Ribonuclease S redux

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Experimental

Materials

DTNB, guanidine–HCl, ampicillin (sodium salt), isopropyl β-D-1-thiogalactopyranoside (IPTG), and dithiothreitol (DTT) were from Research Products International (Mount Prospect, IL). Enterokinase was from EMD Chemicals (Gibbstown, New Jersey). 6-carboxyfluorescein–dArU$(dA)_2$–6-tetramethylrhodamine was from Integrated DNA Technology (Coralville, IA). All other reagents, including subtilisin A (type VIII from bacillus licheniformis), RNase S, and RNase A were from Sigma–Aldrich (St. Louis, MO) and were used without further purification. Total yeast RNA was from Boehringer Mannheim (Damstadt, Germany).

Analytical methods

DNA was sequenced with a 3730xl DNA Analyzer (Applied Biosystems, Carlsbad, CA) at the Biotechnology Center of the University of Wisconsin–Madison. Peptides were synthesized with a Symphony (Protein Technologies, Tucson, AZ) automated synthesizer at the Biotechnology Center, and purified by semi-preparative HPLC using an UltiMate 3000 instrument (Dionex, Bannockburn, IL). Analytical HPLC was performed with a system from Waters (Milford, MA) equipped with two 515 pumps, a 717 plus autosampler, a 996 photodiode array detector, and a C-18 column from Varian (Palo Alto, CA). Non-kinetic ultraviolet/visible measurements were recorded with a Cary 50 spectrophotometer (Varian). An AKTA system (Amersham–Pharmacia, Piscataway, NJ) was used for fast protein liquid chromatography (FPLC), and the results were analyzed with the UNICORN Control System. A Voyager-DE-PRO Biospectrometry Workstation (Applied Biosystems) at the Biophysics Instrumentation Facility of the University of Wisconsin–Madison was used for matrix-assisted laser desorption ionization time of flight (MALDI–TOF) mass spectrometry. A Cary Bio400 spectrophotometer (Varian) and an Infinite M1000 microplate reader (Tecan, Männedorf, Switzerland) were used for kinetic assays.

Site-directed mutagenesis

Plasmids encoding variants of RNase A were generated from plasmid pBXR[1] by Quikchange site-directed mutagenesis (Stratagene, La Jolla, CA) using these oligonucleotides and their reverse complements from Integrated DNA Technology or the Biotechnology Center: DDDDK insertion between residues 20 and 21, GCACTTCCGCTGCCGATGATGATGATAAAAGCA GCTCCAACTAC; H12A, CCAAGTTTGAGCGGCAGGCTATGGACTCCAGCACTTCC; V118C, GCATCAAAGTGACATG GCACATACGGGTTTCC. All mutated genes were verified by DNA sequencing.

Production of ribonucleases

RNase A variants were produced and purified essentially as described elsewhere.[2] Briefly, transformed BL21(DE3) cells were grown at 37 °C with shaking in Terrific Broth containing ampicillin (400 μg/mL) until *OD* = 1.8–2.2 at 600 nm. Gene expression was induced by the addition of IPTG (0.5 mM). After 3–4 h, cells were harvested by centrifugation and lysed with a French pressure cell. Inclusion bodies were recovered after centrifugation and solubilized for 2 h at room temperature with denaturing solution (20 mM Tris–HCl buffer, pH 8.0, containing 7 M guanidine–HCl, 0.10 M DTT, and 10 mM EDTA). Solubilized inclusion bodies were then diluted 10-fold with 20 mM HOAc. The precipitate was removed by centrifugation, and the solution was dialyzed overnight against 20 mM HOAc at 4 °C. After removal of further precipitate, the ribonuclease solution was added dropwise to refolding buffer (0.10 M Tris–HCl buffer, pH 7.8, containing 0.5 M L-arginine–HCl, 1.0 mM reduced glutathione, and 0.2 mM oxidized glutathione). After >3 days at 4 °C, the solution was adjusted to pH 5, and concentrated using an Amicon YM10 membrane (Millipore, Billerica, MA). A 10-mL sample was applied to a G75 gel filtration FPLC column (Amersham–Pharmacia). The major peak after isocratic elution (50 mM sodium acetate buffer, pH 5.0, containing 0.10 M NaCl, 10 mM EDTA, and 0.02% w/v NaN_3) was collected and applied to a Mono S cation-exchange FPLC column (Amersham–Pharmacia). Ribonucleases were eluted with a linear gradient of NaCl (0–0.40 M) in 50 mM NaOAc buffer, pH 5.0, containing EDTA (10 mM). Protein concentrations were determined by absorbance at 278 nm using $\varepsilon = 0.72\ mg^{-1}\cdot mL\cdot cm^{-1}$.[3] The identity of each variant was verified by MALDI–TOF mass spectrometry.

Protection of H12A/DDDDK/V118C RNase A with DTNB

After cation-exchange chromatography, the fractions that contained H12A/DDDDK/V118C RNase A were combined, and the pH of the resulting solution was increased by adding (to 8% v/v) 1.0 M Tris–HCl buffer, pH 8.3, containing EDTA (10 mM). To this solution was added 50 mM Tris–HCl buffer, pH 8.0, containing DTNB (5 mM) and EDTA (50 mM) such that the DTNB was in

4-fold molar excess to the protein. After incubation for 10 min, the solution was dialyzed overnight at 4 °C against 20 mM Tris–HCl buffer, pH 7.4, containing NaCl (50 mM).

Synthesis, activation with DTNB, and purification of peptides

S15 (KETAAAKFERQHMDS) and A4C S15 (KETCAAKFERQHMDS) were synthesized on a 50-μmol scale by standard fluorenylmethoxycarbonyl chemistry using HATU activation and a Fmoc-Ser(*t*Bu) Wang resin (EMD Biocsciences, La Jolla, CA). Peptides were deprotected and cleaved from the resin with 4 mL of 92.5:5:2.5 trifluoroacetic acid (TFA)/thioanisole/ethanedithiol for 4 h. Peptides were then precipitated with *tert*-butyl methyl ether and dried under vacuum. S15 and A4C S15 were purified by reversed-phase HPLC on a C-18 semipreparative column. TFA (0.1%) was included as an ion-pairing agent in HPLC solvents. Peptides were eluted with a linear gradient of acetonitrile (10–30% v/v). One-quarter volume 0.10 M Tris–HCl buffer, pH 8.0, containing DTNB (5 mM) was added to fractions containing A4C S-peptide. The solvent was then removed under reduced pressure. NTB-activated A4C S-peptide was then purified again under the same HPLC conditions. The mass of purified peptides was confirmed by MALDI–TOF mass spectrometry.

Digestion of RNase A with subtilisin

Subtilisin (5 μg) was added to a solution of RNase A (0.5 mg, 88 μL) in phosphate-buffered saline (PBS) and incubated at 4 °C. Aliquots were removed over 24 h and subjected to SDS–PAGE (15% w/v acrylamide).

Digestion of ribonucleases with enterokinase

$CaCl_2$ (2 mM final concentration) was added to RNase A and its DDDDK and H12A/DDDDK/V118C variants (~5 mL of a 0.85 mg/mL solution) that had been dialyzed against 20 mM Tris–HCl buffer, pH 7.4, containing NaCl (50 mM). Enterokinase (5 μL, 5 U) was added, and the reaction mixture was incubated at room temperature. Aliquots were removed over 24 h and subjected to SDS–PAGE.

Separation of S-peptide and S-protein components

The S-peptide and S-protein fragment from the enterokinase-digestion of the DDDDK and H12A/DDDDK/V118C variants of RNase A were separated by analytical or semi-preparative reverse-phase HPLC with a C-18 column and two-step linear gradient (Step 1: 20–50% B over 3 min. Step 2: 50–100% B over 25 min. A: 50 mM sodium phosphate buffer, pH 2.7. B: 40% A + 60% acetonitrile).[4] Fractions containing S-protein were pooled and dialyzed overnight against PBS at 4 °C. The concentration of S-protein was determined by absorbance at 280 nm using $\varepsilon = 9055\ M^{-1}\cdot cm^{-1}$.[5] The identity of each peak on the HPLC trace was confirmed by MALDI–TOF mass spectrometry.

Fragment complementation

To V118C S-protein (100 μL of a 1.76 mg/mL solution) was added 1.0 M Tris–HCl buffer, pH 8.0, containing EDTA (10 mM) (8 μL), and DTT (25 mM) (2 μL). After 2 min, the mixture was desalted with a Zeba Spin column, 7K MWCO (Pierce, Rockford, IL). To the collection tube was added S15 or NTB-activated A4C S15 (4 μL of 20 mg/mL). After ~10 min, the reactions were desalted again with the spin columns. SDS–PAGE and MALDI–TOF mass spectrometry confirmed the covalent linkage between A4C S15 and V118C S-protein (*m*/z 13324; expected: 13317).

Zymogram electrophoresis

The potassium salt of poly(cytidylic acid) (final concentration: 0.5 mg/mL) was included during the casting of a 15% w/v polyacrylamide gel. Laemmli buffer (no reducing agent) was added to each sample (2 ng). After SDS–PAGE, the gel was washed with isopropanol (20% v/v) in 10 mM Tris–HCl buffer, pH 7.0 (2×), 10 mM Tris–HCl buffer, pH 7.0 (2×), and 0.10 M Tris–HCl buffer, pH 7.5 (1×) for 10 min each. The gel was then stained for 10 min with 10 mM Tris–HCl buffer, pH 7.5, containing toluidine blue (0.02% w/v). The stained gel was rinsed several times in water, and soaked in water overnight.

Assays of ribonucleolytic activity

Initial velocities for catalysis of RNA cleavage were determined at high and low concentrations of RNase A, RNase S, and RNase–S in 50 mM NaOAc buffer, pH 6.0, containing NaCl (0.10 M). To avoid the contamination apparent in commercial preparations of RNase S (Figure 5, lane 6), the noncovalent complex assayed here was that of S15 and the deprotected S-protein derived from H12A/DDDDK/V118 RNase A.

A fluorogenic ribonuclease substrate, 6-carboxyfluorescein–dArU$(dA)_2$–6-tetramethylrhodamine,[6] was used for assays at low ribonuclease concentration. Briefly, a ribonuclease (50 μL of a 50 pM solution) was equilibrated at 37 °C in a 96-well plate. To each sample was added 6-carboxyfluorescein–dArU$(dA)_2$–6-tetramethylrhodamine (50 μL of a 100 nM solution in sample buffer). Reaction progress was monitored at 37 °C by the increase in fluorescence emission at 515 nm upon excitation at 493 nm over 5 min.

A modified Kunitz assay was used at high ribonuclease concentration.[7] Briefly, to total yeast RNA (100 μL of a 1 mg/mL solution) was added a ribonuclease (100 μL of a 0.29 μM solution). The decrease in absorbance at 300 nm was monitored at 37 °C for 10 min. Data from the first 2 min were used to determine initial velocities (v_o).

Notes and references

1. S. B. delCardayré, M. Ribó, E. M. Yokel, D. J. Quirk, W. J. Rutter and R. T. Raines, *Protein Eng.*, 1995, **8**, 261–273.
2. T. J. Rutkoski, E. L. Kurten, J. C. Mitchell and R. T. Raines, *J. Mol. Biol.*, 2005, **354**, 41–54.
3. M. Sela, C. B. Anfinsen and W. F. Harrington, *Biochim. Biophys. Acta*, 1957, **26**, 502–512.
4. D. N. Brems and R. L. Baldwin, *J. Mol. Biol*, 1984, **180**, 1141–1156.
5. R. Gilmanshin, J. Van Beek and R. Callender, *J. Phys. Chem*, 1996, **100**, 16754–16760.
6. B. R. Kelemen, T. A. Klink, M. A. Behlke, S. R. Eubanks, P. A. Leland and R. T. Raines, *Nucleic Acids Res.*, 1999, **27**, 3696–3701.
7. M. Kunitz, *J. Biol. Chem.*, 1946, **164**, 563–568.