Esterification Delivers a Functional Enzyme into a Human Cell

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EXPERIMENTAL PROCEDURES

General Experimental

Materials. The BL21(DE3) strain of *Escherichia coli* was from Novagen (Madison, WI). Diazo compound **1** (2-diazo-*N*,*N*-dimethyl-2-(*p*-tolueyl)acetamide) was synthesized by Chicago Discovery Solutions (Plainfeld, IL) as described previously.[1] RNase A (bovine) was from Sigma–Aldrich (St. Louis, MO). A fluorogenic ribonuclease substrate (6-FAM–dArU(dA)$_2$–6-TAMRA) and DNA oligonucleotides were from Integrated DNA Technologies (Coralville, IA). A 50,000× solution of SYPRO Orange Protein Gel Stain was from Life Technologies (Grand Island, NY). All other chemicals and reagents were of commercial reagent grade or better and were used without further purification.

Aqueous solutions were made with water that was generated with an arium Pro water purification system from Sartorius (Bohemia, NY) and had resistivity ≥18 MΩ·cm^{-1}. Phosphate-buffered saline (PBS) contained Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), NaCl (137 mM), and KCl (2.7 mM) at pH 7.4. A Spectra Multicolor Broad Range Protein Ladder from Thermo Fisher Scientific (Waltham, MA) was used as a molecular mass standard for SDS–PAGE.

Instrumentation. Chromatography was performed with an ÄKTA Pure system from GE Healthcare Life Sciences (Piscataway, NJ), and the results were analyzed with the UNICORN Control System. HiTrap SPHP and HiLoad® 26/600 Superdex® 75 pg columns for protein purification were from GE Healthcare Life Sciences.

Protein concentrations were determined with a bicinchonic acid (BCA) assay kit from Thermo Fisher Scientific and measured with an infinite M1000 microplate reader from Tecan (Zürich, Switzerland).

Differential scanning fluorimetry (DSF), which requires thermal denaturation and the simultaneous monitoring of fluorescence, was performed with a ViiA Real-Time PCR system from Applied Biosystems (Foster City, CA). Denaturation data were obtained with ViiA 7 version 2.0 software and analyzed further by Protein Thermal Shift version 1.4 software, both from Applied Biosystems.

The intact molecular mass of RNase 1 variants was determined by ESI mass spectrometry using a 6530 Accurate-Mass Q-TOF LC/MS from Agilent (Santa Clara, CA) with a PLRP-S column (1000-Å pore size, 5-μM particle size, 50-mm length, and 2.1-mm ID).

Conditions. All procedures were performed in air at ambient temperature (~22 °C) and pressure (1.0 atm) unless indicated otherwise.

Enzyme Preparation

Plasmid Preparation. cDNA that encodes Met(–1) RNase 1 in a pET22b(+) expression vector was used to generate wild-type RNase 1 in *E. coli* (Figure S1). Two synthetic cDNAs encoding for either the R39D/N67D/N88A/G89D/R91D (DDADD) variant or the inactive H12A/K41A/H119A variant flanked by regions of homology near the T7 promoter and terminator of the pET22b vector were obtained from Integrated DNA Technologies (Figure S1). Linear pET22b was prepared by PCR using primers that complement the DNA that encodes RNase 1 (5′-AAGCCCGAAAGGAAGCTGAGTTGGCTGCTG-3′ and 3′-AAACAAATTGAAATTCTTCCT CTATATGTA-5′). Each gene and plasmid fragment were combined with Gibson assembly for expression in *E. coli*. The corresponding amino acid sequence for each RNase 1 variant is shown in Figure S2.

Enzyme Production and Purification. All enzymes, including wild-type RNase 1, DDADD RNase 1, H12/K41A/H119A RNase 1, and FLAG–RNase 1, were purified from inclusion bodies as described previously.[2] Briefly, induced cells were lysed with a benchtop cell disruptor from Constant Systems (Kennesaw, GA) at 19.0 kpsi. After centrifugation at 10,500 rpm for 45 min, the resulting inclusion bodies were dissolved in 20 mM Tris–HCl buffer, pH 8.0, containing guanidine–HCl (7 M), EDTA (10 mM), and DTT (100 mM). This solution was diluted 10-fold by the slow addition of degassed 20 mM acetic acid, then subjected to centrifugation at 10,500 rpm for 45 min. The resulting supernatant was then dialyzed using 3.5-kDa MWCO tubing from Spectrum Labs (Rancho Dominguez, CA) against 16 L of 20 mM acetic acid overnight. After centrifugation at 10,500 rpm for 40 min, the retentate was added dropwise to re-folding solution, which was 100 mM Tris–HCl buffer, pH 7.8, containing NaCl (100 mM), reduced glutathione (1.0 mM), and oxidized glutathione (0.2 mM), then allowed to re-fold at 4 °C for 5 days. The pH of the solution was adjusted to 5.0, and the resulting solution was concentrated to 10 mL with an Amicon® Stirred Cell concentrator from EMD Millipore (Billerica, MA) with Hydrostart® 10-kDa filters from Sartorius. The resulting protein solution was purified by chromatography on a HiLoad® 26/600 Superdex® 75 pg gel-filtration column from GE Healthcare Life Sciences with 50 mM sodium acetate buffer, pH 5.0, containing NaCl (100 mM) and sodium azide (0.05% w/v). The protein was purified further by passage through a HiTrap SP cation-exchange column from GE Healthcare Life Sciences before being dialyzed against PBS.

*Enzyme Esterification with Diazo Compound **1**.* Wild-type RNase 1 and its variants were esterified under identical reaction conditions. A solution of diazo compound **1** (2.8 mg, 14 μmol or 5.6 mg, 28 μmol) in 200 μL of acetonitrile was added to a solution of enzyme (0.14 μmol) in 200 μL of 10 mM MES–HCl buffer, pH 5.5. The reaction mixture was incubated for 4 h at 37 °C, then diluted by the addition of 40 mL of PBS. The solution was concentrated by using a Vivaspin filtration column (5-kDa MWCO) from GE Healthcare Life Sciences, and the extent of esterification was assessed with Q-TOF LC/MS.

Assays of Ribonucleolytic Activity

The ribonucleolytic activity of wild-type RNase 1 and its variants was determined by measuring the initial velocity of cleavage of ssRNA substrate in a 96-well plate (Corning) at 25 °C. A fluorogenic RNA substrate (0.2 μM of 6-FAM–dArU$(dA)_2$–6-TAMRA) in 100 mM Tris–HCl buffer, pH 7.5, containing NaCl (100 mM) was added to each well. After baseline fluorescent readings were recorded, enzymes were added to a final concentration of 50 pM, and the initial velocity of substrate turnover was measured by the increase in fluorescence over time. After 8 min, substrate cleavage was saturated by the addition of RNase A to a final concentration of 5 μM. Values of k_{cat}/K_M were determined as described previously.[3] Values represent the mean of at least three independent experiments.

Mammalian Cell Culture

HeLa and NCI-H460 cells were obtained from American Type Culture Collection (Manassas, VA) and stored in vials immersed in N_2(l). These cell lines were authenticated by morphology, karyotyping, and PCR-based methods, which included an assay to detect species specific variants of the cytochrome C oxidase I gene (to rule out interspecies contamination) and short tandem repeat profiling (to distinguish between individual human cell lines and rule out intraspecies contamination). To minimize genetic drift, a thawed vial was used for fewer than fifteen passages.

Cell culture medium and added components were the Gibco brand from Thermo Fisher Scientific. Cells were grown in flat-bottomed culture flasks in a cell culture incubator at 37 °C under CO_2(g) (5%v/v). HeLa and NCI-H460 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) or RPMI 1640 medium, respectively, each containing fetal bovine serum (FBS) (10% v/v) and penicillin–streptomycin solution (1% v/v).

Assays of Cell Viability

Cell-viability was assessed with the CellTiter 96 AQueous One Solution Cell Proliferation Assay from Promega (Madison, WI), which is a tetrazolium dye-based assay for metabolic activity. Cells were grown, treated, and assessed as described previously.[4] Briefly, cells were grown in the wells of a 96-well plate. After 24 h, the medium was replaced with FBS-free DMEM containing penicillin–streptomycin solution (1% v/v) and various concentrations of analyte proteins. Cells were then allowed to incubate for another 48 h before the addition of the MTS reagent and data collection. Data were analyzed with the program Prism from GraphPad (La Jolla, CA). Values of EC_{50}, which is the concentration of analyte that gives half-maximal cell viability, were calculated by using the equation:

$$y = y_{\min} + \frac{(y_{\max} - y_{\min})}{1+\left(\frac{EC_{50}}{x}\right)^{h}} \quad \text{(S1)}$$

where y is cell viability, x is the concentration of analyte, and h is the Hill coefficient. All values were the average of at least three biological replicates.

Reversibility of Enzyme Esterification *in Cellulo*

Human RNase 1 is intact after incubation with a detergent-containing cell lysis kit and produces a well-resolved peak in a Q-TOF LC/MS spectrum. A FLAG-tagged variant of RNase 1 binds to a FLAG antibody with high affinity, thus allowing for recovery of this protein from treated cells. We used FLAG-tagged RNase 1 for an assessment of the bioreversibility of protein esterification *in cellulo*.

HeLa cells were plated at 500,000 cells in 10 mL of medium in a 10-cm^2 dish. After 24 h, cells were treated with FLAG–RNase 1 or esterified FLAG–RNase 1 (1 μg/mL) and incubated for 24 h. Then, cells were washed with PBS and lysed with 1.0 mL of M-PER mammalian protein extraction reagent containing a protease inhibitor cocktail, both from Thermo Fischer Scientific. After centrifugation, the clarified lysate was incubated with α-FLAG magnetic beads from Sigma Chemical for 1 h at room temperature. The beads were washed and then treated twice with 150 ng/μL of 3× FLAG peptide from APExBIO (Boston, MA). The combined eluates were subjected to methanol/chloroform protein precipitation to remove detergents prior to further analysis. Briefly, protein eluate was treated with 4 volumes of methanol, then 1 volume of chloroform, and then 3 volumes of water. After centrifugation at 14,000*g* for 2 min, the upper aqueous layer was removed and 4 volumes of methanol were added to the solution. The precipitated protein was then pelleted by centrifugation at 14,000*g* for 2 min. The methanol solution was removed and the protein pellet was allowed to dry at room temperature for 5 min. After resuspension in PBS, protein samples were passed through a 0.2-μm filter from Thermo Fischer Scientific and analyzed by LC/MS.

References

(1) Mix, K. A.; Raines, R. T. Optimized diazo scaffold for protein esterification. *Org. Lett.* **2015**, *17*, 2358–2361.

(2) Thomas, S. P.; Kim, E.; Kim, J.-S.; Raines, R. T. Knockout of the ribonuclease inhibitor gene leaves human cells vulnerable to secretory ribonucleases. *Biochemistry* **2016**, *55*, 6359–6362.

(3) Kelemen, B. R.; Klink, T. A.; Behlke, M. A.; Eubanks, S. R.; Leland, P. A.; Raines, R. T. Hypersensitive substrate for ribonucleases. *Nucleic Acids Res.* **1999**, *27*, 3696–3701.

(4) Thomas, S. P.; Kim, E.; Kim, J.-S.; Raines, R. T. Knockout of the ribonuclease inhibitor gene leaves human cells vulnerable to secretory ribonucleases. *Biochemistry* **2016**, *55*, 6359–6362.

(5) Keller, B. O.; Sui, J.; Young, A. B.; Whittal, R. M. Interferences and contaminants encountered in modern mass spectrometry. *Anal. Chim. Acta* **2008**, *627*, 71–81.

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wild-type RNase 1:                                  ATGAAAGAATCTCGTGCTAAAAAATTCCAG
DDADD RNase 1:                                      ATGAAAGAATCTCGTGCTAAAAAATTCCAG
H12A/K41A/H119A:                                    ATGAAAGAATCTCGTGCTAAAAAATTCCAG
FLAG–RNase 1:      ATGGACTACAAAGACGATGACGACAAGAAAGAATCTCGTGCTAAAAAATTCCAG

wild-type RNase 1:  CGTCAGCATATGGACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAAC
DDADD RNase 1:      CGTCAGCATATGGACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAAC
H12A/K41A/H119A:    CGTCAGGCGATGGACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAAC
FLAG–RNase 1:       CGTCAGCATATGGACTCTGACTCTTCTCCGTCTTCTTCTTCTACTTACTGCAAC

wild-type RNase 1:  CAGATGATGCGTCGTCGTAACATGACTCAGGGTCGTTGCAAACCGGTTAACACT
DDADD RNase 1:      CAGATGATGCGTCGTCGTAACATGACTCAGGGTGATTGCAAACCGGTTAACACT
H12A/K41A/H119A:    CAGATGATGCGTCGTCGTAACATGACTCAGGGTCGTTGCGCACCGGTTAACACT
FLAG–RNase 1:       CAGATGATGCGTCGTCGTAACATGACTCAGGGTCGTTGCAAACCGGTTAACACT

wild-type RNase 1:  TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAAAAAGTT
DDADD RNase 1:      TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAAAAAGTT
H12A/K41A/H119A:    TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAAAAAGTT
FLAG–RNase 1:       TTCGTTCATGAACCGCTGGTTGACGTTCAGAACGTTTGCTTCCAGGAAAAAGTT

wild-type RNase 1:  ACTTGCAAAAACGGTCAGGGTAACTGCTACAAATCTAACTCTTCTATGCATATC
DDADD RNase 1:      ACTTGCAAAGACGGTCAGGGTAACTGCTACAAATCTAACTCTTCTATGCATATC
H12A/K41A/H119A:    ACTTGCAAAAACGGTCAGGGTAACTGCTACAAATCTAACTCTTCTATGCATATC
FLAG–RNase 1:       ACTTGCAAAAACGGTCAGGGTAACTGCTACAAATCTAACTCTTCTATGCATATC

wild-type RNase 1:  ACTGACTGCCGTCTGACTAACGGTTCTCGTTACCCGAACTGCGCTTACCGTACT
DDADD RNase 1:      ACTGACTGCCGTCTGACTGCGGATTCTGACTACCCGAACTGCGCTTACCGTACT
H12A/K41A/H119A:    ACTGACTGCCGTCTGACTAACGGTTCTCGTTACCCGAACTGCGCTTACCGTACT
FLAG–RNase 1:       ACTGACTGCCGTCTGACTAACGGTTCTCGTTACCCGAACTGCGCTTACCGTACT

wild-type RNase 1:  TCTCCGAAAGAACGTCATATCATCGTTGCTTGCGAAGGTTCTCCGTACGTTCCG
DDADD RNase 1:      TCTCCGAAAGAACGTCATATCATCGTTGCTTGCGAAGGTTCTCCGTACGTTCCG
H12A/K41A/H119A:    TCTCCGAAAGAACGTCATATCATCGTTGCTTGCGAAGGTTCTCCGTACGTTCCG
FLAG–RNase 1:       TCTCCGAAAGAACGTCATATCATCGTTGCTTGCGAAGGTTCTCCGTACGTTCCG

wild-type RNase 1:  GTTCATTTCGACGCTTCTGTTGAAGACTCTACT
DDADD RNase 1:      GTTCATTTCGACGCTTCTGTTGAAGACTCTACT
H12A/K41A/H119A:    GTTGCGTTCGACGCTTCTGTTGAAGACTCTACT
FLAG–RNase 1:       GTTCATTTCGACGCTTCTGTTGAAGACTCTACT
```

Figure S1. DNA sequences that encode the proteins used this work. DDADD RNase 1 refers to the R39D/N67D/N88A/G89D/R91D variant. DNA that encodes a FLAG-tag sequence is in a gray box. The codon for the initial amino acid of each mature protein is in bold typeface. Red sequences indicate mutation sites.

```
wild-type RNase 1:          MKESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNT
DDADD RNase 1:              MKESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGDCKPVNT
H12A/K41A/H119A             MKESRAKKFQRQAMDSDSSPSSSSTYCNQMMRRRNMTQGRCAPVNT
FLAG–RNase 1:      MDYKDDDDKKESRAKKFQRQHMDSDSSPSSSSTYCNQMMRRRNMTQGRCKPVNT

wild-type RNase 1:  FVHEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRLTNGSRYPNCAYRT
DDADD RNase 1:      FVHEPLVDVQNVCFQEKVTCKDGQGNCYKSNSSMHITDCRLTADSDYPNCAYRT
H12A/K41A/H119A:    FVHEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRLTNGSRYPNCAYRT
FLAG–RNase 1:       FVHEPLVDVQNVCFQEKVTCKNGQGNCYKSNSSMHITDCRLTNGSRYPNCAYRT

wild-type RNase 1:  SPKERHIIVACEGSPYVPVHFDASVEDST
DDADD RNase 1:      SPKERHIIVACEGSPYVPVHFDASVEDST
H12A/K41A/H119A:    SPKERHIIVACEGSPYVPVAFDASVEDST
FLAG–RNase 1:       SPKERHIIVACEGSPYVPVHFDASVEDST
```

Figure S2. Amino acid sequences of the proteins used in this work. DDADD RNase 1 refers to the R39D/N67D/N88A/G89D/R91D variant. FLAG-tag sequences are in a gray box. Substitutions from wild-type RNase 1 are indicated in red typeface.

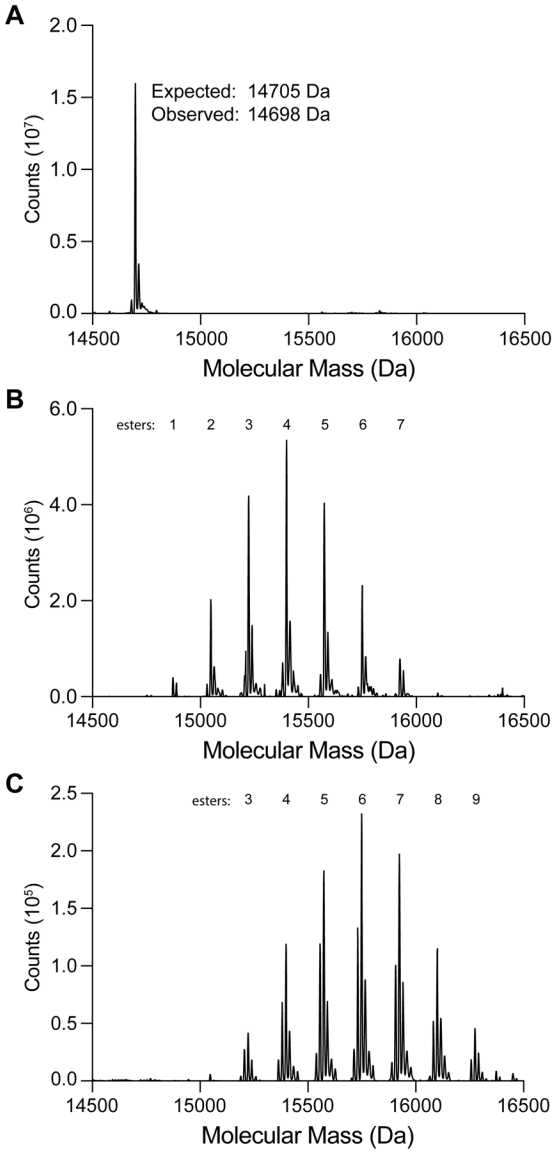

Figure S3. Deconvoluted ESI mass spectra of wild-type RNase 1. (A) Untreated wild-type RNase 1. (B) Wild-type RNase 1 treated with 100 equiv of diazo compound **1**. (C) Wild-type RNase 1 treated with 200 equiv of diazo compound **1**. The numbers above the spectral peaks report the number of ester groups, each of which adds 175 Da. The most prevalent number of ester groups is listed in Table 1.

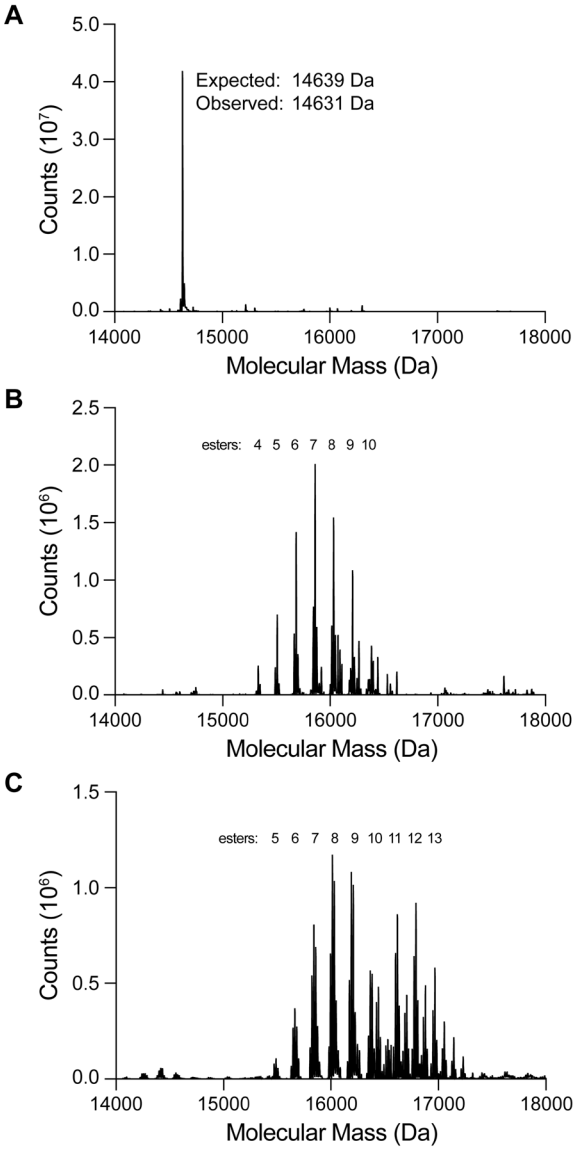

Figure S4. Deconvoluted ESI mass spectra of DDADD RNase 1. (A) Untreated DDADD RNase 1. DDADD RNase 1 treated with 100 equiv of diazo compound **1**. (C) DDADD RNase 1 treated with 200 equiv of diazo compound **1**. DDADD RNase 1 refers to the R39D/N67D/N88A/G89D/R91D variant. The numbers above the spectral peaks report the number of ester groups, each of which adds 175 Da. The most prevalent number of ester groups is listed in Table 1.

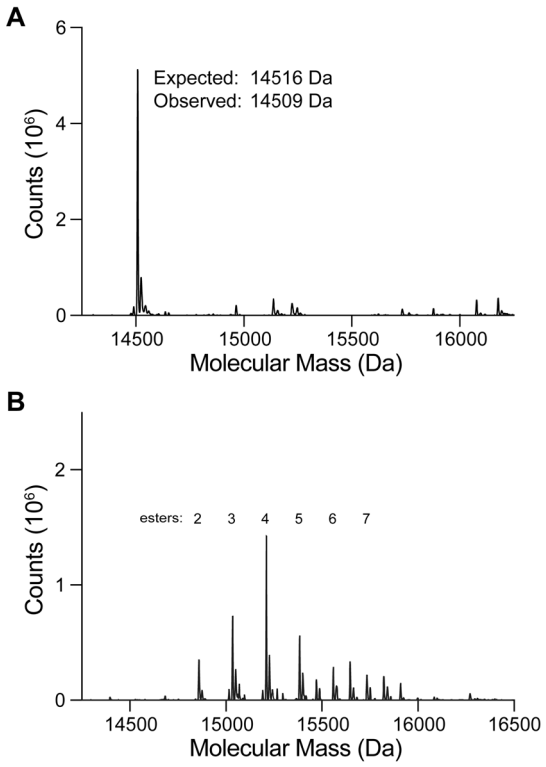

Figure S5. Deconvoluted ESI mass spectra of H12A/K41A/H119A RNase 1. (A) Untreated H12A/K41A/H119A RNase 1. (B) H12A/K41A/H119A RNase 1 treated with 100 equiv of diazo compound **1**. The numbers above the spectral peaks report the number of ester groups, each of which adds 175 Da. The most prevalent number of ester groups is listed in Table 1.

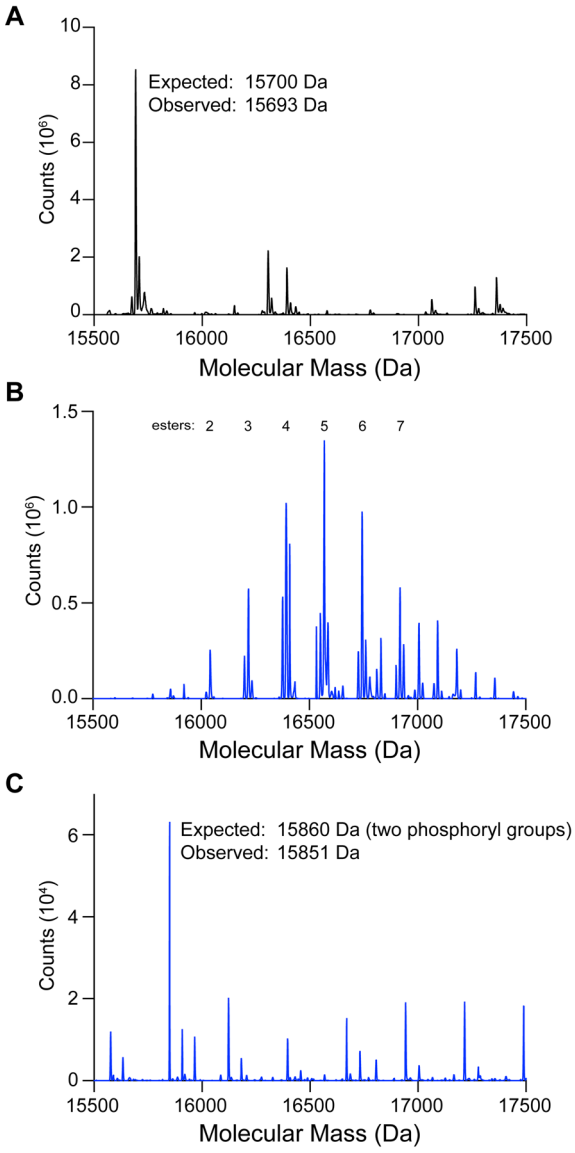

Figure S6. Deconvoluted ESI mass spectra of FLAG–RNase 1 before and after recovery from human cells. (A) Untreated FLAG–RNase 1. (B) FLAG–RNase 1 treated with 100 equiv of diazo compound **1**. (C) Esterified FLAG–RNase 1 after recovery from HeLa cells after a 24-h incubation. The numbers above the spectral peaks report the number of ester groups, each of which adds 175 Da. The most prevalent number of ester groups is listed in Table 1. Additional peaks observed in panel C are likely due to detergents necessary for cell lysis.[5]

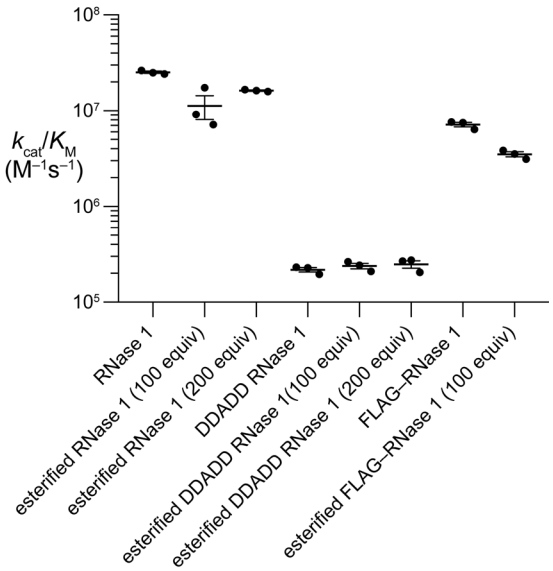

Figure S7. Graph of the values of k_{cat}/K_M for the enzymes used in this work, determined for the cleavage of 6-FAM–dArU(dA)$_2$–6-TAMRA at pH 7.5 and 25 °C.