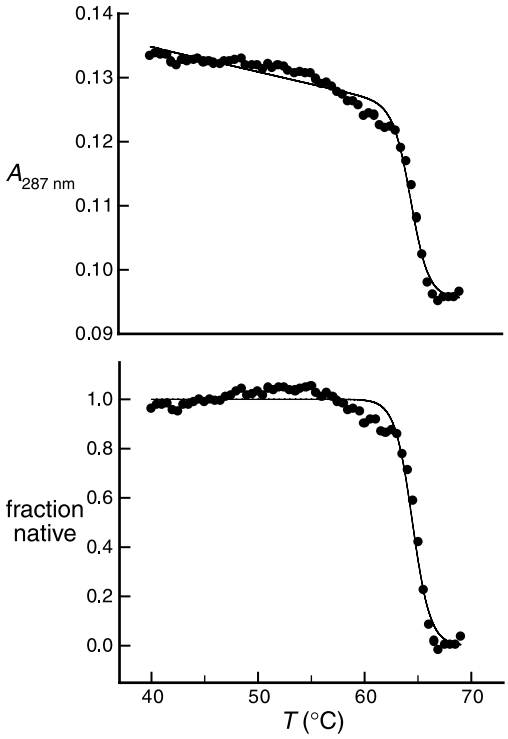
13,809
semisynthetic
wild-type ribonuclease A
8000
10000
12000
14000
16000
m/z

Figure S3


0.14
0.13
0.12
$A_{287\ nm}$
0.11
0.10
0.09
1.0
0.8
0.6
fraction native
0.4
0.2
0.0
40
50
60
70
T (°C)