Creation of a zymogen

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Cells produce proteases as inactive zymogens. Here, we demonstrate that this tactic can extend beyond proteases. By linking the N and C termini of ribonuclease A, we obstruct the active site with the amino acid sequence recognized by plasmepsin II, a highly specific protease from *Plasmodium falciparum*. We generate new N and C termini by circular permutation. In the presence of plasmepsin II, a ribonuclease zymogen gains ~10³-fold in catalytic activity and maintains high conformational stability. We conclude that zymogen creation provides a new and versatile strategy for the control of enzymatic activity, as well as the potential development of chemotherapeutic agents.

The cleavage of target proteins by proteases is critical for many biological processes[1,2]. However, the unbridled enzymatic activity of proteases can inflict catastrophic damage. Accordingly, proteolytic activity is under strict spatial and temporal control. This control is achieved by the binding of protease-inhibitor proteins, as well as the biosynthesis and transport of proteases as inactive zymogens[3–6]. These zymogens are activated upon cleavage by other proteases or in an autocatalytic manner.

Similar to proteases, ribonucleases manifest an enzymatic activity that can be cytotoxic[7]. By analogy to the control of proteolytic activity by protease-inhibitor proteins, ribonucle-olytic activity is controlled by ribonuclease-inhibitor proteins[8]. The resulting complexes can have K_d values in the femtomolar range, indicative of an evolutionary imperative to control ribonucleolytic activity. However, no zymogens are known for ribonucleases. Here, we demonstrate that a ribonuclease can be transformed into a zymogen that unleashes its enzymatic activity only in the presence of a highly specific protease.

Zymogen design

A functional zymogen must have two attributes. First, the enzymatic activity of the activated zymogen must be both high and much greater than that of the unactivated zymogen. Second, the zymogen must have high conformational stability, both before and after activation. We suspected that a zymogen of bovine pancreatic ribonuclease (RNase A)[9,10] could be both created and given these attributes. Specifically, we envisioned that a bridge of amino acids connecting the original N and C termini would span the active site and interfere with the binding of substrate RNA (Fig. 1a,b). This bridge could contain the recognition sequence for a specific protease. New N and C termini could be
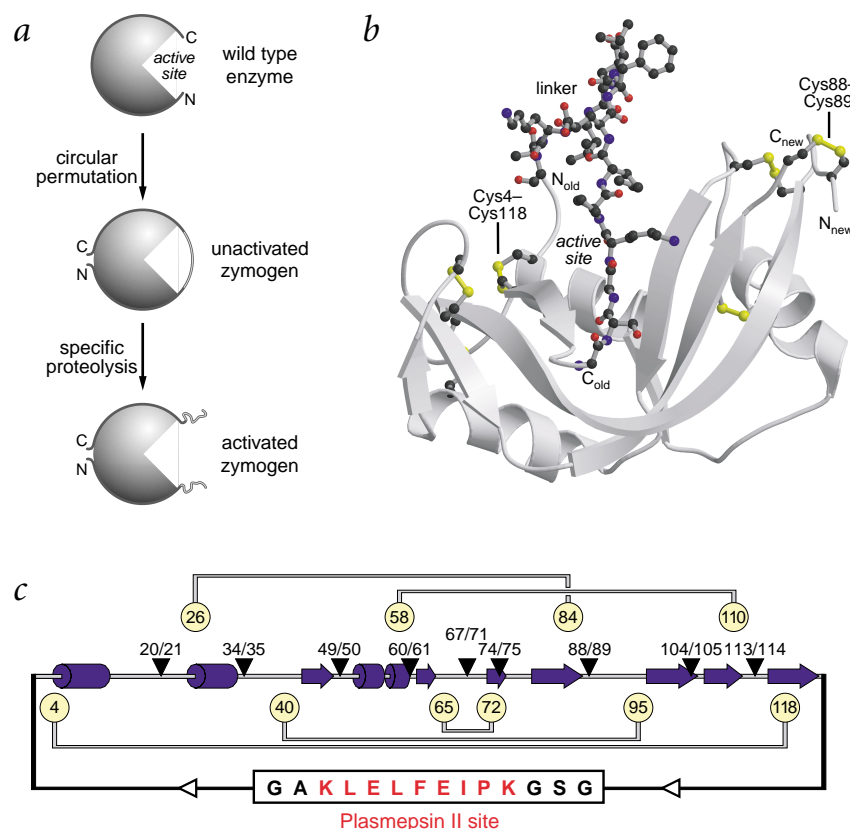

Fig. 1 Design of a ribonuclease zymogen. *a*, Scheme for creating a zymogen in which a circular permutation creates a steric block of the active site. *b*, Structural model of the unactivated ribonuclease A zymogen with 88/89 termini, 14-residue linker and six disulfide bonds. The conformational energy of the non–wild type residues was minimized with the program SYBYL (Tripos). Atoms of the linker and cystines are shown explicitly, and the two non-native cystines are labeled. *c*, Scheme of the primary sequence of ribonuclease A zymogens. The location of α-helices (cylinders) and β-strands (arrows) are indicated. The nine new termini (black triangles), 14-residue linker (boxed) and four native and one non-native (Cys4–Cys118) cystine are indicated.

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Fig. 2 Activation of ribonuclease A zymogen with 88/89 termini. Activation was monitored at different times after addition of plasmepsin II at a molar ratio of 1:100 (plasmepsin II:zymogen) by *a*, SDS-PAGE or *b*, ribonucleolytic activity. 'std' is the protein molecular weight standard; n, 0 min incubation without plasmepsin II; i0, 0 min incubation with unactivated plasmepsin II; and i10, 10 min incubation with unactivated plasmepsin II.

created by circular permutation of the polypeptide chain[11], which is a natural mechanism by which active sites migrate along a polypeptide chain[12] and new protein folds evolve[13].

The distance between the original N and C termini of RNase A is ~30 Å, which could be spanned by as few as eight residues. A linker connecting the original N and C termini must, however, be long enough to leave intact the structure of RNase A and to allow access by a protease but short enough to prevent the binding of substrate RNA. Our molecular modeling suggested that a linker of 14 residues would meet these criteria (Fig. 1*b*).

As a protease, we chose plasmepsin II[14,15]. This highly specific aspartyl protease is produced by *Plasmodium falciparum*, which is responsible for most cases of malaria. The protease catalyzes the hydrolysis of the αPhe33-Leu34 peptide bond in human hemoglobin[16,17]. Plasmepsin II itself is biosynthesized as a zymogen that is activated *in vivo* by another protease[18,19].

The location of the new termini is crucial to the properties of a circular permutation. We selected nine sites for new termini (Fig. 1*c*). Each of these sites lies between different cysteine residues, so that each permuted protein has a distinct disulfide-bonding pattern. In addition, most of the new termini are in β-turns or surface loops, which are probably more tolerant of change than α-helices or β-strands. We used the most stable known variant of RNase A as a template for our zymogen. This variant, A4C/V118C RNase A, has all four native disulfide bonds plus a nonnative, fifth disulfide bond between an N- and C-terminal residue[20].

Zymogen optimization

We produced each ribonuclease zymogen in *Escherichia coli* as an inclusion body and then subjected each to oxidative folding *in vitro*[21,22]. Most of the nine zymogens — those with termini at residues 20/21, 67/71, 88/89, 104/105 and 113/114 — were able to fold properly. These zymogens were purified by gel filtration and cation-exchange chromatography. Treatment with plasmepsin II increased the ability of each ribonuclease zymogen to catalyze the cleavage of tetranucleotide substrate (Table 1). Apparently, the intact linker excludes even a small substrate from the active site. The relative activity of an activated and unactivated zymogen is likely to increase with a longer RNA substrate or an RNA substrate with secondary structure. The increase in ribonucleolytic activity after addition of plasmepsin II was rapid and catalytic (Fig. 2).

The activated zymogens have dissimilar ribonucleolytic activity (Table 1), indicating that the location of the new termini can have a detrimental effect on catalysis. For example, the zymogen with 67/71 termini has a perturbation near a nucleobase-binding subsite[10]. The ribonucleolytic activity of that zymogen is low and increases by only three-fold after activation by plasmepsin II. In contrast, the zymogen with 88/89 termini gains nearly 10^3-fold activity, and has a k_{cat} / K_m value almost equal to that of wild type RNase A. Residues 88 and 89 are remote from the active site (Fig. 1*b*) and have no known role in catalysis[9,10]. Moreover, the enzymatic activity of the zymogen with 88/89 termini did not increase in the presence of a HeLa cell extract (data not shown), which contains nonspecific proteases. Thus, activation requires specific proteolysis.
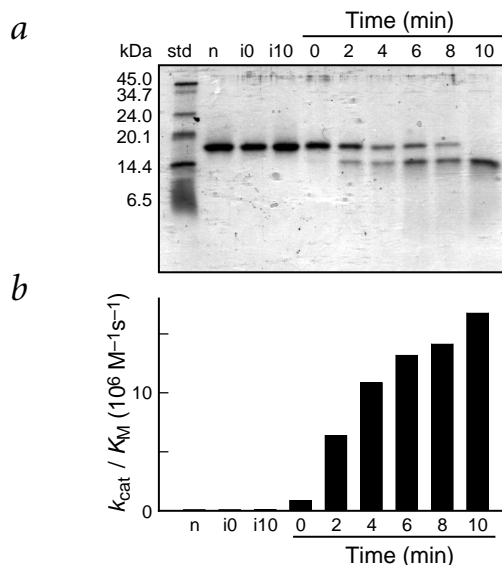
All of the ribonuclease zymogens had a T_m value that was greater than physiological temperature (37 °C) but lower than that of the wild type enzyme (62 °C) (Table 1). Activation by plasmepsin II increased the value of T_m by 5–9 °C. Such an increase in conformational stability after activation is a desirable characteristic. Less stable proteins are more susceptible to nonspecific proteolytic degradation[20,23]. Accordingly, the ribonuclease zymogens are probably degraded more quickly in the absence of their activating protease than in its presence.

The ideal zymogen should have low enzymatic activity before activation. All of the zymogens met this criterion (Table 1), as all have k_{cat} / K_m values $<2 \times 10^4$ M^{-1}s^{-1}. Such low activity is of no known biological consequence for a variant of RNase A. For example, G88R RNase A has $k_{cat} / K_m = 1.4 \times 10^7$ M^{-1}s^{-1} and has well-characterized toxicity to human myelogenous leukemia cells[22,24]. In contrast, K41A/G88R RNase A has $k_{cat} / K_m = 6.5 \times 10^3$ M^{-1}s^{-1} and is not toxic to these cells[24]. Likewise, we find that the zymogen with 20/21 termini (which has $k_{cat} / K_m = 1.02 \times 10^4$ M^{-1}s^{-1} and $T_m = 48$ °C; Table 1) is not cytotoxic at concentrations up to 25 μM.

The ideal zymogen should also have high (that is, nearly wild type) enzymatic activity after activation. The ribonuclease zymogen with 88/89 termini best met this criterion (Table 1). Therefore, we subjected this zymogen to further modification. Decreasing the linker from 14 to 13 residues had little effect on the ribonucleolytic activity or conformational stability of the zymogen with 88/89 termini, either before or after activation (Fig. 3*a*).

Table 1 Values of k_{cat} / K_M (10³ M⁻¹s⁻¹) and T_m (°C) for wild type ribonuclease A and zymogens with five disulfide bonds and various termini, before and after activation by plasmepsin II

Parameter	Wild type	20/21	67/71	88/89	104/105	113/114
$(k_{cat} / K_m)_{unactivated}$[1]	—	10.2	8.0	16.7	12.6	5.1
$(k_{cat} / K_m)_{activated}$[1]	36,000	496	24.0	16,100	1,160	298
$(k_{cat} / K_m)_{activated} / (k_{cat} / K_m)_{unactivated}$	—	48.6	3.0	964	92.1	58
$(T_m)_{unactivated}$[2]	—	48	52	42	42	45
$(T_m)_{activated}$[2]	62	52	58	48	51	53

[1]Values of k_{cat} / K_m were determined for catalysis of 6-FAM–dArU(dA)$_2$–6-TAMRA cleavage at 25 °C in 0.10 M MES-NaOH buffer, pH 6.0, containing 0.10 M NaCl[32].
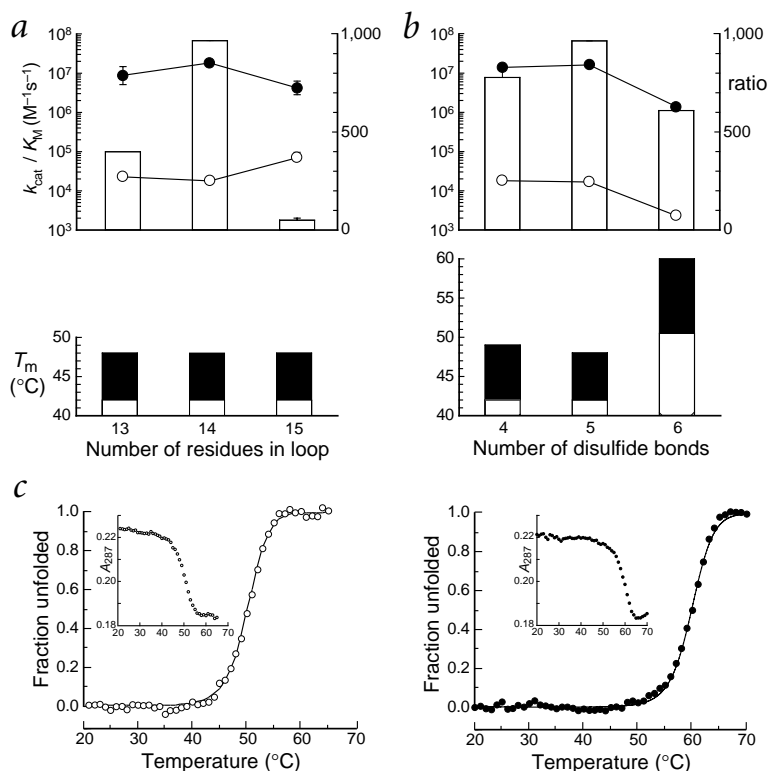[2]Values of T_m were determined in PBS by UV spectroscopy[33].

Fig. 3 Optimization of ribonuclease A zymogen. **a**, Effect of linker length of ribonucleolytic activity and conformational stability of ribonuclease A zymogen with 88/89 termini, before (open) and after (filled) activation by plasmepsin II. Zymogens have linkers of 13 (GS**KPIE FLELK**AG), 14 (GSG**KPIEFLELK**AG) or 15 (GSG**KPIEFLELK** GAG) residues[34]. Bars in the top panel depict the $(k_{cat} / K_m)_{activated} / (k_{cat} / K_m)_{unactivated}$ ratio. **b**, Effect of number of disulfide bonds on the ribonucleolytic activity and conformational stability of ribonuclease A zymogen with 88/89 termini, before (open) and after (filled) activation by plasmepsin II. Zymogens have four (native), five (native plus Cys4–Cys118) or six (native plus Cys4–Cys118 and Cys88–Cys89) disulfide bonds. Bars in the top panel depict the $(k_{cat} / K_m)_{activated} / (k_{cat} / K_m)_{unactivated}$ ratio. **c**, Thermal unfolding of ribonuclease A zymogen with 88/89 termini and four native and two nonnative (Cys4–118 and Cys88–Cys89) disulfide bonds, before (left; T_m 50 °C) and after (right; T_m 60 °C) activation by plasmepsin II. Insets, raw data from UV spectroscopy

which relies on the function of an enzyme rather than its inhibition, could evade known mechanisms of microbial resistance.
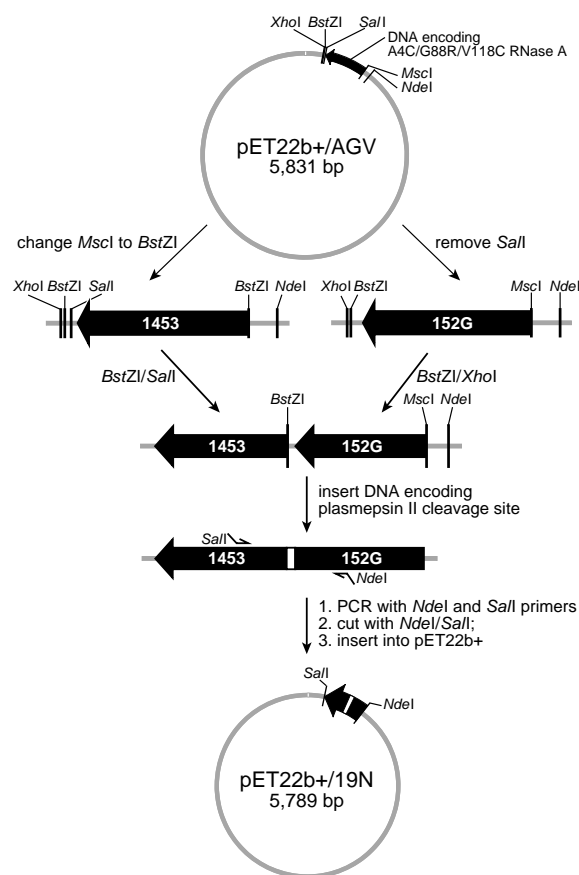
Methods

Instrumentation. UV absorbance measurements were made on a Cary Model 3 or 50 spectrophotometer (Varian) equipped with a Cary temperature controller. Fluorescence measurements were made on a QuantaMaster1 photon-counting fluorometer (Photon Technology International) equipped with sample stirring.

Molecular modeling. The atomic coordinates of wild type RNase A were obtained from the Protein Data Bank (accession code 7RSA)[29]. A model of the structure of each RNase A zymogen was created by using SYBYL (Tripos) on an Octane computer (Silicon Graphics). The program was used to link the N and C termini by a bridge of 13–15 residues, replace residues (for example, A4C and V118C), break the polypeptide chain in one of nine places and minimize the conformational energy of the new residues in the resulting structures.

Preparation of zymogens. A scheme showing the construction of a plasmid pET22b+/19N, which directs the expression of an RNase A zymogen, is shown in Fig. 4. Plasmid pET22b+/AGV, which directs the expression of A4C/G88R/V118C RNase A[20], served as the starting material. Therefore, all of the RNase A zymogens (except those with termini at residues 88/89) had an Arg 88. The G88R mutant of RNase A is able to manifest its ribonucleolytic activity in the presence of the cytosolic ribonuclease inhibitor protein[20,22,26].

The *Msc*I site in plasmid pET22b+/AGV was replaced with a *Bst*ZI site (underlined) by single-stranded DNA mutagenesis using the oligonucleotide 5'-CACAAGTTTCCTTGC<u>CGGCCG</u>CCGGCTGGGCAGC-GAG-3', resulting in plasmid p1453. The *Sal*I site was removed by using the oligonucleotide 5'-CCGCAAGCTTGCGAGGATCCCACTGAA-GCATCAAA-3', resulting in plasmid p152G. Plasmid p1453 was subjected to digestion with *Bst*ZI and *Sal*I endonucleases, and a 385 base pair (bp) fragment was purified after electrophoresis in an agarose gel. Plasmid p152G was subjected to restriction enzyme digestion with *Bst*ZI and *Xho*I endonucleases, and a 5,805 bp fragment was purified. The two DNA fragments were ligated (*Xho*I and *Sal*I digestion yield compatible cohesive ends), resulting in plasmid pSMFII. Plasmid pSMFII was then subjected to digestion with the *Bam*HI and *Bst*ZI endonucleases, and a 6,190 bp fragment was purified. A phosphorylated double-stranded oligonucleotide encoding a plasmepsin II cleavage sequence within 13, 14 or 15 amino acids and having *Bst*ZI (italics)- and *Bam*HI (bold)-compatible cohesive ends was ligated to the pSMFII/*Bst*ZI/*Bam*HI fragment (5'-**GATC-**TAAACCGATTGAATTTCTGGAACTGAA-3' and 5'-*GGCC*TTCAGTTCCA-GAAATTCAATCGGTTTA-3' for the 13-residue linker, 5'-**GATC**TGG CAAACCGATTGAATTTCTGGAACTGAA-3' and 5'-*GGCC*TTCAGTTCCA GAAATTCAATCGGTTTGCCA-3' for the 14-residue linker, and 5'-**GA**

In contrast, increasing the linker from 14 to 15 residues had the undesirable effect of increasing the ribonucleolytic activity before plasmepsin II activation. This finding validates our concern that a long linker could allow a substrate greater access to the active site.

The introduction of a new disulfide bond that links the 88/89 termini increased the conformational stability of the zymogen. The T_m value of the zymogen with 88/89 termini and six disulfide bonds was 50 °C before activation and 60 °C after activation (Fig. 3b). This zymogen unfolded in a cooperative, two-state transition (Fig. 3c), as expected for a globular protein with a single domain. Accordingly, the protein (Fig. 1b) has all of the attributes associated with known zymogens, except that its activation yields a ribonuclease.

A zymogen as a precursor

Organisms could have created zymogens to control the activity of their nonproteolytic enzymes. The near absence of natural nonprotease zymogens could indicate that creating a zymogen from an existing enzyme (Fig. 1a) is more difficult than evolving alternative mechanisms to control enzymatic activity. In addition, the unique ability of proteases to undergo autocatalytic activation could facilitate the evolution of protease zymogens but not zymogens of other enzymes.

Zymogen creation provides a versatile option for the control of enzymatic activity. This strategy avails new applications for enzymes in biotechnology and medicine; for example, a 'Trojan horse' based on a ribonuclease zymogen could have pharmacological use. RNase A can enter human cells, and that process is enhanced by the fusion of a protein transduction domain[25]. Simple variants of both RNase A[22,26] and its human homolog[27] are toxic to cancer cells *in vitro*. An amphibian homolog, Onconase, is now in Phase III clinical trials for the treatment of malignant mesothelioma[7,28]. RNase A zymogens activated by pathogenic proteases could extend the chemotherapeutic utility of ribonucleases to maladies other than cancer. Such a chemotherapeutic strategy,

articles

Fig. 4 Scheme for the creation of plasmid that directs the expression of a ribonuclease A zymogen.

and cooled slowly (over ≥4 h) to room temperature. The resulting double-stranded oligonucleotides were subjected to 5′ phosphorylation by treatment for 1 h with T4 polynucleotide kinase.

The production, folding and purification of RNase A zymogens were done as described for other variants of RNase A[21,22], except that the oxidative folding was done at pH 7.8 for at least 48 h.

Preparation of protease. The production, folding and purification of proplasmepsin II were done as described[30,31]. Proplasmepsin II was activated by the addition of 1 μl of 1.0 M sodium citrate buffer, pH 4.7, to 9 μl of a solution of proplasmepsin II (10 μM in 20 mM Tris-HCl buffer, pH 8.0) and incubation of the resulting solution at 37 °C for 45 min.

Activation of zymogens. RNase A zymogens were activated by mixing 19.5 μl of a solution of 25 μM zymogen with 0.5 μl of a solution of 10 μM activated plasmepsin II and then incubating the resulting mixture at 37 °C for 15 min. Activation was stopped by the addition of pepstatin A to a final concentration of 1 μM. To assess zymogen activation, reaction mixtures were subjected to electrophoresis in a 15% (w/v) polyacrylamide gel containing 1% (w/v) SDS and assayed for ribonucleolytic activity as described[32].

Enzymatic activity of zymogens. The ribonucleolytic activity of RNase A zymogens was evaluated before and after activation with an assay using 6-FAM–dArU(dA)$_2$–6-TAMRA (Integrated DNA Technologies), where 6-FAM is 6-carboxyfluorescein and 6-TAMRA is 6-carboxytetramethylrhodamine[32]. Cleavage of this fluorogenic substrate results in a ~200-fold increase in fluorescence intensity (excitation at 492 nm and emission at 515 nm). Assays were performed at 23 °C in 2.0 ml of 0.10 M MES-NaOH buffer, pH 6.0, containing 0.10 M NaCl, 50 nM 6-FAM–dArU(dA)$_2$–6-TAMRA and 0.1 nM–1 μM zymogen. Data were fit to the equation $k_{cat} / K_m = (\Delta I / \Delta t) / ((I_f - I_0)[E])$, where $\Delta I / \Delta t$ is the initial velocity of the reaction, I_0 is the fluorescence intensity before the addition of enzyme, I_f is the fluorescence intensity after complete hydrolysis with excess wild type enzyme and [E] is the ribonuclease concentration.

Cytotoxicity of zymogens. The toxicity of the unactivated zymogen with 20/21 termini for K-562 cells, which derive from a continuous human chronic myelogenous leukemia line, was assayed as described[22].

Conformational stability of zymogens. The conformational stability of RNase A zymogens was assessed before and after activation by recording the change in absorbance at 287 nm with increasing temperature[33]. The temperature of a solution of 0.15–0.25 mg ml^{-1} RNase A zymogen in PBS was increased continuously from 20 to 70 °C at 0.15 °C min^{-1}. The absorbance was recorded at 1 °C intervals and fit to a two-state model for denaturation. The temperature at the midpoint of the transition is defined as T_m.

TCTGGCAAACCGATTGAATTTCTGGAACTGGGCAA-3′ and 5′-*GGC*-CTTGCCCAGTTCCAGAAATTCAATCGGTTTGCCA-3′ for the 15-residue linker). Oligonucleotide primers corresponding to different new N termini were engineered to have an *Nde*I-compatible cohesive end, and those corresponding to different new C termini were engineered to have a *Sal*I-compatible cohesive end. These pairs of primers were used in the PCR, and the resulting products were purified and subjected to digestion with the *Nde*I and *Sal*I endonucleases. The resulting fragments were inserted into the *Nde*I and *Sal*I sites of plasmid pET22b+ to produce plasmid pET22b+/19N (Fig. 4).

The Cys4–Cys118 cystine was removed from the circularly permuted RNase A with 88/89 termini by PCR-based site-directed mutagenesis using oligonucleotides 5′-AAGGAAA**CTGCAG**CAGCCAAG TTTGAGCGGCAGC-3′ and 5′-GCTGCCGCTCAAACTTGGCTG**CTGCAG** TTTCCTT-3′ for the C4A substitution and 5′-GCATCAAAGTGGACTG-GCA*CGTACG*GGGTTTCCC-3′ and 5′-GGGAAACCC*GTACG*TGCCAGTC-CACTTTGATGC 3′ for the C118V substitution. Digestion with *Pst*I endonuclease (bold) was used to screen for the C4A substitution, and digestion with *Bsi*WI endonuclease (italics) was used to screen for the C118V substitution.

The permuted RNase A with 88/89 termini and a sixth disulfide bond was created by PCR of plasmid pSMFII using oligonucleotide primers 5′-CGTGAG**CATATG**TGTTCCAAGTACCCC-3′ and 5′-GTTGG-G*GTCGAC*CTACTAGCACGTCTCACGGCAGTC-3′ with the *Nde*I (bold) and *Sal*I (italics) restriction sites. The PCR product was purified, digested with the *Nde*I and *Sal*I endonucleases, and inserted into the *Nde*I and *Sal*I sites of plasmid pET22b+. The resulting plasmid encodes a permuted variant with the eight native cysteine residues plus Cys4, Cys88, Cys89 and Cys118.

Oligonucleotides were annealed by dissolving them to 0.25 mM in 10 mM Tris-HCl buffer, pH 8.0, containing 50 mM NaCl and 1 mM EDTA. The resulting solution was heated to 95 °C in a water bath

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