Supplementary information

RimJ-mediated context-dependent N-terminal acetylation of the recombinant Z-domain protein in *Escherichia coli*

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Materials and Methods

General

XL1-Blue *E. coli* cells (Stratagene) were used for cloning and maintaining plasmids. BL21(DE3), JM109(DE3) and DH10B *E. coli* cells were used for protein expression. *PfuUltra* High-Fidelity DNA polymerase (Stratagene) was used for polymerase chain reaction (PCR). Quikchange II site-directed mutagenesis kit (Stratagene) was used for site-directed mutagenesis.

Construction of plasmids

pET-Z, a plasmid that encodes the Z-domain C-terminal hexa-histidine fusion gene under control of T7 promoter, was generated by inserting the fusion gene between the NdeI and XhoI sites of pET-21a(+) (Novagen). pBAD-Z, a plasmid that encodes the Z-domain C-terminal hexa-histidine fusion gene under control of *araBAD* promoter, was constructed by cloning the fusion gene between the NcoI and HindIII sites of pBAD/*myc*-His (Invitrogen). The *rimJ* gene was amplified from MG1655 *E. coli* genomic DNA by PCR. pACYCDuet-RimJ, a p15A plasmid that encodes the *rimJ* gene under control of T7 promoter, was generated by inserting the PCR-amplified *rimJ* gene between the NdeI and XhoI sites of pACYCDuet-I (Novagen). All plasmids constructed were verified by sequencing.

Protein expression and purification

BL21(DE3) or JM109(DE3) *E. coli* cells were transformed with pET-Z or pBAD-Z. Either of these two plasmids was co-transformed with pSup-JYRS-6TRN or pACYCDuet-RimJ into BL21(DE3) or JM109(DE3) *E. coli* cells. DH10B *E. coli* cells were transformed with pBAD-Z, or co-transformed with pBAD-Z and pSup-JYRS-6TRN. The transformed cells were grown overnight at 37°C in the PA-5052 auto-induction medium containing 100 μg/mL carbenicillin (and 50 μg/mL chloramphenicol for the double transformants). Cells were harvested by centrifugation and lysed by sonication in lysis buffer (50

mM sodium phosphate, 300 mM NaCl, pH8.0). The Z-domain protein in the cell lysate was purified with Ni-NTA metal affinity resin under native conditions according to the manufacturer's protocol. Purified protein was concentrated by ultrafiltration and analyzed by SDS-PAGE.

Expression of the Z-domain Bpa mutants

The Z-domain Bpa mutants were produced based on the procedure previously reported.[1] The codons for Ser-3, Val-4, and Asp-5 of the Z-domain in pET-Z were mutated to an amber (TAG) codon by site-directed mutagenesis to generate pET-Z(S3TAG), pET-Z(V4TAG) and pET-Z(D5TAG), respectively. These plasmids were co-transformed with pSup-BpaRS-6TRN into BL21(DE3) cells. The transformed cells were grown in the presence of 1mM p-benzoyl-L-phenylalanine (Bpa) overnight at 37°C in the PA-5052 medium containing 100 μg/mL carbenicillin and 50 μg/mL chloramphenicol. The mutant proteins were isolated and purified as described above for the wild type protein.

Deletion of the rimJ gene from the BL21(DE3) E. coli strain

The *rimJ* gene was deleted from the chromosome of BL21(DE3) *E. coli* cells according to the method of Datsenko and Wanner.[2] The kanamycin resistance cassette of pKD13 was amplified by PCR using two oligonucleotides: 5'-ATG TTT GGC TAT CGC AGT AAC GTG CCA AAA GTG CGC TTA ACC ACA GAC CGG TGT AGG CTG GAG CTG CTT C-3' and 5'-TTA GCG GCC GGG CGT CCA GTC TGG GGT AGT TAA TGC CGT CAG TAC ATG GGA ATT AGC CAT GG TCC-3'. The PCR product was transformed into electrocompetent BL21(DE3) *E. coli* cells containing pKD46, a plasmid encoding the phage λ Red recombinase. The transformants were incubated at 30^{o}C for 12 h and plated on a large kanamycin Luria Bertani (LB) agar plate. The plate was incubated at 43^{o}C for 24 h. Single colonies were picked and amplified in LB media. The genomic DNA was isolated and amplified by PCR using two oligonucleotides: 5'-ATG TTT GGC TAT CGC AGT AA CG-3' and 5'- TTA GCG GCC GGG CGT CCA GTC-3'. The successful integration of the knockout cassette was confirmed by examining the size of the PCR product.

Mass spectrometry

Electrospray ionization mass spectrometry was performed on quadrupole (LCQ) or linear ion trap (LTQ) mass spectrometers (both from Thermo Scientific, San Jose, CA) equipped with an electrospray ionization (ESI) source and operated with the Xcalibur data acquisition software. ESI spray voltage and capillary temperature were maintained at 2.5-3.0 kV and 200 °C, respectively. Protein solutions of 10 to 50 ng/μL concentration were sprayed at an infusion rate of 5 μL/min. At least ten scans covering mass-

to-charge (*m/z*) ratios of 200 to 2000 Th were averaged to obtain ESI mass spectra from which multiple-charged protein ions were deconvoluted by using the ProMass for XCalibur program (Novatia, Monmouth Junction, NJ).[3] Peak heights in the resultant graphs were considered proportional to the abundance of the proteins with the corresponding molecular mass (m) present in the samples. High resolution ESI mass spectra, obtained by using the Fourier-transform ion cyclotron resonance (FTICR) operating in tandem with the LTQ linear ion trap (LTQ-FT, Thermo), were used to confirm deconvolution by ProMass. FTICR full-scan mass spectra were acquired at 100,000 nominal mass resolving power (M/ΔM at m/z 400 and taking the full width at half maximum intensity, FWHM, as ΔM) from *m/z* 400 to 2000 Th using the automatic gain control mode of ion trapping.

Theoretical mass calculations

The theoretical mass of the Z-domain was calculated based on its amino acid sequence: MTSVDNKINKEQQNAFYEILHLPNLNEEQRDAFIQSLKDDPSQSANLLAEAKKLNDAQAPKGS HHHHH. The calculated average mass of the Z-domain with the cleavage of the N-terminal methionine (the Z form) is 7762.4 Da. The calculated average mass of the N^{α}-acetylated Z-domain with the removal of the N-terminal methionine (the Ac-Z form) is 7804.5 Da. The molecular weight of p-benzoyl-L-phenylalanine (Bpa) is 269.3. The theoretical mass of the Z-domain Bpa mutants were calculated based on the molecular weight difference between the wild type amino acid residue and Bpa at each position as shown in the table below.

The Z-domain mutant	Molecular weight difference	Calculated average mass of the Z-form (Da)	Calculated average mass of the Ac-Z form (Da)
Ser3Bpa	+164.2	7926.6	7968.7
Val4Bpa	+152.1	7914.5	7956.6
Asp5Bpa	+136.2	7898.6	7940.7

Trypsin proteolysis and high resolution ESI-MS analysis

A 120 μL solution of the isolated protein (20 μg) in 50 mM ammonium bicarbonate buffer (pH 8.5) containing 50% 2,2,2-trifluoroethanol was heated at 60°C for 1 h, cooled to room temperature, and diluted 10-fold with 50 mM ammonium bicarbonate buffer. Digestion was performed at 37°C for 20 h in the presence of 1 μg trypsin. The reaction mixture was evaporated to the dryness and re-dissolved in 700 μL 0.1% aqueous acetic acid. A 3 μL aliquot of the digested sample was directly injected to Agilent 6224 Accurate-Mass TOF LC/MS system and analyzed by MassHunter Workstation software.

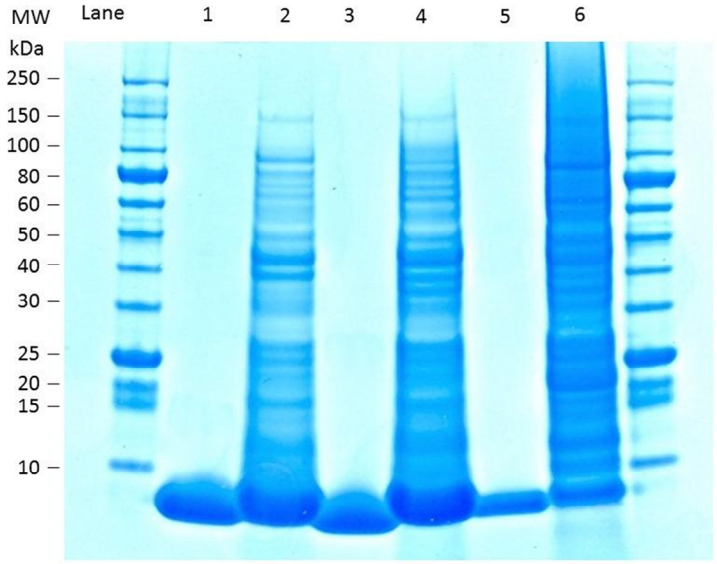

Fig. S1. SDS-PAGE analysis of the Z-domain in *E. coli* cell lysates and the protein purified by immobilized Ni ion-affinity chromatography. Lanes 1 and 2: the Z-domain purified from BL21(DE3) *E. coli* cells co-transformed with pET-Z and pSup-JYRS-6TRN, and its corresponding cell lysate, respectively; Lanes 3 and 4: the Z-domain purified from BL21(DE3) *E. coli* cells transformed with pET-Z alone and its corresponding cell lysate, respectively; Lanes 5 and 6: the Z-domain purified from BL21(DE3) *E. coli* cells co-transformed with pBAD-Z and pACYCDuet-RimJ, and its corresponding cell lysate, respectively.

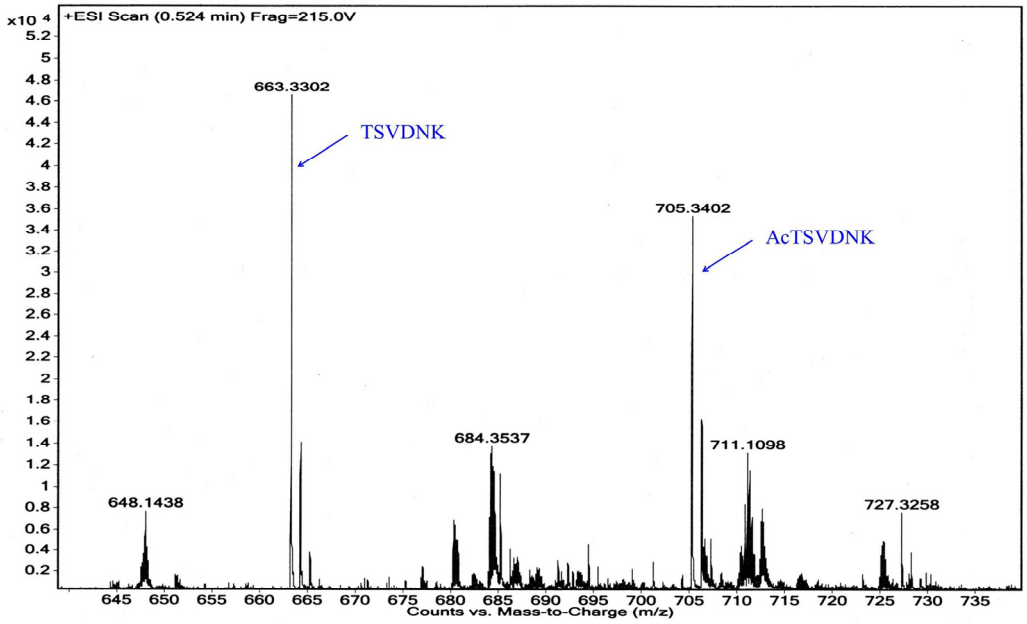

Fig. S2-1. High Resolution ESI-MS analysis of the N-terminal peptide fragments obtained by the trypsin proteolysis of the Z-domain, which was expressed in BL21(DE3) *E. coli* cells in the presence of pET-Z and pSup-JYRS-6TRN.

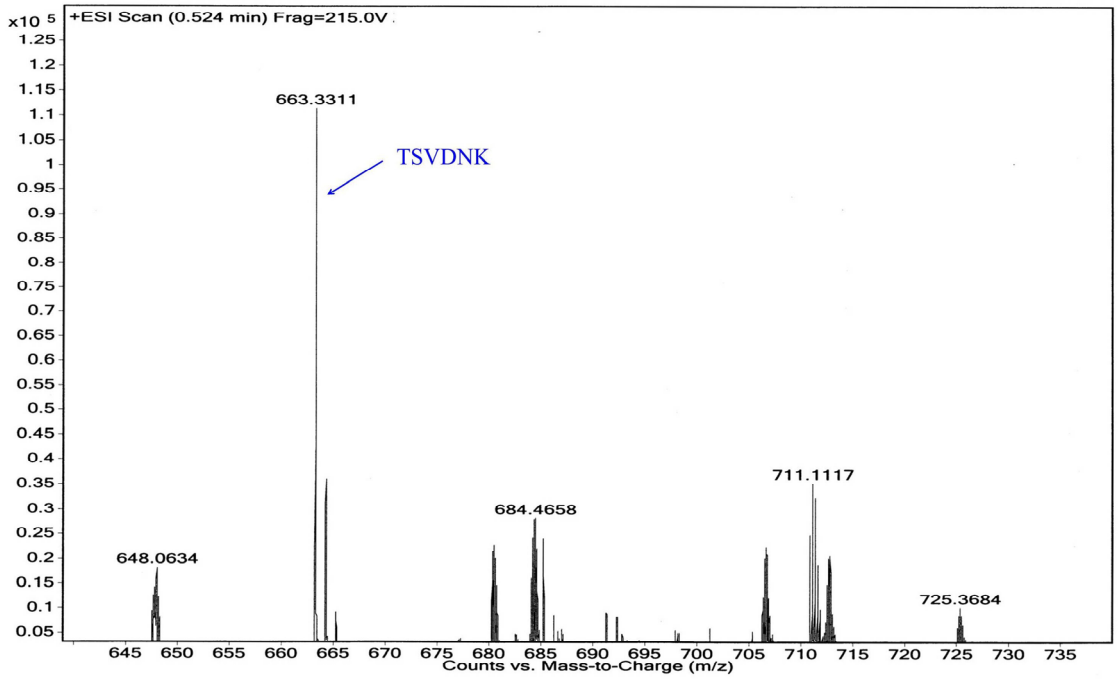

Fig. S2-2. High Resolution ESI-MS analysis of the N-terminal peptide fragments obtained by the trypsin proteolysis of the Z-domain, which was expressed in BL21(DE3) *E. coli* cells in the presence of pET-Z.

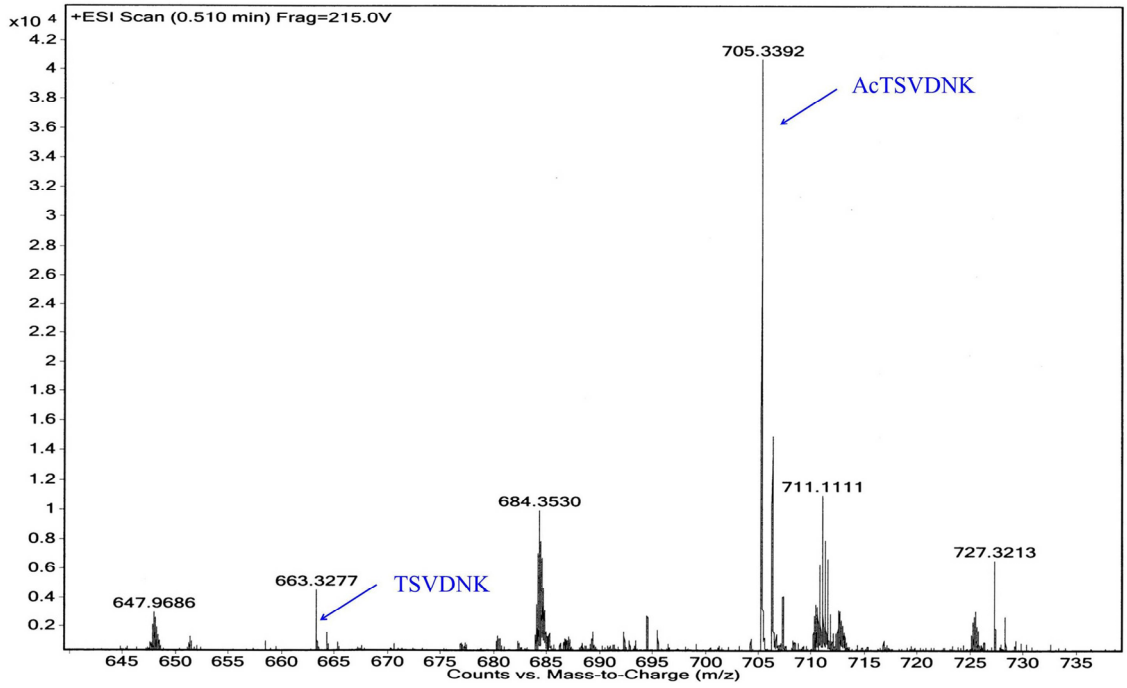

Fig. S2-3. High Resolution ESI-MS analysis of the N-terminal peptide fragments obtained by the trypsin proteolysis of the Z-domain, which was expressed in BL21(DE3) *E. coli* cells in the presence of pBAD-Z and pACYCDuet-RimJ.

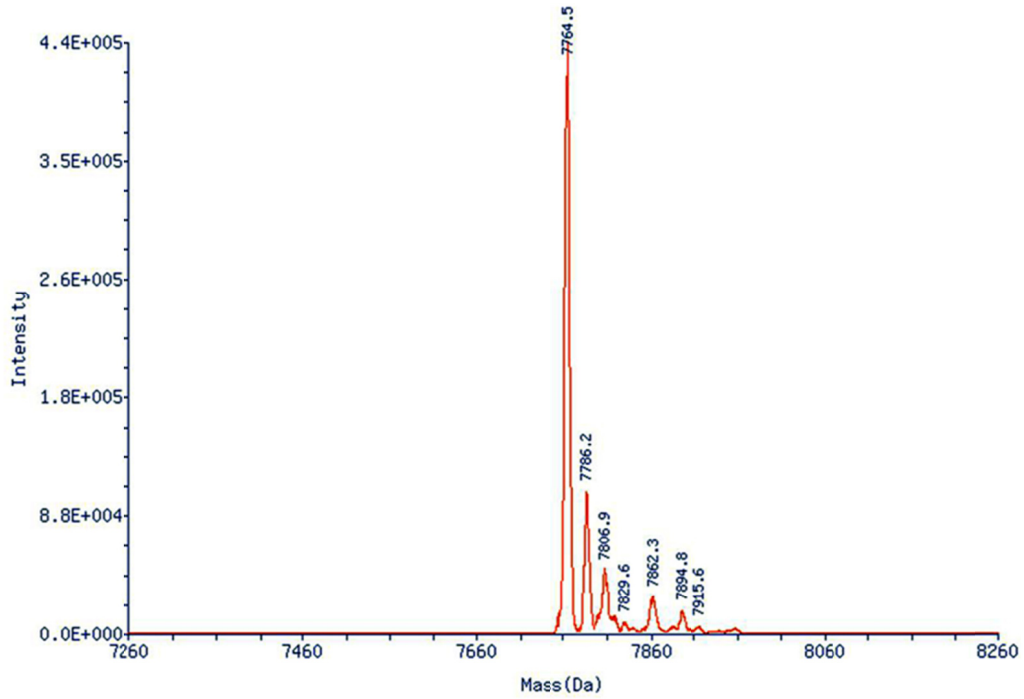

Fig. S3-1. Mass-transformed ESI-MS spectrum of the Z-domain protein that was expressed in JM109(DE3) cells transformed with pET-Z.

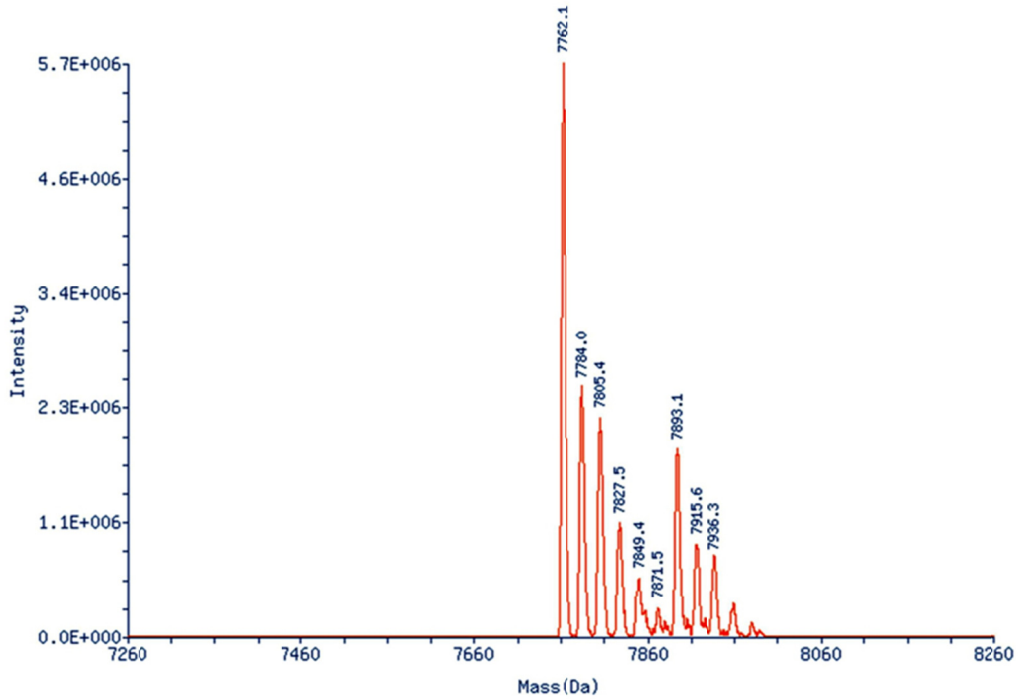

Fig. S3-2. Mass-transformed ESI-MS spectrum of the Z-domain protein that was expressed in JM109(DE3) cells co-transformed with pET-Z and pSup-JYRS-6TRN.

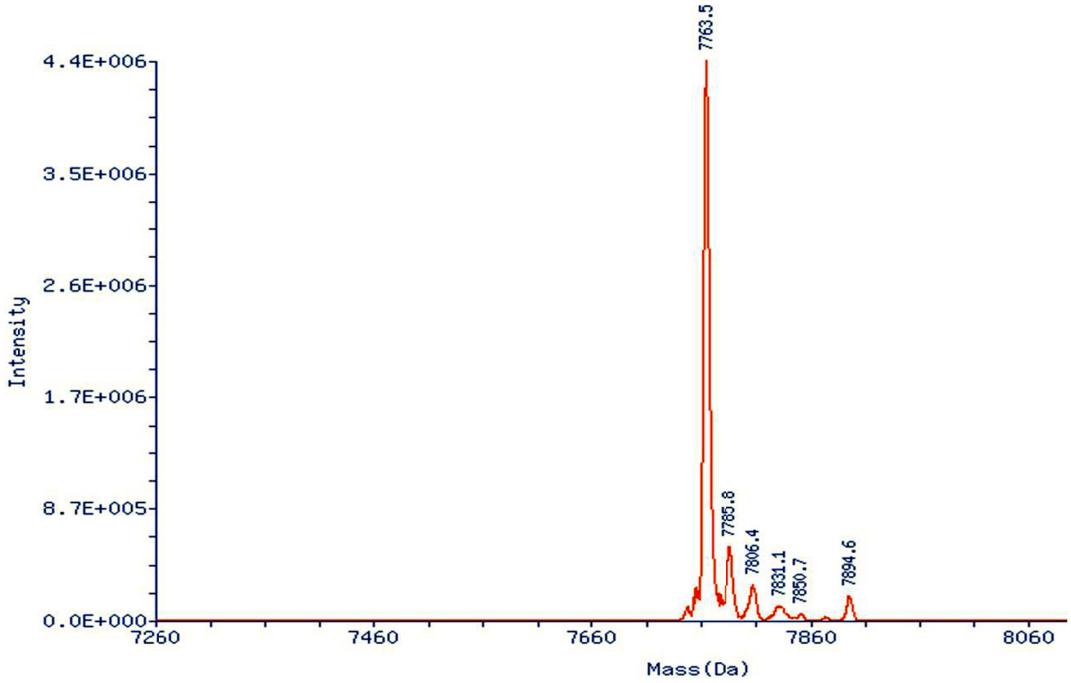

Fig. S3-3. Mass-transformed ESI-MS spectrum of the Z-domain protein that was expressed in DH10B cells transformed with pBAD-Z.

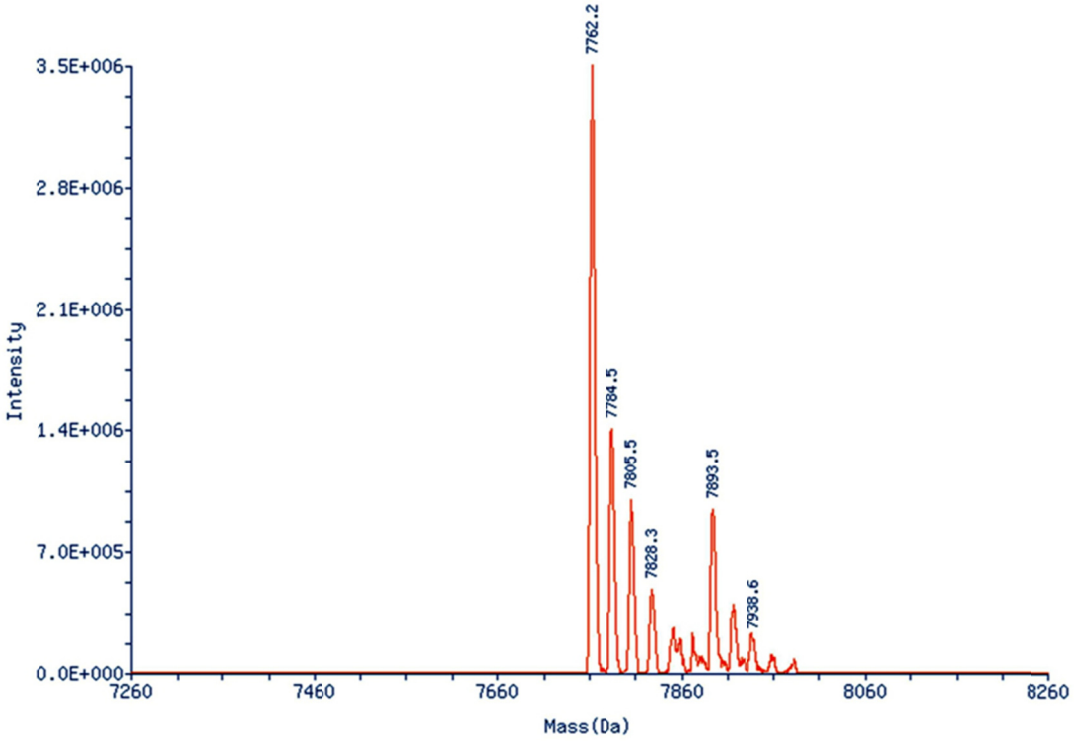

Fig. S3-4. Mass-transformed ESI-MS spectrum of the Z-domain protein that was expressed in DH10B cells co-transformed with pBAD-Z and pSup-JYRS-6TRN.

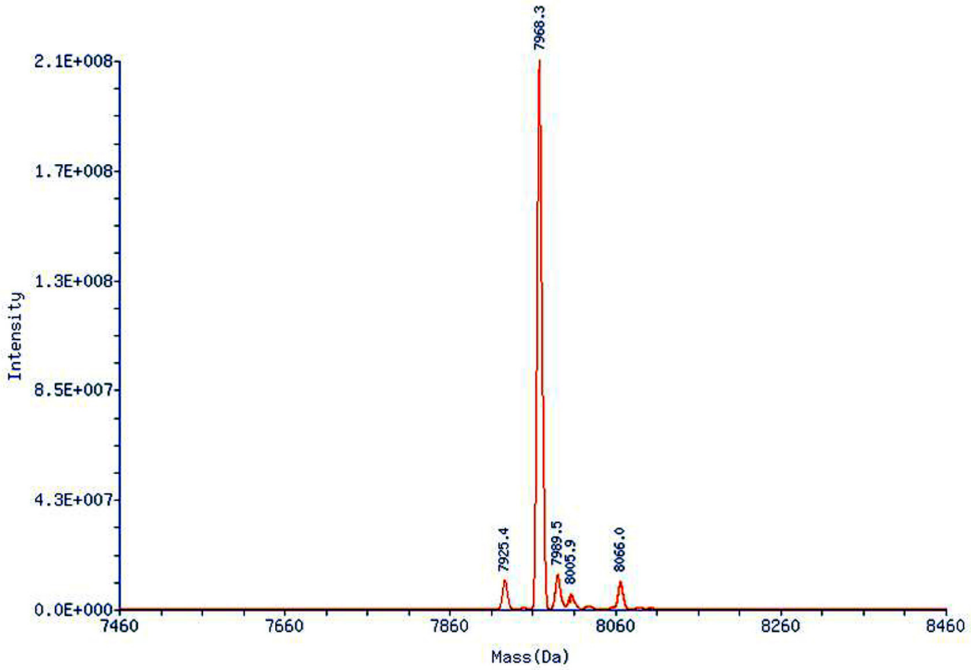

Fig. S3-5. Mass-transformed ESI-MS spectrum of the Z-domain Ser3Bpa mutant that was expressed in the presence of 1mM Bpa in BL21(DE3) cells co-transformed with pET-Z(S3TAG) and pSup-BpaRS-6TRN.

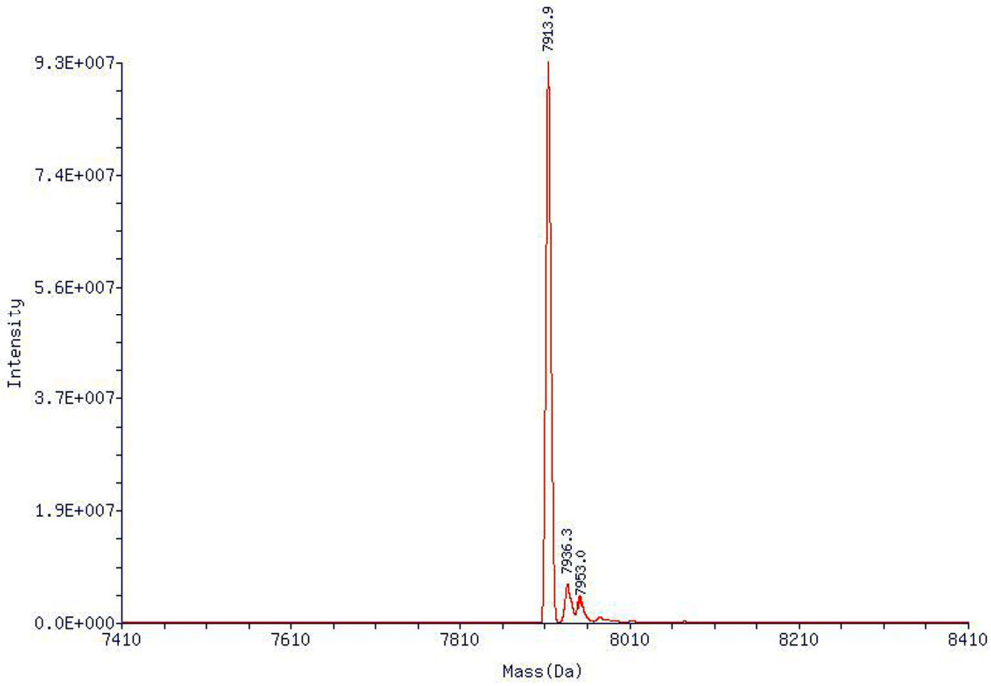

Fig. S3-6. Mass-transformed ESI-MS spectrum of the Z-domain Val4Bpa mutant that was expressed in the presence of 1mM Bpa in BL21(DE3) cells co-transformed with pET-Z(V4TAG) and pSup-BpaRS-6TRN.

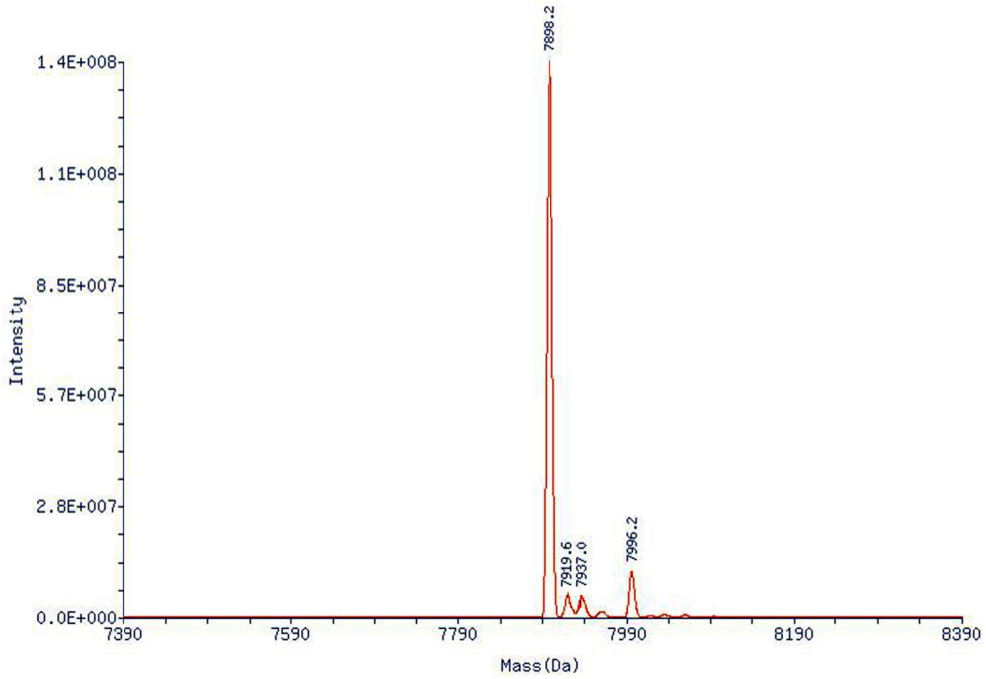

Fig. S3-7. Mass-transformed ESI-MS spectrum of the Z-domain Asp5Bpa mutant that was expressed in the presence of 1mM Bpa in BL21(DE3) cells co-transformed with pET-Z(D5TAG) and pSup-BpaRS-6TRN.

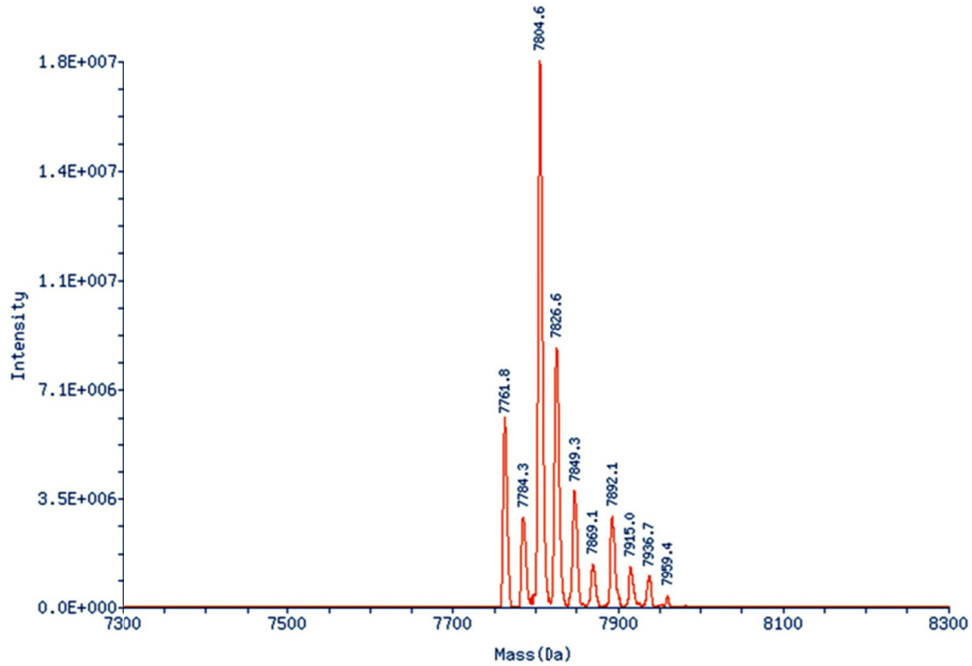

Fig. S3-8. Mass-transformed ESI-MS spectrum of the Z-domain protein that was expressed in BL21(DE3) *E. coli* cells co-transformed with pET-Z and pACYCDuet-RimJ.

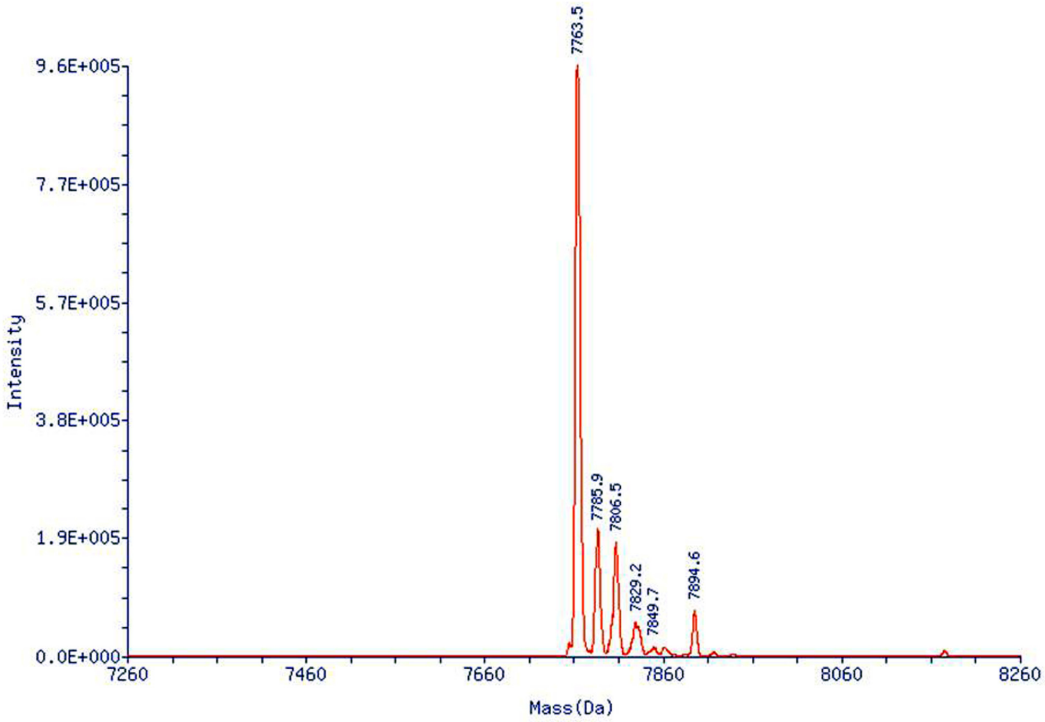

Fig. S3-9. Mass-transformed ESI-MS spectrum of the Z-domain that was expressed in BL21(DE3) *ΔrimJ::kan* cells co-transformed with pET-Z and pSup-JYRS-6TRN.

References

1. Y. Ryu and P. G. Schultz, *Nat. Methods*, 2006, **3**, 263-265.

2. K. A. Datsenko and B. L. Wanner, *Proc. Natl. Acad. Sci. USA*, 2000, **97**, 6640-6645.

3. M. Mann, C. K. Meng and J. B. Fenn, *Anal. Chem.*, 1989, **61**, 1702-1708.