Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Chemical Synthesis of Human Selenoprotein F and Elucidation of Its Thiol-Disulfide Oxidoreductase Activity

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1 Representative synthetic selenoproteins or Sec-containing proteins

Table S1. Representative synthetic selenoproteins or Sec-containing proteins via native chemical ligation or related methods.

Entry	Protein	# of aa	# of ligations	# of Cys/Sec	Post-ligation treatment	Refs
			Selenoprotein			
1	SelW	86	1	2Cys, 1Sec	None	[1]
2	SelM	122	3	1Cys, 1Sec	$MeONH_2$ treatment; Deselenization	[1]
3	SelK	90	1	1Sec	Deselenization	[2]
4	Thioredoxin Reductase	490	1	3Cys, 1Sec	None	[3]
5	SelF	134	2	7Cys, 1Sec	Desulfurization; Acm-removal/Mob-removal	This work
			Sec-containing protein			
6	Seleno-Grx3	82	1	1Cys, 1Sec or 2Sec	Thiol alkylation at the ligation site	[4]
7	Seleno-Grx1	85	2	1Sec	Thiol alkylation at the ligation site	[5]
8	RNaseA C110U	124	1	7Cys, 1Sec	None	[6]
9	[7Sec-81Sec]-Cp-5	82	1	4Cys, 2Sec	None	[7]
10	Sec-analogues of BPTI	58	1 or 2	5Cys, 1Sec or 4Cys, 2Sec	None; or $MeONH_2$ treatment	[8-10]
11	Sec112-azurin	128	1	2Cys, 1Sec	None	[11]
12	Sec-analogues of SDF-1	72	1	4Cys, 1Sec	None	[12]
13	Se-TSTD1	114	2	1Sec	Deselenization	[13]
14	Seleno–Fd	97	2	4Sec,1Cys	None	[14]

2 General reagents and methods

Commercially available materials were obtained from Adamas, Energy Chemicals, or Sigma-Aldrich. Standard Fmoc-amino acids, Fmoc-Sec(Mob)-OH, 2-Cl-(Trt)-Cl resins, Rink amide MBHA resin, 1-hydroxybenzotriazole (HOBt), 2-(1h-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) were purchased from GL Biochem (Shanghai). The reagents acetylacetone (acac) and inslin (from bovine pancreas) were purchased from Aladdin. Sodium ascorbate, ethylene diamine tetraacetic acid (EDTA) and 2,2′-(ethylenedioxy)diethanethiol (DODT) were purchased from TCI. Palladium chloride ($PdCl_2$) and 2,2'-Azobis[2-(2-imidazolin-2-yl) propane] dihydrochloride (VA-044) were obtained from J&K Scientific. N,N-diisopropylethylamine (DIPEA), N,N'-diisopropylcarbodiimide (DIC), dithiothreitol (DTT), tris(2-carboxyethyl)phosphine (TCEP), trifluoroacetic acid (TFA), 2-methyl-2-propanethiol (t-BuSH), and guanidine hydrochloride (Gdn·HCl) were obtained from Adamas. Methoxylamine (CH_3ONH_2·HCl) and triisopropylsilane (TIPS) were purchased from Energy Chemicals. Ribonuclease A from bovine pancreas (RNase A), trypsin (from bovine pancreas), L-arginine hydrochloride (Arg·HCl), tris(hydroxymethyl)aminomethane (Tris), glutathione reduced (GSH) and glutathione oxidized (GSSG) were purchased from Sangon. The reagents N,N-dimethylformamide (DMF) and dichloromethane (DCM) were purchased from GHTCH (Guangdong). 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 4-mercaptophenylacetic acid (MPAA) were purchased from Alfa Aesar. His_6-Ulp1 was recombinantly expressed using a reported procedure[15]. Acetonitrile (MeCN) used in analytical HPLC was obtained from Fisher. α-chymotrypsin (from bovine pancreas, type II) and acetonitrile (MeCN) used in preparative HPLC were obtained from Sigma-Aldrich. Analytical HPLC (Agilent 1260) was performed on a Phenomenex Jupiter C4 column (4.6 × 250 mm, 300 Å, 5 μm particle size) running at a flow rate of 1 mL/min with UV detection at 214 and 254 nm. Semi-preparative HPLC (Shimadzu AR-20) was performed using Waters a XBridge® peptide BEH C18 OBD™ Prep column (300 Å, 5 μm, 10 × 250 mm) or a Welch Ultimate XB-C4 column Prep column (300 Å, 5 μm, 10 × 250 mm) running at a flow rate of 4.7 mL/min with UV detection at 214 and 254 nm. Preparative HPLC (Ruihe® Tech) was performed using a Welch Ultimate XB-C4 column Prep column (300 Å, 5 μm, 30 × 250 mm) running at a flow rate of 40 mL/min with UV detection at 214 and 254 nm. Solvent A: 0.1 % TFA in water; Solvent B: 0.1 % TFA in MeCN. LC-MS was performed on an Agilent LC/MSD (ESI) system on ACE 5 C4 column (150 × 4.6 mm). MALDI-TOF mass spectra (Shimadzu 8020) were obtained in the linear positive mode using a matrix of 10 mg/mL α-cyano-4-hydroxy-cinnamic acid (CHCA) in water/MeCN (1: 1, v/v) with 0.1% TFA.

3 General synthesis procedures

3.1 Preloading of 2-Cl-(Trt)-$NHNH_2$ resin

2-Chlorotrityl chloride resin (0.9 mmol/g, 1 g) was swollen in DMF for 20 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2× 5 mL). The resin was treated with freshly prepared 5% hydrazine monohydrate in DMF (2× 20 mL) for 30 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). The resin was treated with freshly prepared 5% MeOH in DMF (20 mL) for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL). DIPEA (1.2 mmol) was added to a solution of Fmoc-AA-OH (0.6mmol) and TBTU (0.6 mmol) in DMF (5 mL). After 2 min of pre-activation, the mixture was added to the resin, which was then shaken for 2 h at 25 °C. The resin was washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL), and then capped with 20% acetic anhydride in DMF (10 mL) for 20 min, and washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL) again.[16]

3.2 Estimation of amino acid loading

The resin (10 mg) loaded with the first amino acid was treated with 2% DBU/DMF (2 mL) for 30 min at 25 °C to remove the Fmoc group. The blank group was set up. The deprotection solution (2 mL) was diluted to 10 ml with MeCN, and then 0.8 mL was further diluted to 10 mL with MeCN. The UV absorbance of the resulting piperidine-fulvene adduct solution was measured (λ = 304 nm) to estimate the amino acid loading on the resin.

3.3 Fmoc deprotection

The resin was treated with 20% piperidine in DMF (5 mL, 2 × 10 min) at 25 °C and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.4 Coupling of general amino acids

Peptides were synthesized on a CS Bio 136XT synthesizer using Fmoc solid phase peptide synthesis (SPPS) chemistry. The following Fmoc amino acids with side-chain protecting groups were used: Fmoc-Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(tBu)-OH, Fmoc-Thr(tBu)-OH, Fmoc-Tyr(tBu)-OH, Fmoc-Val-OH, etc. SPPS was performed on 2-Cl-(Trt)-Cl or Rink amide MBHA resins. Fmoc deprotections were performed with 20% piperidine in DMF (10 min × 2). Couplings were performed with Fmoc amino acid (4.0 equiv to resin substitution), TBTU (3.9 equiv) and DIPEA (8.0 equiv) in DMF for 60 min (45 °C). After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.5 Coupling of Fmoc-Phe-Ser(Psi(Me, Me)Pro)-OH or Fmoc-Asp(OtBu)-Ser(Psi(Me, Me)Pro)-OH

A solution of Fmoc-Phe-Ser(Psi(Me, Me)Pro)-OH or Fmoc-Asp(OtBu)-Ser(Psi(Me, Me)Pro)-OH (4 equiv), HOBT (3.9 equiv), and DIC (8 equiv) in DMF was added to the resin. The reaction was shaken for 4 h at 30 °C. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.6 Coupling of Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH

A solution of Fmoc-Cys(Trt)-OH or Fmoc-Cys(Acm)-OH (4 equiv), HOBT (3.9 equiv), and DIC (8 equiv) in DMF was added to the resin. The reaction was shaken for 1 h at 45 °C. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

3.7 Coupling of Fmoc-Sec(Mob)-OH

A solution of Fmoc-Sec(Mob)-OH (2.5 equiv), HOBT (2.45 equiv), and DIC (5 equiv) in DMF was added to the resin. The reaction was shaken for overnight at RT. After coupling, unreacted free amine was capped by treatment with 20% acetic anhydride in DMF for 10 min and then washed with DMF (2 × 5 mL), DCM (2 × 5 mL), and DMF (2 × 5 mL).

4 Synthesis of the peptide fragments

4.1 Synthesis of SelF(1–41) thioester fragment 1

Amino acid sequence of S1:

FGAE*FS*SEAC[10] R ELG*FS*SNLL CSSCDLLGQF NLLQLDPDCR G[41]

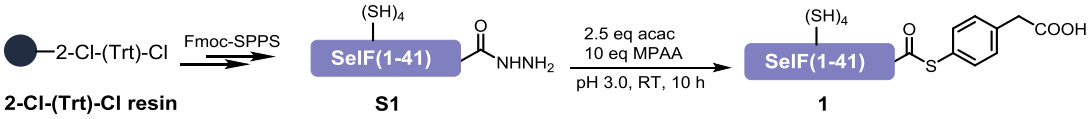


Figure S1. Synthesis of SelF(1–41) thioester **1**, and the pseudo-proline dipeptide building blocks used during SPPS are shown in italic.

SelF(1–41)-$NHNH_2$ (**S1**) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) using Fmoc-Gly-OH with 0.4 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h a t 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide SelF(1–41)-$NHNH_2$ (**S1**).

The crude peptide **S1** (400 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 10 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form thioester[17] fragment SelF(1–41)-SR (**1**). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 70% MeCN (with 0.1% TFA) in 25 min to obtain 8 mg of segment **1** (1 g resin; 2.5%). Overall, 18 mg of segment **1** was obtained. The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **1** with the observed mass 4593.0 Da, calcd 4593.0 Da (average isotopes).

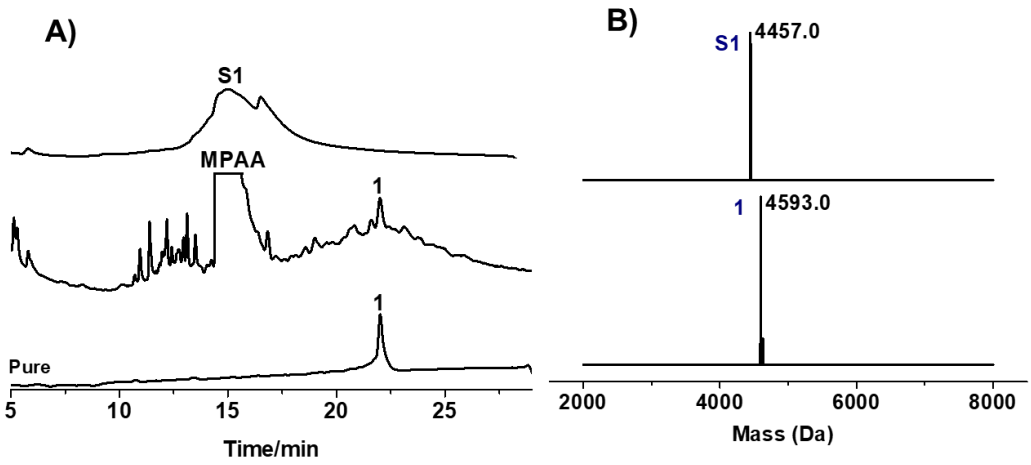


Figure S2. (A) Analytical HPLC traces (25 to 70% MeCN (with 0.1% TFA) in 25 min, λ = 214 nm) of crude SelF(1–41)-$NHNH_2$ **S1** and purified SelF(1–41) thioester **1**. (B) ESI-MS analysis of **S1** with the observed mass 4457.0 Da, calcd 4457.0 Da (average isotopes). ESI-MS analysis of **1** with the observed mass 4593.0 Da, calcd 4593.0 Da (average isotopes).

4.2 Synthesis of SelF(42–74) thioester fragment 2

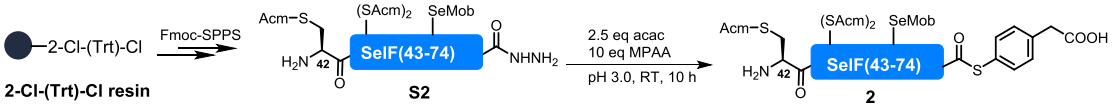


Figure S3. Synthesis of SelF(42–74) thioester **2**.

SelF(42–74)-$NHNH_2$ (**S2**) was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) Fmoc-Ala-OH with 0.35 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved[18, 19] using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2–3 mL per 100 mg of resin) for 1.5 h at 4 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2 about 30 min at 4 °C. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide SelF(42–74)-$NHNH_2$ (**S2**).

The crude peptide **S2** (300 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 10 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form thioester fragment SelF(42–74)-SR (**2**). The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to obtain 25.9 mg of segment **2** (1 g resin; 8.6%). Overall, 102 mg of segment **2** was obtained. The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **2** with the observed mass 4164.0 Da, calcd 4163.8 Da (average isotopes).

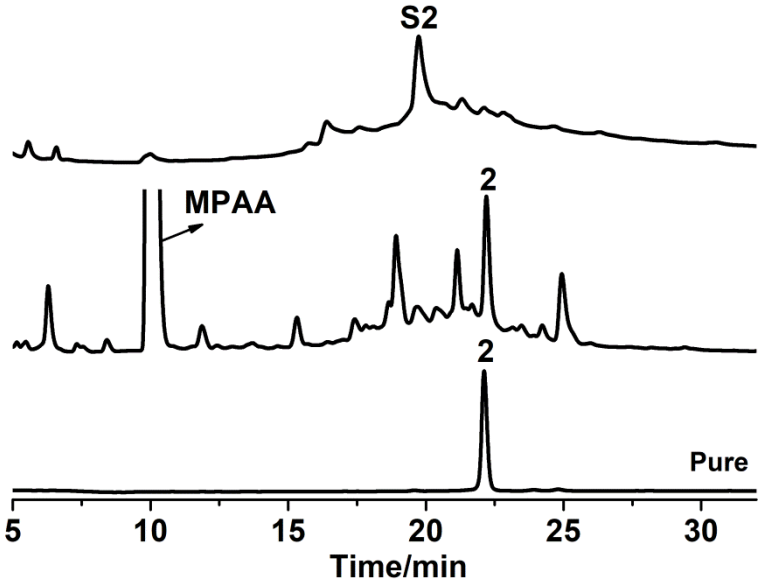


Figure S4. (A) Analytical HPLC traces of crude SelF(42–74)-$NHNH_2$ **S2** and purified SelF(42–74) thioester **2**.

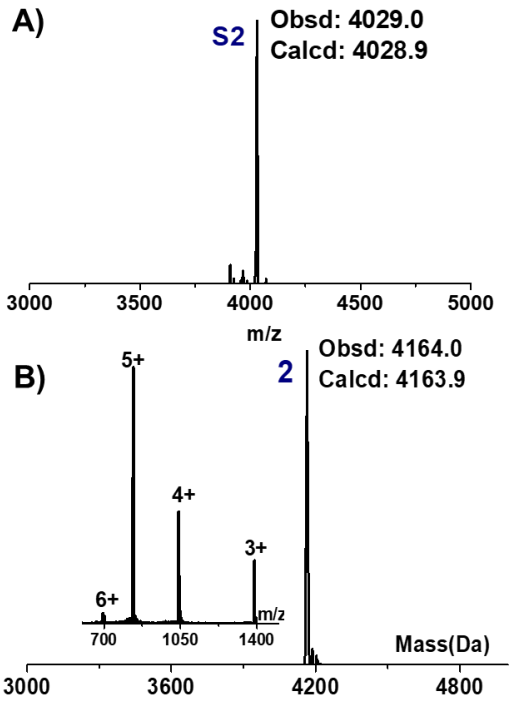
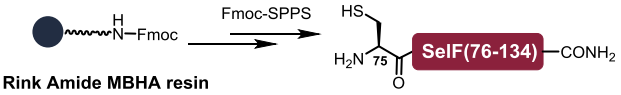


Figure S5. (A) MALDI-TOF analysis of **S2** with the observed mass 4029.0 Da, calcd 4028.9 Da (average isotopes). (B) ESI-MS analysis of **2** with the observed mass 4164.0 Da, calcd 4163.8 Da (average isotopes).

4.3 Synthesis of SelF(75–134) fragment 3

4.3.1 Attempted synthesis of SelF(75–134) fragment 3 by Fmoc-SPPS

C^{75}FVRSDKPKLFRGLQIKYVRGSDPVL^{100}KLLDDNGNIAEELSILKWNT*DS*VEEFLSEKLERI^{134}



The SelF(75-134)-$CONH_2$ fragment 3 was synthesized on Rink amide MBHA resin (theoretical loading: 1.2 mmol/g) using Fmoc-Ile-OH with 0.46 mmol/g loading and elongated according to standard Fmoc-SPPS protocol to afford resin-bound peptide. The pseudoproline dipeptides of Fmoc-Asp(OtBu)-Ser(Psi(Me, Me)Pro)-OH was coupled according to the protocol 3.5. The peptide was cleaved using TFA/H_2O/TIPS (95:2.5:2.5 v/v/v) (2-3 mL per 100 mg of resin) for 2 h at 25 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide. To determine the progress of synthesis, the crude peptides of SelF(106-134), SelF(100-134) and SelF(97-134) were analysed using analytical HPLC and MALDI-TOF. It was found that the synthesis was already poor at Leu100 (i.e. 35 aa), while the target peptide peak was low and with significant aspartimide contamination[20], possibly at the Asp-Asp or Asp-Asn sequences. When synthesis continued to Asp97 (i.e. 38 aa), the target peptide content was extremely low. It would be difficult to complete the synthesis of the 60-aa SelF(75-134), fragment 3, we have thus decided to switch to the recombinant expression technology (see below).

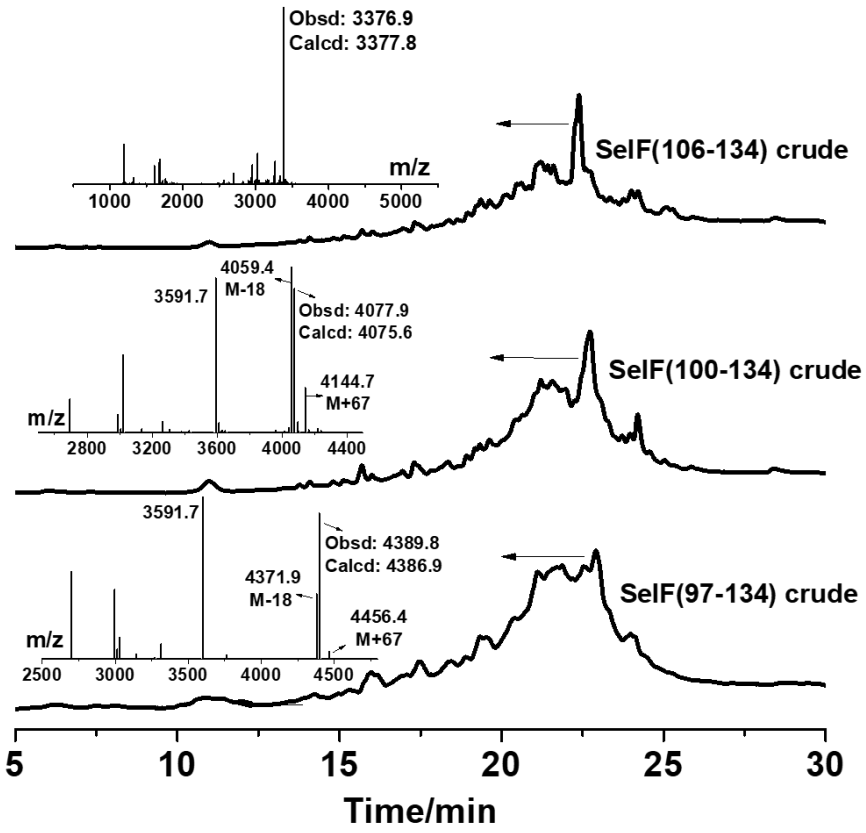


Figure S6. Analytical cleavage and analysis of the attempted synthesis of SelF(75-134) fragment 3 via HPLC (15 to 65% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) and MALDI-TOF.

*4.3.2 Expression of SelF(75–134) fragment **3***

The gene encoding for the His_6-SUMO-SelF(75–134) fusion protein[21] was synthesized and codon-optimized for E. Coli expression (GenScript Inc., Nanjing). The synthetic gene was cloned into the pET-30a expression vector using the *Nde*I/*Eco*RI restriction sites. The vector pET-30a (+)-SelF(75–134) required for the overexpression of the desired His_6-SUMO-SelF(75–134) can be obtained from GL Biochem. The cleavage site between Gly and Cys is marked in bold. The full amino acid sequence of His_6-SUMO-SelF(75–134) was:
MNWSHPQFEKSSGSSGGHHHHHHGGSGGSGSDSEVNQEAKPEVKPEVKPETHINLKVSDGSSEIFFKIKKTTPLRRLMEAFAKRQG
KEMDSLRFLYDGIRIQADQAPEDLDMEDNDIIEAHREQIG**GC**FVRSDKPKLFRGLQIKYVRGSDPVLKLLDDNGNIAEELSILKWNTDSV
EEFLSEKLERI.

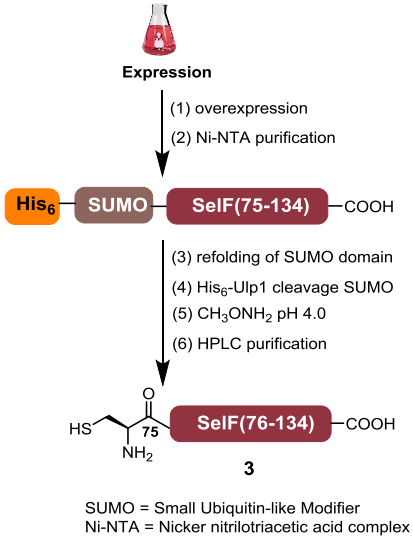


Figure S7. Expression and purification of SelF(75–134) fragment **3**.

Overexpression and purification of His_6-SUMO-SelF(75–134):
LB (2 L): 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, pH 7
Cell lysis buffer: 0.9% NaCl, pH 7
Extraction buffer: 8 M Urea, 20 mM imidazole, 0.5 M NaCl, 20 mM Tris, pH 8
Ni-NTA binding buffer: 8 M Urea, 20 mM Imidazole, 0.5 M NaCl, 20 mM Tris, pH 8
Ni-NTA eluting buffer: 8 M Urea, 250 mM Imidazole, 0.5 M NaCl, 20 mM Tris, pH 8
Refolding buffer: 6 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 4 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 2 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 1 M Urea, 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8; 0.15 M NaCl, 20 mM Tris, 10% glycerol, pH 8, these dialysates were changed every 12 h.
The plasmid was firstly transformed into BL21(DE3) E. coli cells chemically. An overnight culture of the cells harboring an expression vector was inoculated (1:50 dilution) in a 4 L flask containing 30 µg/mL Kanamycin in 2 L LB at 37 °C. After reaching an OD_{600} of 0.6–0.8 overexpression of His_6-SUMO-SelF(75–134) was induced by the addition of 2 mL 1 M IPTG stock solution (final conc. 1 mM) at 37 °C for 4 h. Cells were harvested by centrifugation (8000 rpm, 4 °C, 15 min). Typically, 6 g cell precipitates were resuspended in 50 mL of cell lysis buffer and lysed by ultrasonication (30–40 % power, 3 s on 5 s off, 25 min). The crude lysate was centrifuged (16000 rpm, 4 °C, 20 min) and the supernatant was discarded. The precipitate was stirred at 4 °C overnight with 10 mL of Ni-NTA binding buffer to extract His_6-SUMO-SelF(75–134). The precipitate was removed by centrifugation (16000 rpm, 30 min, 4 °C, 5 cycles) and the supernatant applied to a HisTrap™ FF column (5 mL) at 2 mL/min with a AKTA pure chromatography system. Absorption was monitored at 280 nm. The column was washed with 20 mL of Ni-NTA binding buffer. His_6-SUMO-SelF(75–134) (**S3**) was eluted with 25 mL of Ni-NTA eluting buffer in fractions of 5 mL.
The 10 mL fractions with A280 > 0.1 was gradually dialyzed to the 0.5 L refolding buffer to complete the refolding of SUMO domain. 400 µL of a stock solution of His_6-Ulp (A280 = 0.5) were added to the folded His_6-SUMO-SelF(75–134) (**S4**) (10 mL, A280=1.8) (Ulp1: protein = 2:50, v/v) and the reaction was incubated for 2 h at 30 °C. Equal volume of the buffer containing 6 M Gdn·HCl, 200 mM Na_2HPO_4, 200 mM CH_3ONH_2·HCl, 10 mM TCEP (pH 3), was added to the mixture and the pH was adjusted to 4.0 to remove any N-terminal cyclized byproduct of fragment **3**. After overnight incubation, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 48% MeCN (with 0.1% TFA) in 20 min to obtain 13 mg of SelF(75–134) (**3**) (6.5 mg/L LB). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. ESI-MS analysis of **3** with the observed mass 6965.7 Da, calcd 6965.7 Da (average isotopes).

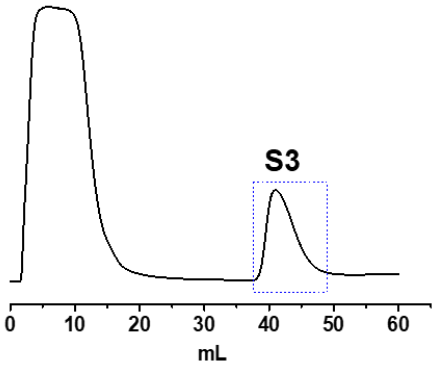


Figure S8. Purification of His_6-SUMO-SelF(75–134) by Histrap^{TM} FF column (λ = 280 nm).

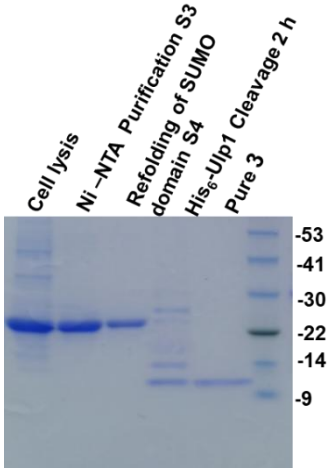


Figure S9. SDS-PAGE of the His_6-SUMO-SelF(75–134) cell lysis, Ni-NTA purification of **S3** and refolding of **S4** followed by cleavage of the His_6-SUMO tag. Lane 1: soluble fraction after cell lysis; lane 2: after Ni-NTA purification; lane 3: refolding of **S4**; lane 4: cleavage of the His_6-SUMO tag; lane 5: pure SelF(75–134) (**3**); lane 6: molecular weight standard.

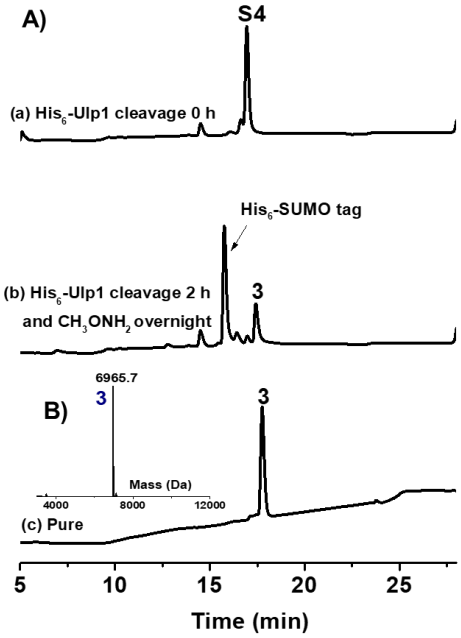


Figure S10. (A) Analytical HPLC traces (15 to 65% MeCN (with 0.1% TFA) in 20 min, λ = 214 nm) of **S4**, the cleavage of the His_6-SUMO tag from refolded **S4** and pure **3.** (B) ESI-MS analysis of **3** with the observed mass 6965.7 Da, calcd 6965.7 Da (average isotopes).

5 1st ligation, desulfurization and Acm/Mob deprotection

5.1 Synthesis of fragment 4 by NCL (on a 5 μmol scale)

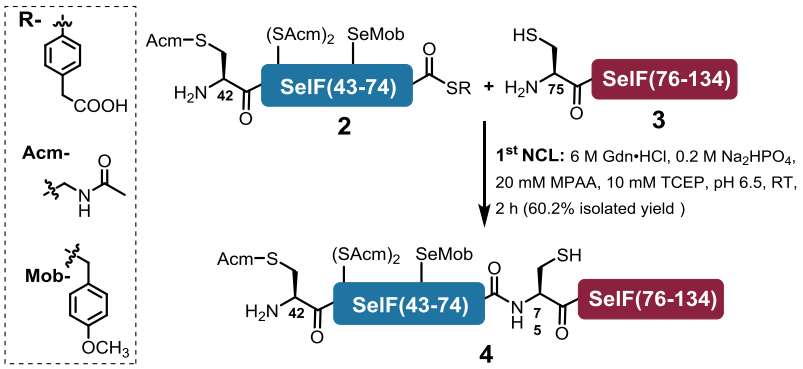


Figure S11. Synthesis of fragment 4 by NCL.

The peptide-thioester **2** (6.5 μmol, 1.3 equiv, 27.1 mg) and Cys-peptide **3** (5 μmol, 1 equiv, 34.8 mg) was dissolved in 2.5 mL of ligation buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 10 mM TCEP and 20 mM MPAA at pH 6.5. The solution was incubated at RT for 2 h (confirmed by LC-MS monitoring). Then, to the ligation reaction mixture was added 5 equiv TCEP to reduce at RT for 15 min. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired fragment **4** as a white amorphous powder (33 mg, 60.2% isolated yield). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively.

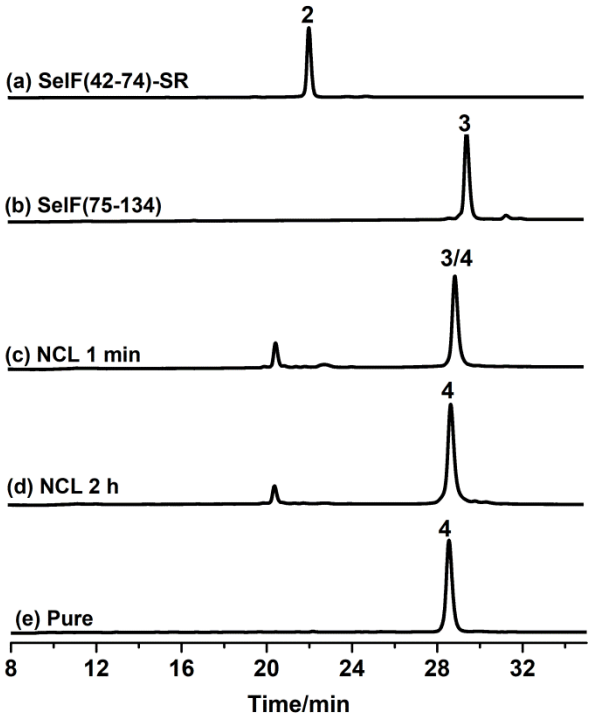


Figure S12. Analytical HPLC traces (25 to 55% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of NCL and the purified **4**.

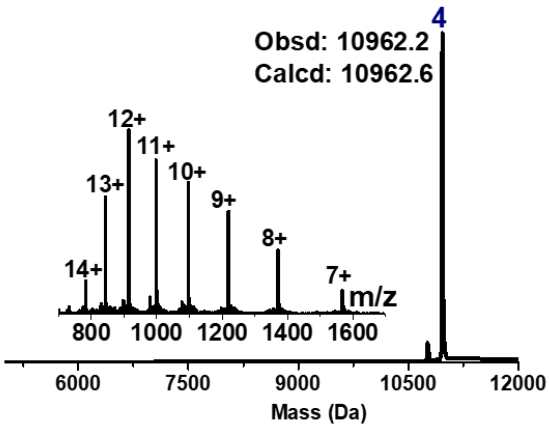


Figure S13. ESI-MS analysis of **4** with the observed mass 10962.2 Da, calcd 10962.6 Da (average isotopes).

5.2 Synthesis of fragment 5 by desulfurization

*5.2.1 Desulfurization of peptide **4** in standard conditions*

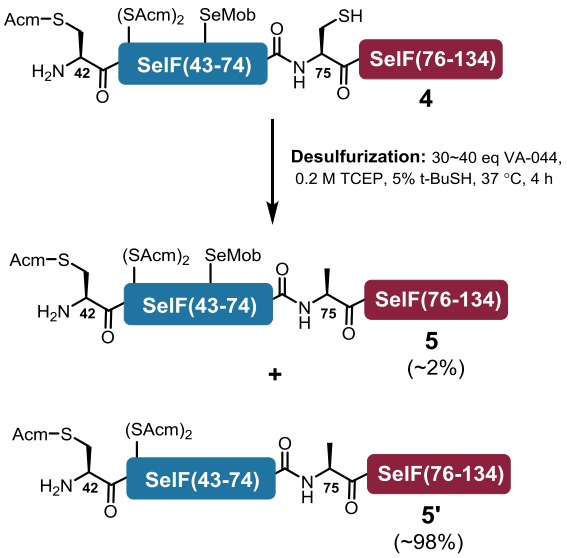


Figure S14. Schematic representation for the desulfurization of peptide **4** with 30-40 eq loadings of VA-044 for 4 h.

The peptide **4** (0.091 μmol, 1 mg) was dissolved in 0.36 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 0.2 M TCEP at pH 6.5 and divided into two portions. Then, to the each of the peptide solution was added t-BuSH (5 μL, v/v), and then 40 equiv and 30 equiv of VA-044 (the stock solution 0.1 M) were added to the each of the peptide solution, which was incubated at 37 °C for 4 h.[22] Then, the mixture was followed by centrifugation and injection of the supernatant into HPLC using a C4 analytical column with a gradient of 25-55% MeCN (with 0.1% TFA) in 20 min. When the high equivalent VA-044 was added, only the by-product **5'** with desulfurization at Cys75 and deselenization at Sec65—despite being Mob-protected, was obtained.

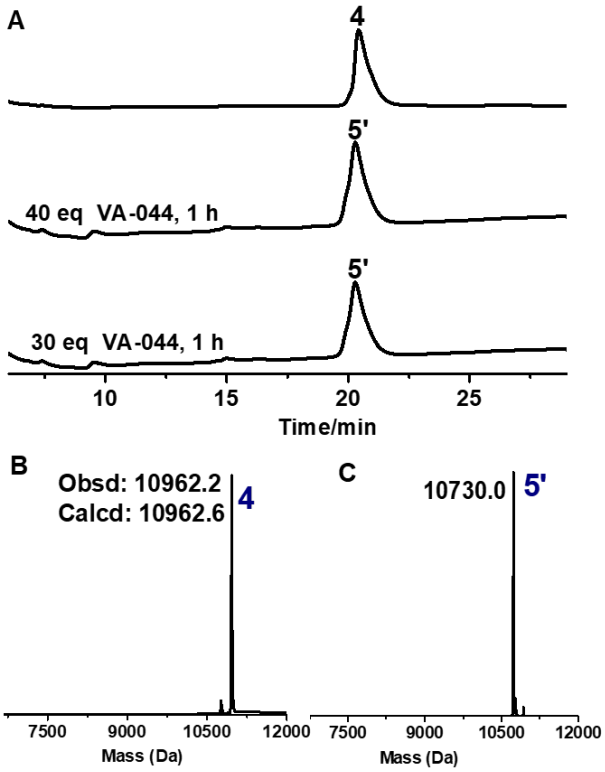


Figure S15. (A) Analytical HPLC traces of the desulfurization of peptide **4** with 30-40 eq loadings of VA-044 for 4 h. (B) ESI-MS analysis of **4** with the observed mass 10962.2 Da, calcd 10962.6 Da (average isotopes); (C) ESI-MS analysis of by-product **5'** with the observed mass 10730.0 Da (average isotopes).

5.2.2 Stability experiment of side chain Se(Mob) group

The Sec-peptide **S2** (0.124 μmol, 0.5 mg) was dissolved in 0.6 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 at pH 6.5 and divided into six portions. The peptide **S2** were put in the following conditions: (a) 5% t-BuSH (5.2 μL, v/v); (b) 0.2 M TCEP (5.72 mg); (c) 20 equiv VA-044 (0.1 M stock solution, 4 μL); (d) 5% t-BuSH (5.2 μL, v/v), 0.2 M TCEP (5.72 mg); (e) 20 equiv VA-044(0.1 M stock solution, 4 μL), 0.2 M TCEP (5.72 mg); (f) 20 equiv VA-044 (0.1 M stock solution, 4 μL), 0.2 M TCEP (5.72 mg), 5% t-BuSH (5.5 μL, v/v), respectively. Then, the each of the peptide solution was incubated at 37 °C for 4 h. The each of mixture was followed by centrifugation and injection of the supernatant into HPLC using a C4 analytical column with a gradient of 25-55% MeCN (with 0.1% TFA) in 20 min. There are no significant changes can be observed when peptide **S2** was incubated with TCEP or t-BuSH, which led us to conclude that VA-044 could most probably be the reason. VA-044 would cause Se(Mob) group unstable and removed. The by-product **S2'** and **S2''** was confirmed using analytical HPLC and ESI-MS, respectively.

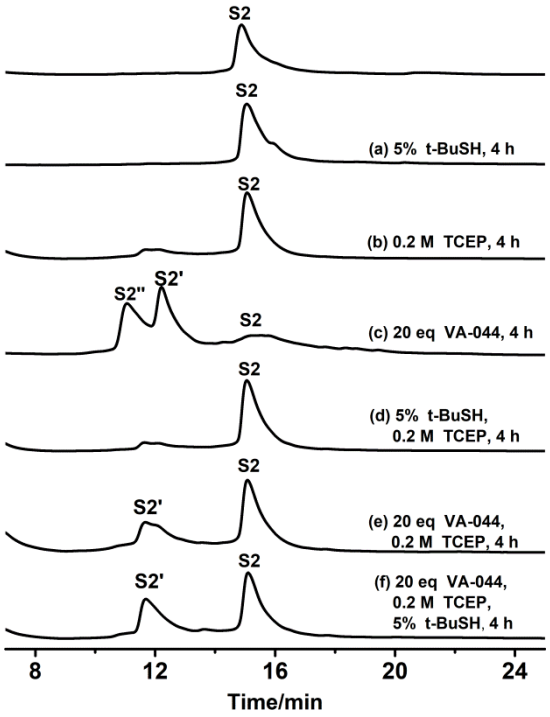


Figure S16. Analytical HPLC traces of peptide **S2** in the conditions of (a)~(f) for 4 h, respectively.

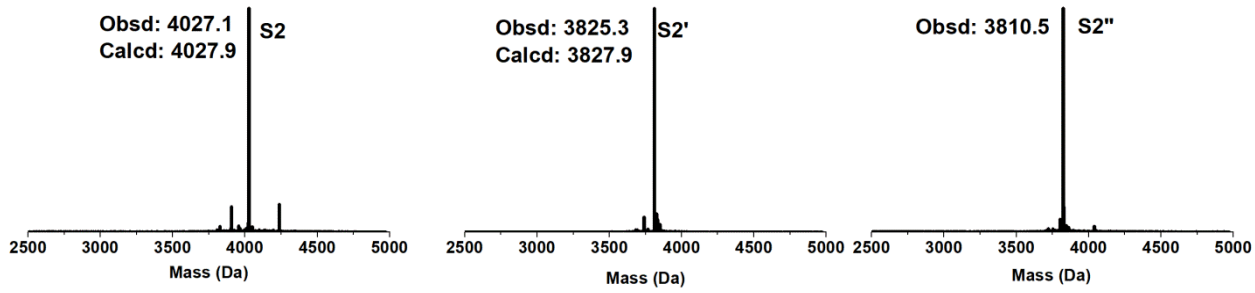


Figure S17. MALDI-TOF analysis of **S2** with the observed mass 4027.1 Da, calcd 4027.9 Da (average isotopes); by-product **S2'** with the observed mass 3825.3 Da, calcd 3827.9 Da (average isotopes); by-product **S2''** with the observed mass 3810.5 Da.

*5.2.3 Effect of different loading of VA-044 on the desulfurization of peptide **4***

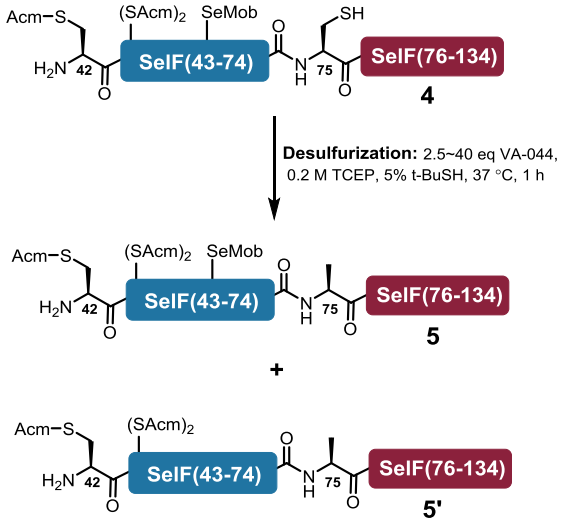


Figure S18. Schematic representation for the desulfurization of peptide **4** with different loadings of VA-044.

The peptide **4** (0.182 μmol, 2 mg) was dissolved in 0.36 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 0.2 M TCEP at pH 6.5 and divided into four portions. Then, to the each of the peptide solution was added t-BuSH (5 μL, v/v), and then 40 equiv, 30 equiv, 15 equiv and 2.5 equiv of VA-044 (the stock solution 0.1 M) were added to the each of the peptide solution, which was incubated at 37 °C for 1 h. Then, the mixture was followed by centrifugation and injection of the supernatant into HPLC using a C4 analytical column with a gradient of 25-55% MeCN (with 0.1% TFA) in 20 min. When the high equivalent VA-044 was present, only the by-product **5'** was obtained, while the equivalent VA-044 was reduced to 2.5 equiv, the desired desulfurized peptide **5** was obtained as the major product.

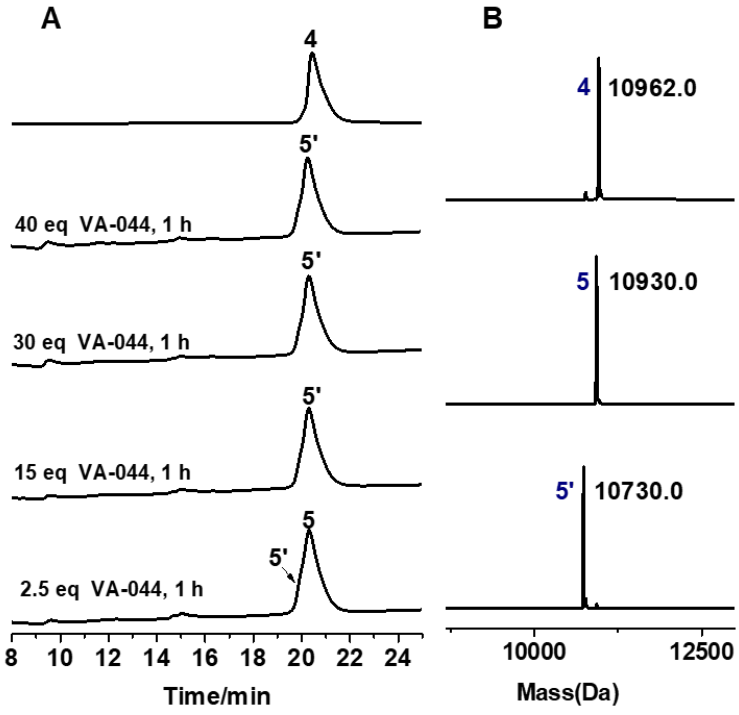


Figure S19. (A) Analytical HPLC traces of the desulfurization of peptide **4** with different loadings of VA-044. (B) ESI-MS analysis of **5** with the observed mass 10930.0 Da, calcd 10930.6 Da (average isotopes); by-product **5'** with the observed mass 10730.0 Da (average isotopes).

*5.2.4 Effect of reaction time on the desulfurization of peptide **4***

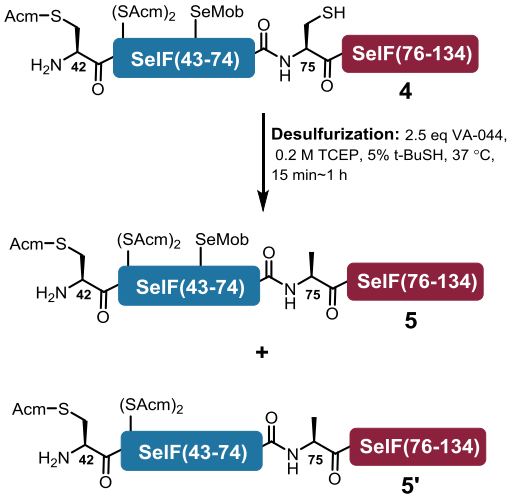


Figure S20. Schematic representation for the desulfurization of peptide **4** with 2.5 equiv VA-044 in different reaction time.

The peptide **4** (0.137 μmol, 1.5 mg) was dissolved in 0.27 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 0.2 M TCEP at pH 6.5. Then, to the peptide solution was added t-BuSH (15 μL, v/v), and then 2.5 equiv of VA-044 (the stock solution 0.01 M, 15 μL) was added to the peptide solution and divided into three portions. The each of the peptide solution was incubated at 37 °C for 1 h, 30 min and 15 min, respectively. Then, the mixture was followed by centrifugation and injection of the supernatant into HPLC using a C4 analytical column with a gradient of 25-55% MeCN (with 0.1% TFA) in 20 min. When the reaction time was 30 min, the best result of the desulfurization was obtained.

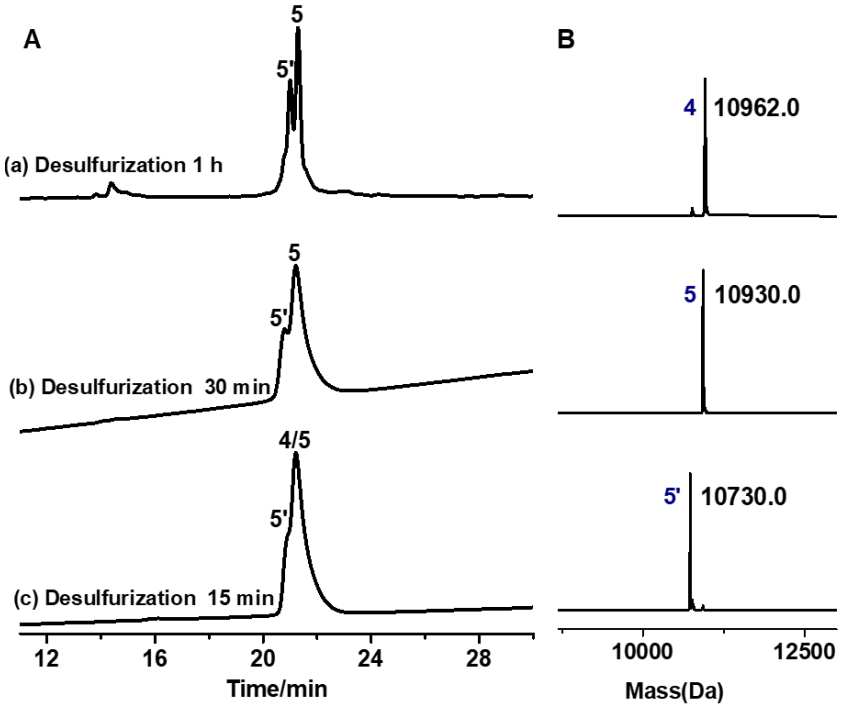


Figure S21. (A) Analytical HPLC traces of desulfurization in 1 h, 30 min and 15 min. (B) ESI-MS analysis of **4** with the observed mass 10962.2 Da, calcd 10962.6 Da (average isotopes); **5** with the observed mass 10930.0 Da, calcd 10930.6 Da (average isotopes); by-product **5'** with the observed mass 10730.0 Da (average isotopes).

*5.2.5 Synthesis of fragment **5** by desulfurization (on a 3 μmol scale)*

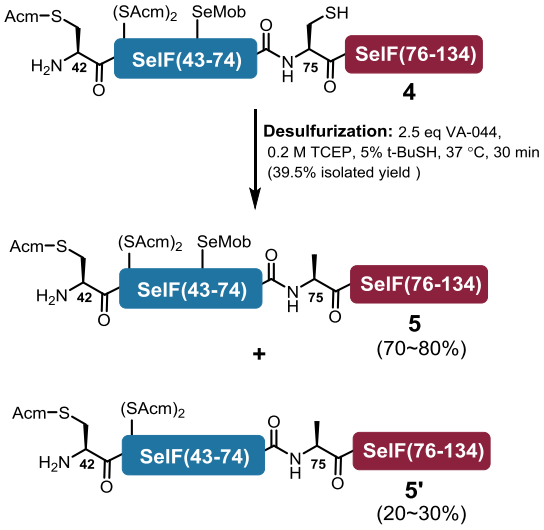


Figure S22. Synthesis of fragment **5** by desulfurization.

The peptide **4** (3 μmol, 1 equiv, 33 mg) was dissolved in 3.4 mL of ligation buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 0.2 M TCEP at pH 6.5. Then, to the mixture was added t-BuSH (215 μL, 5%, v/v), and an aqueous solution (0.75 mL) of 0.01 M VA-044 (2.5 equiv, 7.5 μmol), was incubated at 37 °C (the reaction was monitored by LC-MS). After the desulfurization was completed (30 min), the product **5** and a small amount of Se(Mob) group unexpectedly removed by-product **5'** were obtained, and the by-product **5'** increases with the reaction time. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25°C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired product **5** as a white amorphous powder (13 mg, 39.5% isolated yield). The purity and exact mass of the product was confirmed using analytical HPLC and ESI-MS, respectively.

Note: In order to inhibit Se(Mob) group further removed, after the desulfurization was completed (30 min), the mixture can be placed at 4 °C or ascorbic acid was added to quench the reaction. And the product needs to be purified in time.

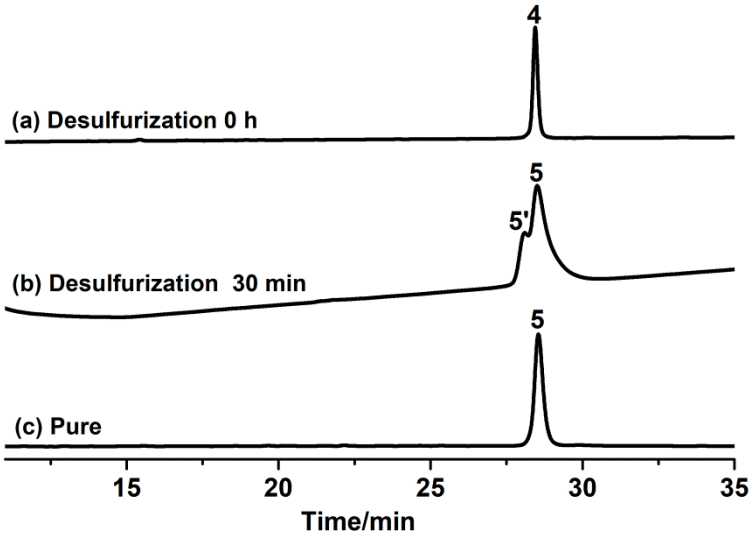


Figure S23. (A) Analytical HPLC traces of desulfurization, and the purified **5**.

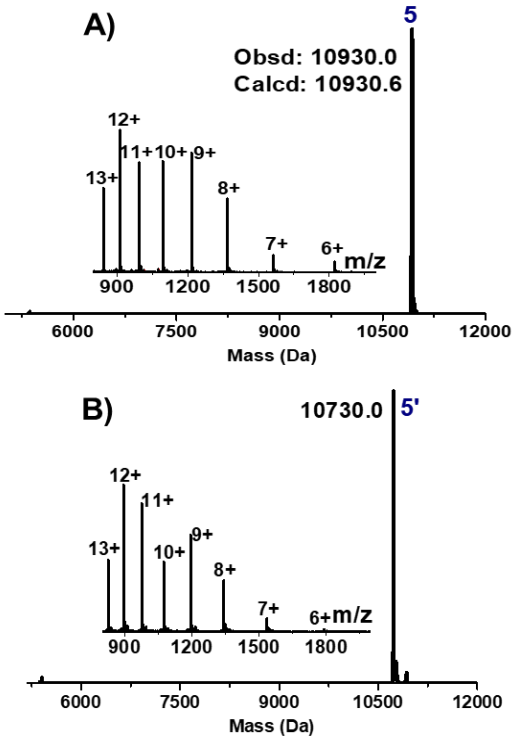


Figure S24. (A) ESI-MS analysis of **5** with the observed mass 10930.0 Da, calcd 10930.6 Da (average isotopes) (B) ESI-MS analysis of by-product **5'** with the observed mass 10730.0 Da.

5.3 Synthesis of fragment 6 by Acm and Mob deprotection

5.3.1 Removal of Acm and Mob using AgOAc method

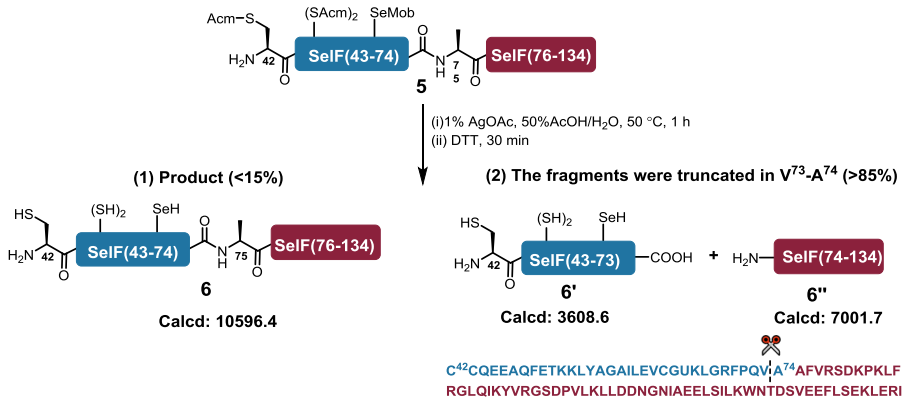


Figure S25. Removal of Acm and Mob using AgOAc method.

The peptide **5** (0.1 μmol, 1.0 equiv, 1.09 mg) was dissolved in a 50% aq. acetic acid (0.5 mL) containing 1% AgOAc, and the mixture was stirred at 50 °C for 1 h in the dark. Then 15.4 mg DTT was added to the mixture, and the formed precipitate was separated by centrifugation. The precipitate was repeatedly washed with 6 M Gdn·HCl solution and the combined supernatant (ca. 0.8 mL) was filtered and using a C4 analytical HPLC column with a gradient of 25-55% MeCN (with 0.1% TFA) in 30 min. The reaction trace and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively. We have found that AgOAc method can simultaneously remove the Acm and Mob groups, but only have little product **6**, most by-products were fragment **6'** and fragment **6''**, which were truncated in the site of V73-A74.

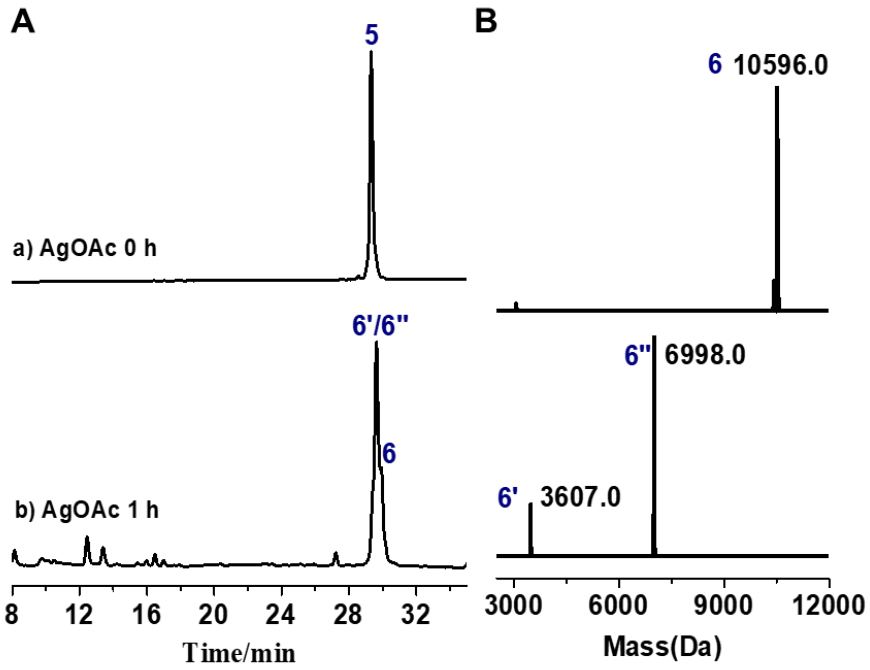


Figure S26. (A) Analytical HPLC traces of peptide **5** with AgOAc for 1 hour. (B) ESI-MS analysis of product **6** with the observed mass 10596.0 Da, calcd 10596.4 Da (average isotopes); the by-product **6'** with the observed mass 3607.0 Da and the by-product **6''** with the observed mass 6998.0 Da.

5.3.2 Removal of Acm and Mob using $PdCl_2$ method

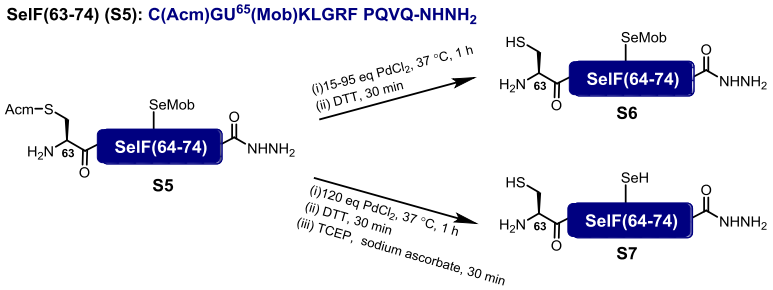


Figure S27. Schematic representation for the deprotection of peptide **S5** with different loading of $PdCl_2$.

The peptide **S5** (1.7 μmol, 1.0 equiv, 2.7 mg) was dissolved in 0.25 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 at pH 6.9 and divided into five portions. 112 mg of $PdCl_2$ dissolved in 1 mL of 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 6.9 buffer and incubated at 37 °C for 15 min to assist dissolution. Then 15 equiv, 30 equiv, 45 equiv, 65 equiv, 95 equiv and 120 equiv of $PdCl_2$ solution[23] were added to the each of the peptide solution, which were incubated at 37 °C for 1 h. Progress of the reaction was monitored by taking aliquot from the reaction mixture and treated with a small amount of DTT, followed by centrifugation. The precipitate was repeatedly washed with 6 M Gdn·HCl solution and reduced by the addition of the buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 0.2 M sodium ascorbate, pH 6.5), incubated for 30 min. And then, the reaction traces and exact mass were confirmed using a C4 analytical HPLC column with a gradient of 15-50% MeCN (with 0.1% TFA) in 30 min and ESI-MS, respectively. We have found that 15–95 equiv $PdCl_2$ method can only remove the Acm group to obtain **S6**, while the equivalent $PdCl_2$ was increased to 120 equiv, **S7** was obtained with Acm and Mob groups both removed. In all, 15 equiv $PdCl_2$ for one Acm group and 100 equiv $PdCl_2$ for one Mob group.

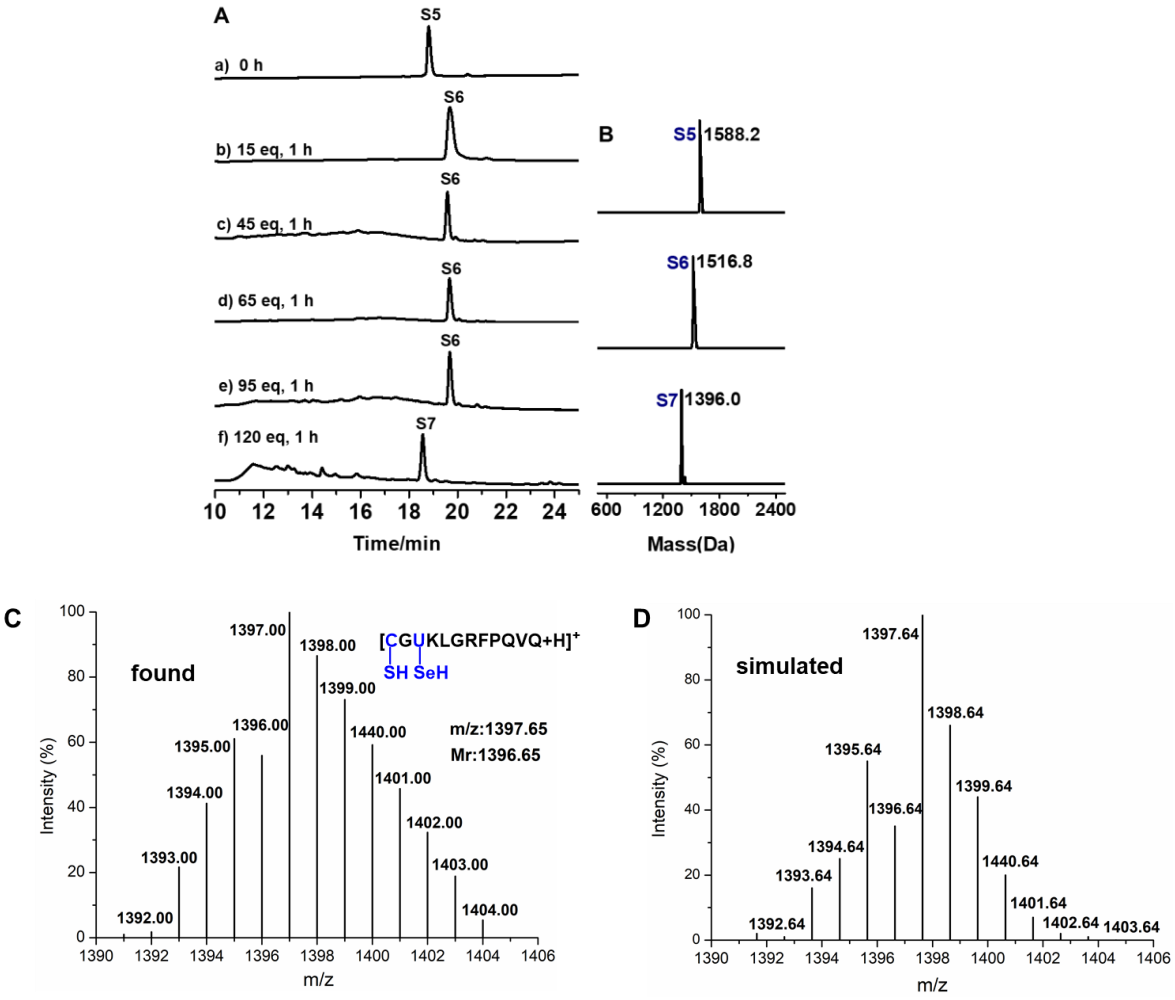


Figure S28. (A) Analytical HPLC traces of peptide **S5** with different loading of $PdCl_2$ for 1 h. (B) ESI-MS analysis of the Model peptide **S5**, the Acm removed product **S6** and both Acm and Mob groups removed product **S7**, respectively. **S5**: observed mass 1588.2 Da, calcd 1588.2 Da (average isotopes); **S6**: observed mass 1516.8 Da, calcd 1516.7 Da (average isotopes); **S7**: observed mass 1396.0 Da, calcd 1396.5 Da (average isotopes). (C-D) Identification of the free selenol/thiol form of S7 via comparison of the experimental and theoretical isotopic distributions of **S7.**

*5.3.3 Synthesis of fragment **6** by deprotection with $PdCl_2$ (on a 1 μmol scale)*

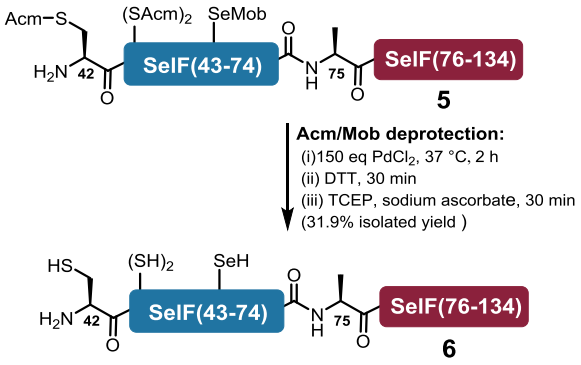


Figure S29. Schematic representation for the deprotection of fragment **5** with $PdCl_2$.

The peptide **5** (1 μmol, 1.0 equiv, 10.9 mg) was dissolved in 1.5 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 at pH 6.9. $PdCl_2$ (150 equiv, 150 μmol, 26.6 mg) dissolved in 0.2 mL of 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 6.9 buffer and incubated at 37 °C for 15 min and added to the peptide solution, which was incubated at 37 °C for 2 h. Then 231 mg DTT was added to the mixture, and the formed precipitate was separated by centrifugation. Due to the extremely low redox potential of the selenol group, Sec containing proteins can easily exist in the oxidized state such as Se–Se or Se–S dimers. To facilitate subsequent purification, the precipitate was repeatedly washed with 6 M Gdn·HCl solution and the combined supernatant (ca. 5 mL), which was reduced by the addition of 100 μL buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 0.2 M sodium ascorbate, pH 6.5) and incubated for 30 min. The mixture was filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired product **6** powder (3.4 mg, 31.9% isolated yield). The purity and exact mass of the product was confirmed using analytical HPLC and ESI-MS, respectively.

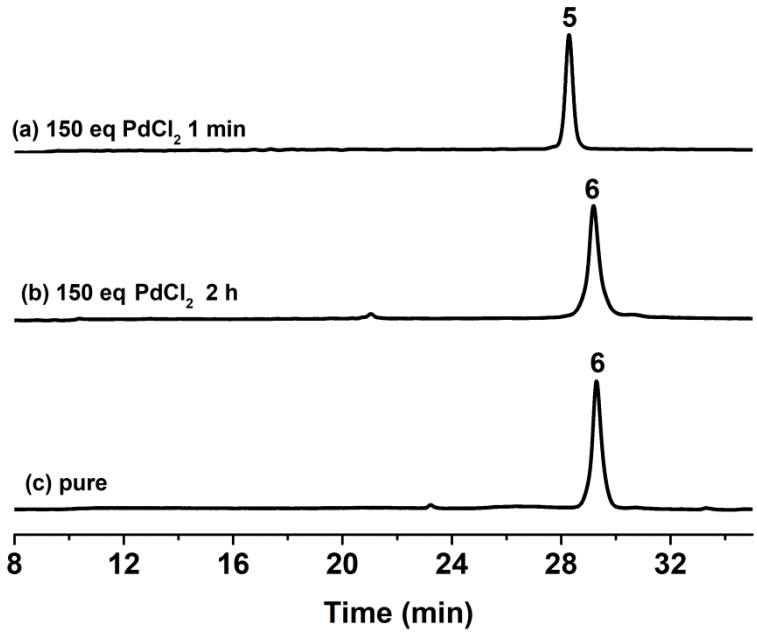


Figure S30. Analytical HPLC traces of peptide **5** with $PdCl_2$ and the purified **6**.

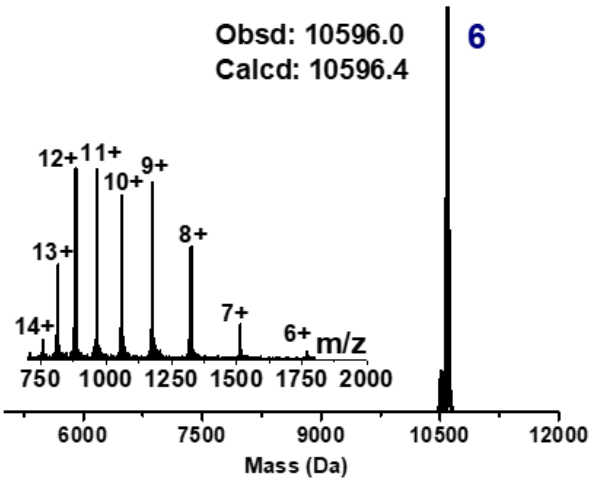


Figure S31. ESI-MS analysis of the fragment **6**, observed mass 10596.0 Da, calcd 10596.4 Da.

5.4 Synthesis of SelF(Q74A) 8 by 2nd ligation and protein folding (one-pot)

*5.4.1 Synthesis of SelF(Q74A) **8** by 2nd ligation and attempted sequential dialysis folding approach*

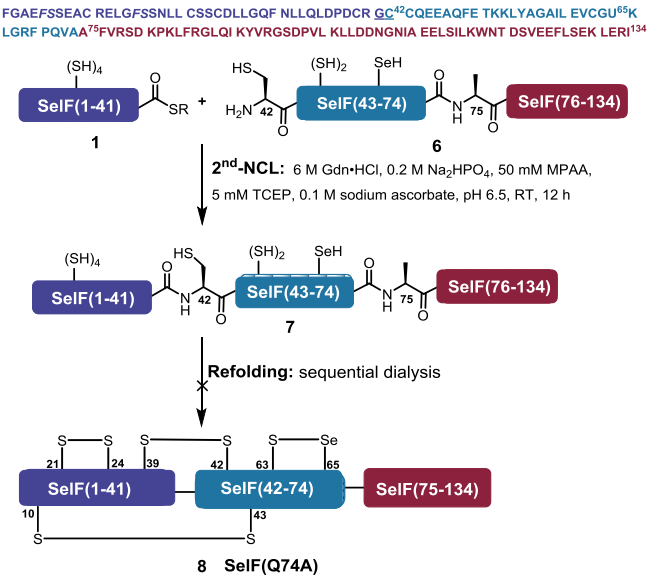


Figure S32. Synthesis of the SelF(Q74A) **8** by native chemical ligation and attempted sequential dialysis folding approach.

A series of buffers were prepared as:

I) 6 M Gdn·HCl, 0.15 M NaCl, 20 mM Tris, 1 mM GSH, pH 8.5

II) 4 M Gdn·HCl, 0.15 M NaCl, 20 mM Tris, 1 mM GSH, pH 8.5

III) 2 M Gdn·HCl, 0.15 M NaCl, 20 mM Tris, 1 mM GSH, 0.1 mM GSSG, pH 8.5

IV) 0.15 M NaCl, 20 mM Tris, 1 mM GSH, 0.1 mM GSSG, pH 8.5

The peptide-thioester **1** (0.3 µmol, 2 equiv, 1.38 mg) and Cys-peptide **6** (0.15 µmol, 1 equiv, 1.57 mg) was dissolved in 75 µL of the ligation buffer[1] (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 5 mM TCEP, 50 mM MPAA, 0.1 M sodium ascorbate, pH 6.5). The solution was incubated at RT for 12 h (confirmed by LC-MS monitoring). The ligation reaction mixture was reduced by the addition of 45 µL buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 1 M sodium ascorbate, pH 6.5) and incubated for 15 min. The 120 µL of the ligation mixture was exchanged into a 2 mL buffer containing 6 M Gdn·HCl, 0.15 M NaCl, 20 mM Tris 1 mM GSH, pH 8.5 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 10000 rpm, 4 °C, 3 times) and was added into a dialysis cassette. The dialysis cassette was transferred to the subsequent renaturation buffers II-IV (1 L) sequentially ensuring a minimum of 12 hours under each condition. Following the final renaturation, the sample was analysed by HPLC and ESI-MS, revealing no product related species.

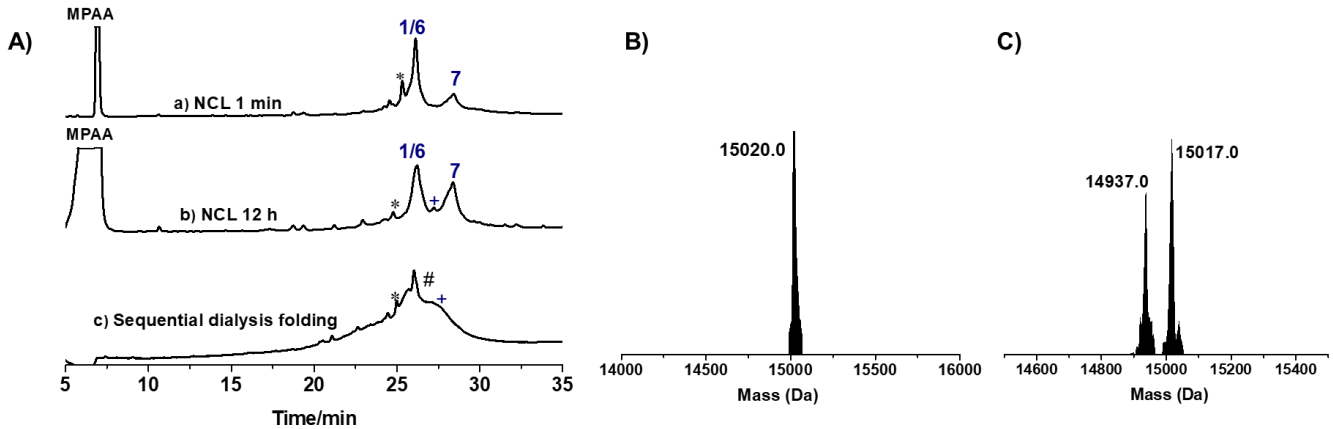


Figure S33. (A) Analytical HPLC traces of ligation and attempted refolding of SelF(Q74A) by sequential dialysis. The presence of thiolactones (indicated with *) resulting from the thiol-exchange between the C-thioester and thiol side-chains in peptide **1**. The peak # contains hydrolysis by-product of **1**, unreacted peptide **6** and the misfolded by-products, etc. The peak + denotes a small amount of deselenized by-product. (B) Mass analysis of ligation reaction product **7**, observed mass 15020.0 Da, calcd 15020.4 Da (average isotopes). (C) Mass analysis of the misfolded by-product (corresponding to the peak #) (observed mass 15017.0 Da, calcd 15012.5 Da) and deselenized by-product (corresponding to the peak + in Fig. S33A-c) (observed mass 14937.0 Da).

*5.4.2 Synthesis of SelF(Q74A) **8** by 2nd ligation and rapid dilution folding method*

(1) *Ligation followed by attempted rapid dilution folding method without arginine in the refolding buffer*

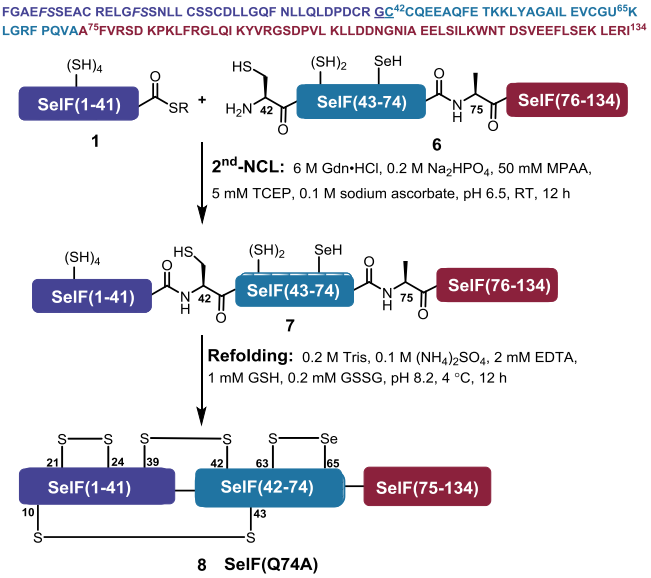


Figure S34. Synthesis of the SelF(Q74A) **8** by native chemical ligation and refolding.

The peptide-thioester **1** (0.3 µmol, 2 equiv, 1.38 mg) and Cys-peptide **6** (0.15 µmol, 1 equiv, 1.57 mg) was dissolved in 75 µL of the ligation buffer[1] (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 5 mM TCEP, 50 mM MPAA, 0.1 M sodium ascorbate, pH 6.5). The solution was incubated at RT for 12 h (confirmed by LC-MS monitoring). The ligation reaction mixture was reduced by the addition of 45 µL buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 1 M sodium ascorbate, pH 6.5) and incubated for 15 min. Then, the mixture was exchanged into a 200 µL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, 3 times). The resulting ligation mixture (~200 µL) was added dropwise to a 5 mL refolding buffer (0.2 M Tris, 0.1 M $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mM GSSG, 1 mM GSH, pH 8.2) (final protein conc. was ~0.25 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at 4 °C for 12 h. The mixture was analysed by analytical HPLC and ESI-MS, revealing small amounts of refolding products and deselenized by-product with poor refolding conversion.

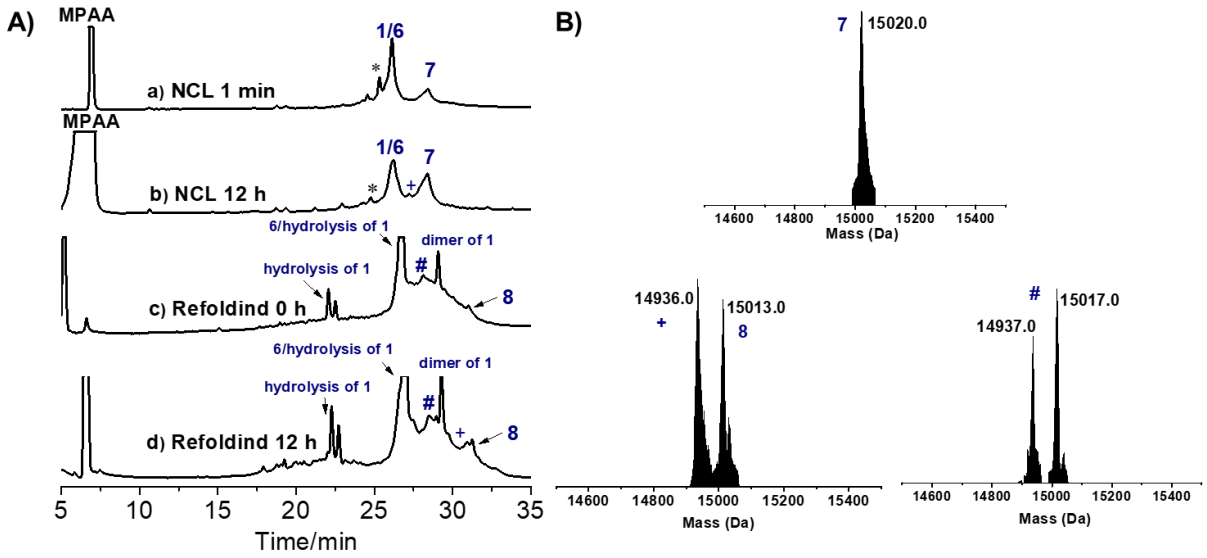


Figure S35. (A) Analytical HPLC traces of ligation and attempted refolding reaction of SelF(Q74A) **8** by rapid dilution. The presence of thiolactones (indicated with *) resulting from the thiol-exchange between the C-thioester and thiol side-chains in peptide **1**. The peak # contains the misfolded by-product and deselenized by-product. The peak + denotes the deselenized by-product. (B) Mass analysis of ligation reaction product **7**, deselenized and misfolded by-products (corresponding to the peak # in Fig. S35A-d), deselenized by-product (corresponding to the peak + in the Fig. S35A-d) and the folded product **8**, respectively. **7**: observed mass 15020.0 Da, calcd 15020.4 Da (average isotopes); the deselenized by-product (corresponding to the peak # in Fig. S35A-d): observed mass 14937.0 Da; the misfolded by-product (corresponding to the peak # in Fig. S35A-d): observed mass 15017.0 Da; the deselenized by-product (corresponding to the peak + in the Fig. S35A-d): observed mass 14936.0 Da; the folded product SelF(Q74A) **8**: observed mass 15013.0 Da, calcd 15012.5 Da.

(2) *Ligation followed by optimised rapid dilution folding method with arginine in the refolding buffer*

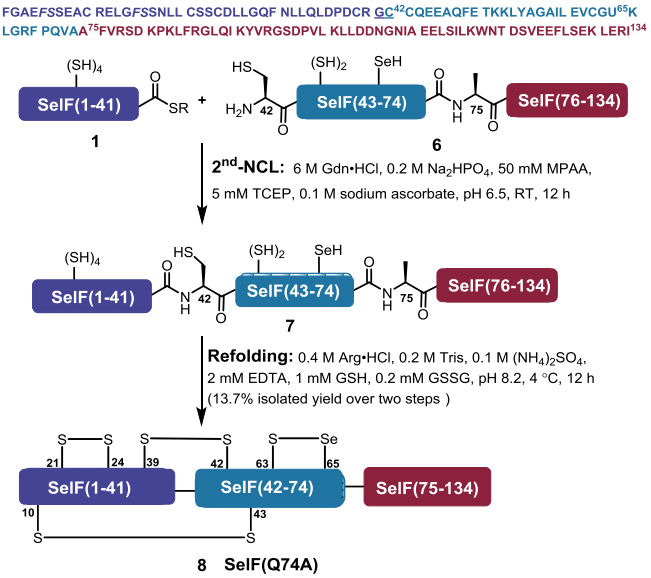


Figure S36. Synthesis of the SelF(Q74A) **8** by native chemical ligation and refolding.

The peptide-thioester **1** (0.9 μmol, 2 equiv, 4.13 mg) and Cys-peptide **6** (0.45 μmol, 1 equiv, 4.70 mg) was dissolved in 225 μL of the ligation buffer[1] (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 5 mM TCEP, 50 mM MPAA, 0.1 M sodium ascorbate, pH 6.5). The solution was incubated at RT for 12 h (confirmed by LC-MS monitoring). The ligation reaction mixture was reduced by the addition of 75 μL buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 1 M sodium ascorbate, pH 6.5) and incubated for 15 min. Then, 300 μL of the ligation mixture (in 100 μL portions) was exchanged into a 300 μL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, 0.1 M sodium ascorbate at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, 2 times). Then, the mixture (in 100 μL portions) was exchanged into a 300 μL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, 3 times).The resulting ligation mixture (~500 μL) was added dropwise to a 15 mL refolding buffer (0.4 M Arg·HCl, 0.2 M Tris, 0.1 M $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mM GSSG, 1 mM GSH, pH 8.2) (final protein conc. was ~0.25 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at 4 °C for 12 h. The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a two-step gradient: 10 to 30% MeCN in 5 min, then 30 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and lyophilized, and the desired folded protein SelF(Q74A) (**8**) powder (0.92 mg, 13.7% isolated yield over two steps) was obtained. The purity and exact mass of the SelF(Q74A) **8** was confirmed using analytical HPLC and ESI-MS, respectively.

Inductively coupled plasma mass spectrometry (ICP-MS) experiment for detection of Pd content: 0.2 mg of purified SelF(Q74A) **8** were dissolved in 3 mL of HCl/HNO_3 (3:1) for overnight and evaporate at 130 °C for 1.5 h. The resulting mixture was dissolved in 2% nitric acid. Protein solutions and a series of concentrations of Pd standard (0.5, 1, 2, 5, 10 and 20 ppb) were subjected to ICP-MS experiment in an iCAP RQ ICP-MS (Thermo Scientific). Plots and calibration curve fits were made with Origin. The detected value of 243289 corresponds to an average amount of Pd content of 0.061% in the protein.

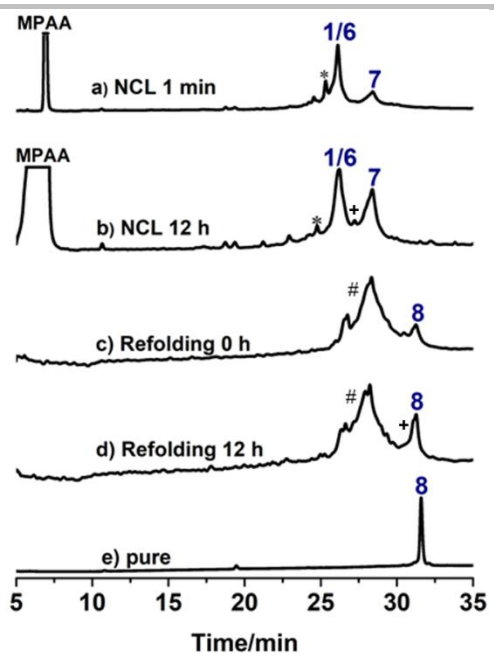


Figure S37. Analytical HPLC traces of ligation and optimised refolding reaction of SelF(Q74A) **8**. The presence of thiolactones (indicated with *) resulting from the thiol-exchange between the C-thioester and thiol side-chains in peptide **1**. The peak # contains hydrolysis by-product of **1**, unreacted peptide **6** and the misfolded by-products, etc. The peak + denotes a small amount of deselenized by-product.

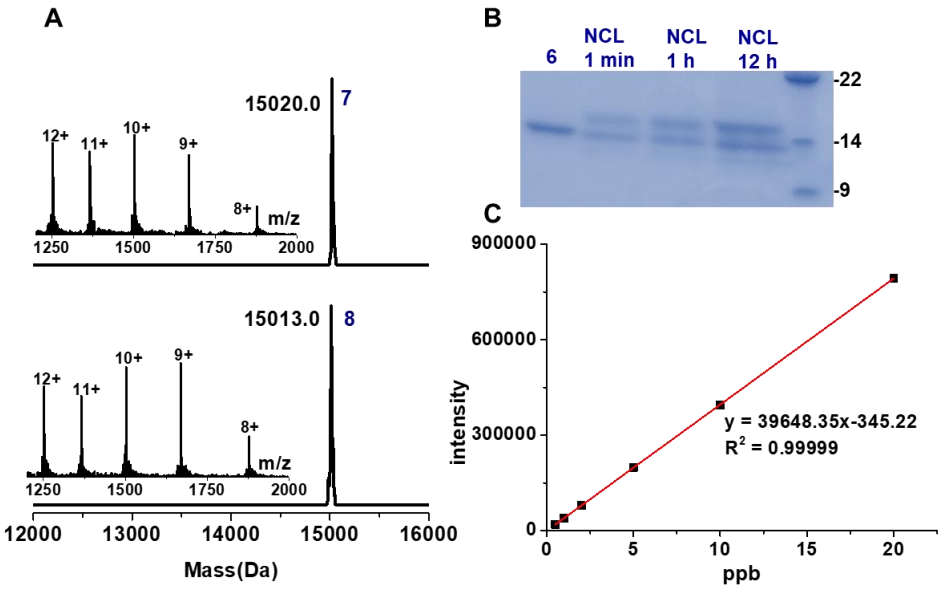


Figure S38. (A) Mass analysis of ligation reaction product **7** and its folded protein **8**, respectively. **7:** observed mass 15020.0 Da, calcd 15020.4 Da (average isotopes); **8:** observed mass 15013.0 Da, calcd 15012.5 Da (average isotopes). (B) Analytical SDS-PAGE analysis of the ligation reaction. (C) The calibration curve derived from the ICP-MS analysis of Pd standard solutions.

6 Synthesis of the Trx-like domain SelF(63–134)(Q74A) 9

6.1 Synthesis of SelF(63–74) thioester fragment S9

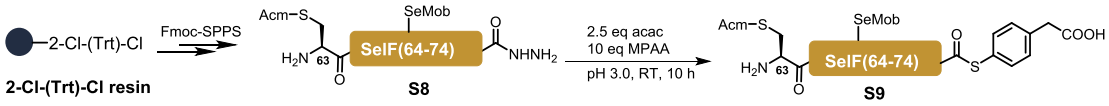


Figure S39. Synthesis of SelF (63-74) thioester **S9**.

SelF(63–74)-$NHNH_2$ (**S8)** was synthesized on 2-Cl-(Trt)-Cl resin (theoretical loading: 0.9 mmol/g) Fmoc-Ala-OH with 0.4 mmol/g loading and elongated according to standard Fmoc-SPPS protocols to afford resin-bound peptide. The peptide was cleaved using TFA/H_2O/DODT (95:2.5:2.5 v/v/v) (2–3 mL per 100 mg of resin) for 1.5 h at 4 °C. After filtration, the filtrate was concentrated by blowing with a gentle flow of N_2 about 30 min at 4 °C. Add the precooled diethyl ether to precipitate crude peptides. The resulted suspension was centrifuged (8000 rpm, 5 min, 4 °C), and the ether layer was decanted. Air-dry the peptide product in the open centrifuge tube for about 30 min to acquire the crude peptide SelF(63–74)-$NHNH_2$ (**S8)**.

The crude peptide **S8** (80 mg, assumed 100% purity) was dissolved to 40 mg/mL in 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 3.0, with 10 equiv MPAA, 2.5 equiv acac (from a 0.1 M stock in water) were added to the mixture, and the reaction mixture was stirred for 10 h to form thioester fragment SelF(63–74)-SR (**S9)**. The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 15 to 50% MeCN (with 0.1% TFA) in 25 min to obtain 16 mg of segment **S9** (0.4 g resin; 20%). The purity and exact mass of the peptide was confirmed using analytical HPLC and ESI-MS, respectively.

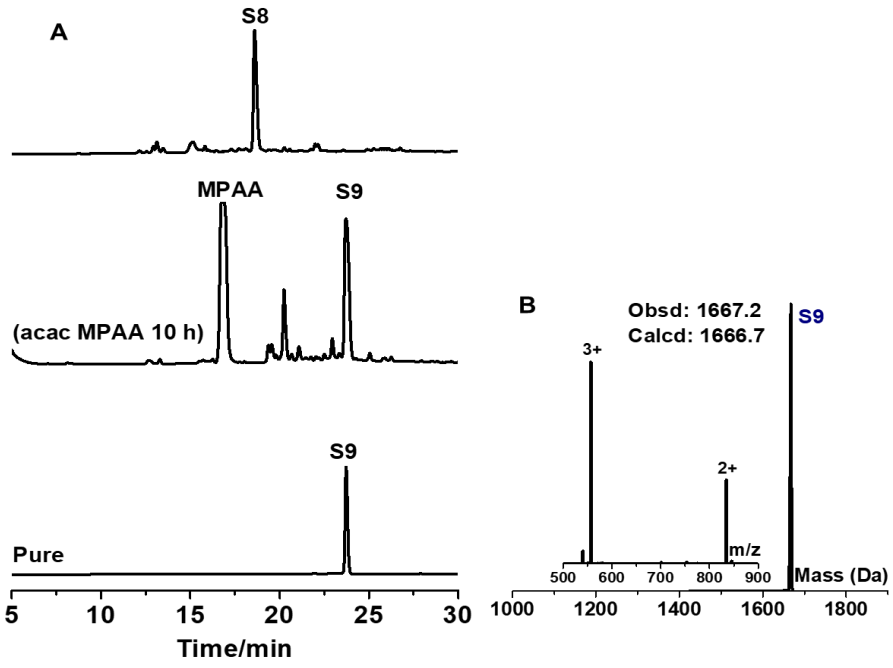


Figure S40. (A) Analytical HPLC traces of crude SelF(63–74)-$NHNH_2$ **S8** and purified SelF(63–74) thioester **S9**. (B) ESI-MS analysis of the fragment **S9**, observed mass 1667.2 Da, calcd 1666.7 Da (average isotopes).

6.2 Synthesis of fragment S10 by NCL

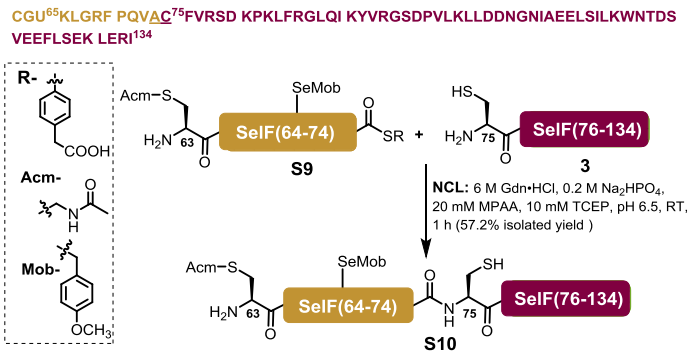


Figure S41. Synthesis of the fragment **S10** by native chemical ligation.

The peptide-thioester **S9** (3.3 μmol, 2 equiv, 5.5 mg) and Cys-peptide **3** (1.66 μmol, 1 equiv, 11.6 mg) was dissolved in 0.8 mL of ligation buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 10 mM TCEP and 20 mM MPAA at pH 6.5. The solution was incubated at RT for 1 h (confirmed by LC-MS monitoring). Then, to the ligation reaction mixture was added 5 equiv TCEP to reduce at RT for 15 min. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25 °C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired product **S10** as a white amorphous powder (8 mg, 57.2% isolated yield). The purity and exact mass of the product was confirmed using analytical HPLC and ESI-MS, respectively.

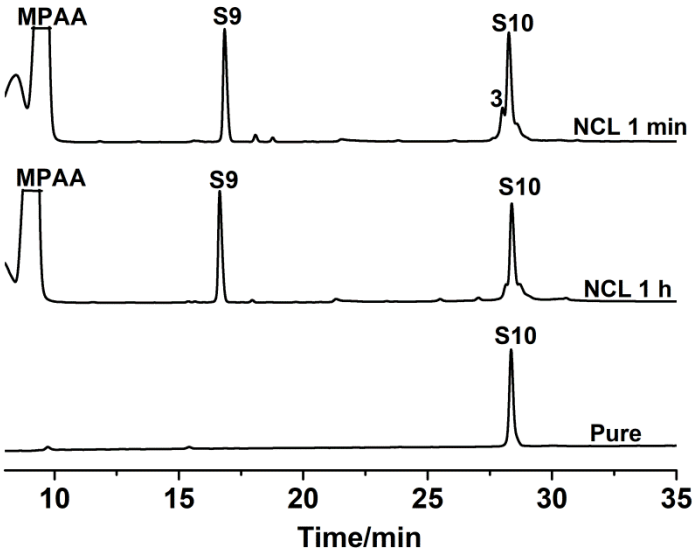


Figure S42. Analytical HPLC traces (25 to 55% MeCN (with 0.1% TFA) in 30 min, λ = 214 nm) of NCL and the purified **S10**.

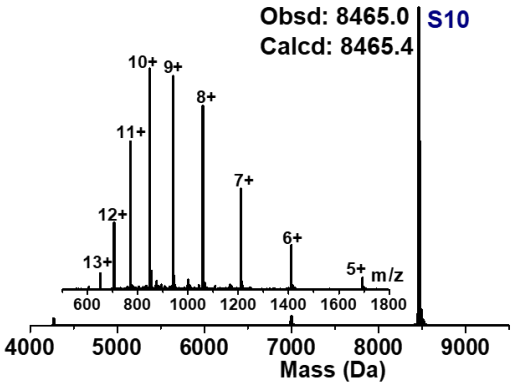


Figure S43. ESI-MS analysis of **S10** with the observed mass 8465.0 Da, calcd 8465.4 Da (average isotopes).

6.3 Synthesis of fragment S11 by desulfurization

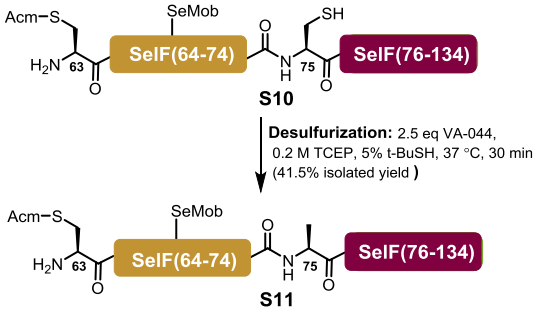


Figure S44. Synthesis of fragment **S11** by desulfurization.

The peptide **S10** (0.95 μmol, 1 equiv, 8 mg) was dissolved in 1.19 mL of ligation buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 with 0.2 M TCEP at pH 6.5. Then, to the mixture was added t-BuSH (75 μL, 5%, v/v), and an aqueous solution (238 μL) of 0.01 M VA-044 (2.5 equiv, 2.38 μmol), was incubated at 37 °C (the reaction was monitored by LC-MS). After the desulfurization was completed (30 min), the product **S11** and a small amount of Se(Mob) group unexpectedly removed by-products **S11'** (mass data not shown) were obtained. Then, the mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by preparative HPLC at 25°C with a gradient of 25 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and immediately lyophilized, affording the desired product **S11** as a white amorphous powder (3.32 mg, 41.5% isolated yield). The purity and exact mass of the product was confirmed using analytical HPLC and ESI-MS, respectively.

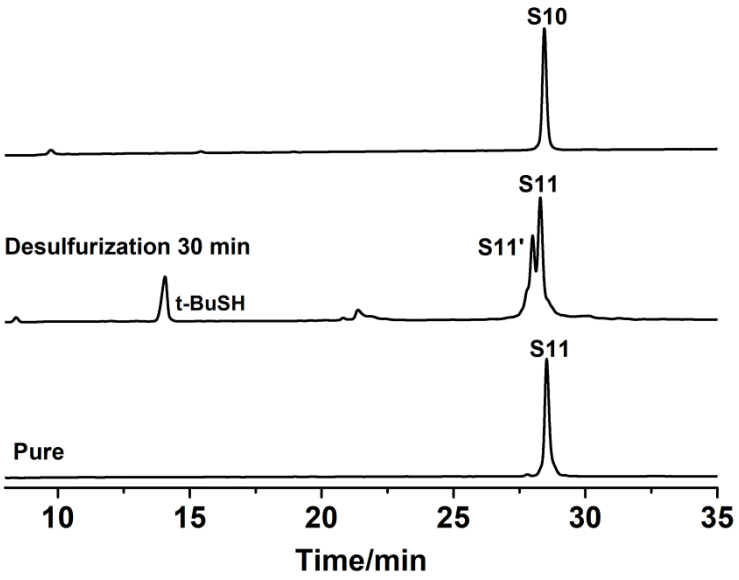


Figure S45. Analytical HPLC traces of desulfurization, and the purified **S11**.

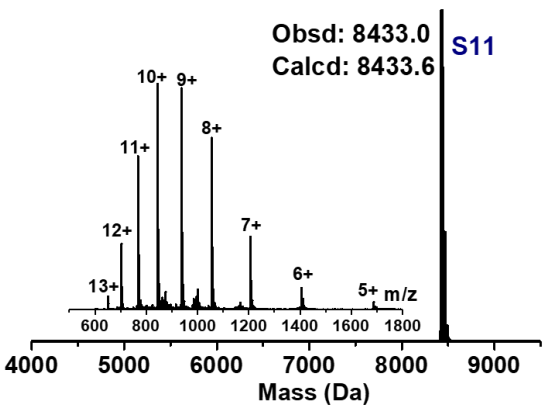


Figure S46. ESI-MS analysis of **S11** with the observed mass 8433.0 Da, calcd 8433.6 Da (average isotopes).

6.4 Synthesis of SelF(63–134)(Q74A) domain 9 by deprotection and folding (one-pot)

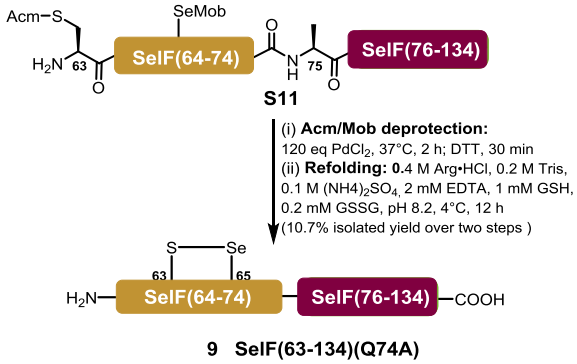


Figure S47. Schematic representation for the deprotection of fragment **9** with $PdCl_2$ and refolding.

The peptide **S11** (0.39 µmol, 1.0 equiv, 3.3 mg) was dissolved in 0.2 mL of buffer of 6 M Gdn·HCl and 0.2 M Na_2HPO_4 at pH 6.9. $PdCl_2$ (120 equiv, 47 µmol, 8.33 mg) dissolved in 0.4 mL of 6 M Gdn·HCl, 0.2 M Na_2HPO_4, pH 6.9 buffer and incubated at 37 °C for 15 min and added to the peptide solution, which was incubated at 37 °C for 2 h. Then 72 mg DTT was added to the mixture for 30 min, and the formed precipitate was separated by centrifugation. The precipitate was repeatedly washed with 6 M Gdn·HCl solution and the combined supernatant (ca. 2.5 mL), which was reduced by the addition of 50 µL buffer (6 M Gdn·HCl, 0.2 M Na_2HPO_4, 50 mM TCEP, 0.2 M sodium ascorbate, pH 6.5) and incubated for 30 min. Then, the mixture (in 500 µL portions) was exchanged into a 500 µL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, 0.1 M sodium ascorbate at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 ml, 3 K MWCO, 11000 rpm, 4 °C, 2 times). Then, the mixture was exchanged into a 500 µL buffer containing 6 M Gdn·HCl, 0.2 M Na_2HPO_4, at pH 6 (Amicon® Ultra-0.5 concentrator, 0.5 mL, 3 K MWCO, 11000 rpm, 4 °C, 4 times).The resulting mixture (~500 µL) was added dropwise to a 10 mL refolding buffer (0.4 M Arg·HCl, 0.2 M Tris, 0.1 M $(NH_4)_2SO_4$, 2 mM EDTA, 0.2 mM GSSG, 1 mM GSH, pH 8.2) (final protein conc. was ~0.3 mg/mL) at 4 °C in 30 min, and was stirred in a refrigerator at 4 °C for 12 h. The mixture was centrifuged (8000 rpm, 5 min, 4 °C), filtered and purified by semi-preparative HPLC with a two-step gradient: 10 to 30% MeCN in 5 min, then 30 to 55% MeCN (with 0.1% TFA) in 30 min to collect the desired fractions and lyophilized, and the desired folded protein SelF(63–134) (Q74A) (**9)** powder (0.35 mg, 10.7% isolated yield over two steps) was obtained. The purity and exact mass of the product was confirmed using analytical HPLC and ESI-MS, respectively.

ICP-MS experiment for detection of Pd content was repeated and the detected value of 272320 corresponds to an average amount of Pd content of 0.092% in the protein **9**.

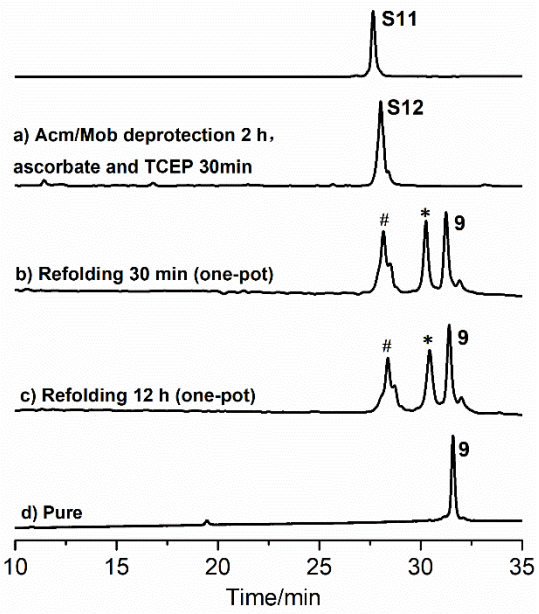


Figure S48. Analytical HPLC traces of the treatment of peptide **S11** with $PdCl_2$ affording the Acm/Mob groups removed product **S12** (a), the refolding process at 30 min (b) and 12 h (c), and the purified product **9** (d). The peak # denotes unidentified (misfolded) by-product and * denotes the dimer by-product.

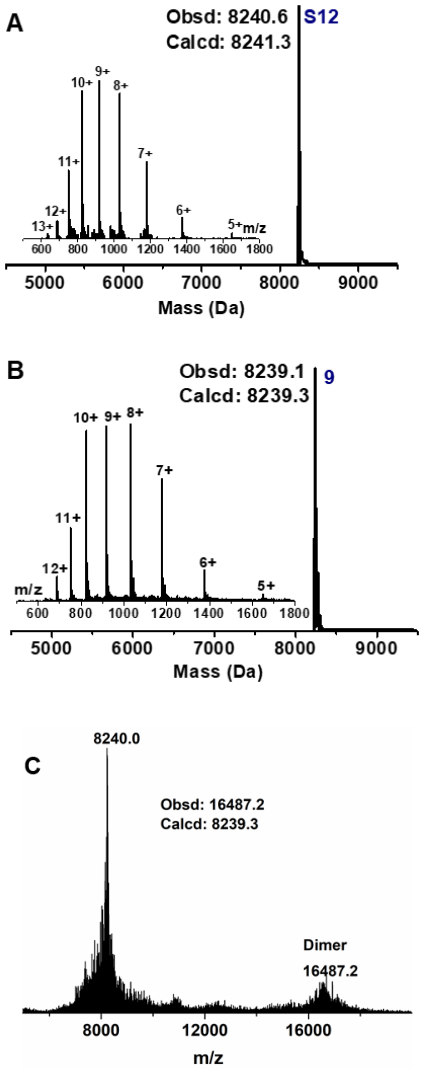


Figure S49. (A) ESI-MS analysis of Acm/Mob groups removed product **S12** with observed mass 8240.6 Da, calcd 8241.3 Da (average isotopes). (B) ESI-MS analysis of the folded protein **9** with observed mass 8239.1 Da, calcd 8239.3 Da (average isotopes). (C) MALDI-TOF analysis of the dimer by-product (corresponding to the peak * in Fig. S48), with an observed mass of 16487.2 Da.

7 Synthesis of SelF(U65C/Q74A) 10 and SelF(U65C) 11

The Cys analogues, SelF(U65C/Q74A) (**10**) and SelF(U65C) (**11**) were prepared according to the previous work[21].

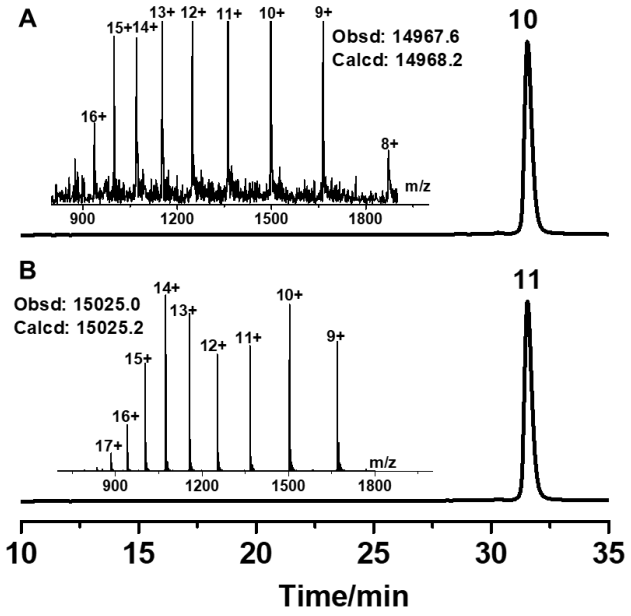


Figure S50. (A) HPLC and ESI-MS analysis of SelF(U65C/Q74A) (**10**). (B) HPLC and ESI-MS analysis of SelF(U65C) (**11**).

8 Circular dichroism (CD)

The secondary structure content of the synthetic SelF(Q74A) (**8**) and SelF(63-134)(Q74A) (**9**) using far-UV CD spectroscopy (200 to 260 nm). Spectra were recorded on a Chirascan™-Plus Circular Dichroism Spectrometer (Applied Photophysics Ltd, U.K.), using a quartz cuvette with a path length of 0.1 cm, and obtained by averaging 3 wavelength scans in 1 nm steps, with a signal averaging time of 1 s and a bandwidth of 1 nm. Each purified protein was dissolved separately in NH_4HCO_3 buffer. Measuring conditions: SelF(Q74A) (**8**) conc.: ~30 µM; SelF(63-134) (Q74A) (**9**) conc.: ~15 µM; Buffer: 100 mM NH_4HCO_3, pH 8.

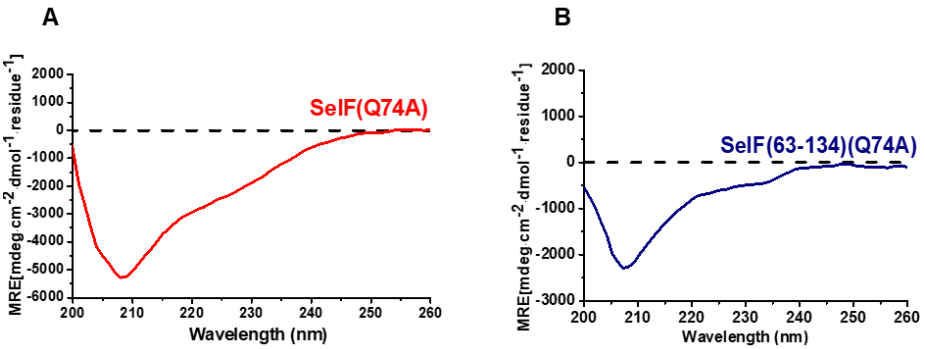


Figure S51. (A) CD spectra of the synthetic SelF(Q74A) (**8**) (B) CD spectra of the SelF(63–134)(Q74A) (**9**).

9 Enzyme digestion

SelF(Q74A) (**8**) was dissolved in a solution of 25 mM NH_4HCO_3 (1 mg/mL, 100 µL). Trypsin (0.5 mg/mL, 10 µL) was firstly added to the mixture to digest the protein. The reaction was carried out at 37 °C for 2 h. Next, chymotrypsin (0.16 mg/mL, 11 µL) was added to the reaction mixture for further digestion. The reaction was carried out at 30 °C for 2 h. Finally, the enzymatic reaction was analysed by LC-MS. As shown in Figure S52, following trypsin and chymotrypsin treatment of the protein SelF(Q74A), **8**, a peptide fragment bearing the Cys63–Sec65 selenenylsulfide bond can be clearly observed mass 1108.5 Da (calcd mass 1108.5 Da); another peptide fragment containing Cys21–Cys24 can be clearly observed mass 1583.3 Da (calcd mass 1583.7 Da), which agrees well with the reported structure of a mutant protein—SelF(U65C) (**11**).[21] Based on the previous work on mutant research,[21] we infer the remaining the other two disulfide bonds were established as Cys10–Cys43 and Cys39–Cys42, respectively.

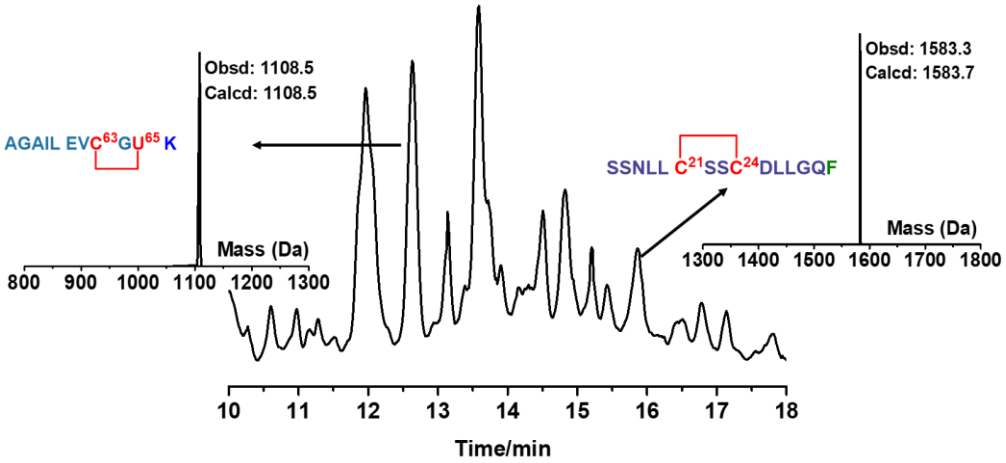


Figure S52. LC-MS analysis of the trypsin and chymotrypsin digest of SelF(Q74A) (**8**).

10 Measurements of protein reduction potential determined by analytic HPLC

10.1 Measurements of SelF(U65C) reduction potential determined by analytic HPLC

The fully oxidized protein SelF(U65C) (20 μg) was dissolved in a buffer (0.5 mL, 100 mM NH_4HCO_3, 1 mM EDTA, pH 7.0) with different ratios of GSH/GSSG[24, 25] ((a) 20 mM GSH/0.1 mM GSSG; (b) 10 mM GSH/0.1 mM GSSG; (c) 10 mM GSH/0.2 mM GSSG; (d) 10 mM GSH/0.4 mM GSSG; (e) 10 mM GSH/0.5 mM GSSG; (f) 10 mM GSH/1mM GSSG; (g) 20 mM GSH/4 mM GSSG; (h) 10 mM GSH/2.5 mM GSSG; (i) 5 mM GSH/10 mM GSSG; (j) 10 mM GSH/10 mM GSSG); (k) 0.5 mM GSH/10 mM GSSG; (l) 20 mM GSSG, respectively). The GSH/GSSG ratio was used to poise the reduction potential of the buffer. The protein solutions were degassed to prevent excessive oxidation. The samples were incubated at 25 °C for 15 h to ensure full equilibration. The equilibrated reaction mixtures were quenched by adding 10 μL of 20% TFA and mixed immediately. The quenched reaction mixture was then spun for 10 min at 8000 rpm. The supernatant was injected into analytical HPLC (C4 column) using a gradient of 30-55% MeCN (with 0.1% TFA) in 30 min. The percentage of reduced and oxidized protein was determined by integrating the relevant peak area.

The reaction of SelF(U65C) with the GSSG/GSH redox pair (Reaction I) and the corresponding equilibrium constant (K_{eq}) are given in Reaction I:

$$\mathrm{SelF(U65C)_{red}} + 4\mathrm{GSSG} \longleftrightarrow \mathrm{SelF(U65C)_{ox}} + 8\mathrm{GSH} \qquad \text{(Reaction I)}$$

$$K_{eq} = {[\mathrm{SelF(U65C)}_{ox}][GSH]^8}\Big/{[\mathrm{SelF(U65C)}_{red}][GSSG]^4} \qquad \text{(Eq.1)}$$

The reduction potential of SelF(U65C) at pH 7.0 and 25°C [E'_0(SelF(U65C))] was then calculated from the Nernst equation (Eq.2):

$$E'_0(SelF(U65C)) = E'_0(GSH/GSSG) - \frac{RT}{nF} \times \ln(k_{eq}) \qquad \text{(Eq.2)}$$

Where E'_0(GSH/GSSG) = −240 mV (pH 7.0 and 25 °C), R is the gas constant (8.315 $J \cdot K^{-1} \cdot mol^{-1}$), T is the absolute temperature (298 K), n is the number of electrons transferred in the reaction (n = 8), and F is the Faraday constant (9.649×10^4 $C \cdot mol^{-1}$). Plots and curve fits were made with Origin.

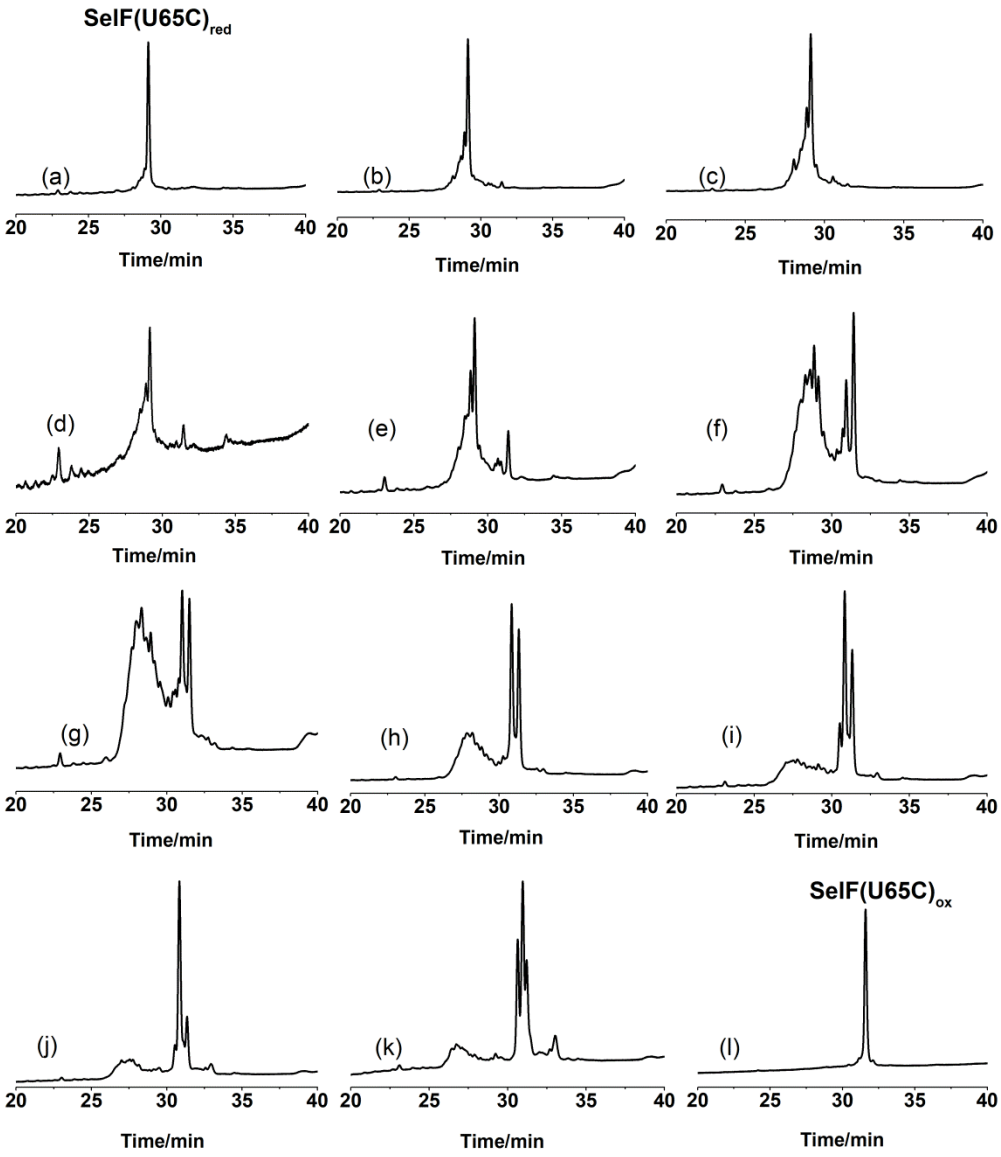


Figure S53. Analytical HPLC traces of the SelF(U65C) following incubation in various redox buffers. Each panel shows the reduced and oxidized protein. The calculated redox potential of SelF(U65C) under each condition: (a) –274 mV, (b) -253 mV, (c) -243 mV, (d) -230 mV, (e) -225 mV, (f) -214 mV, (g) -211 mV, (h) -197 mV, (i) -160 mV, (j) -118 mV, (k) -99 mV, respectively.

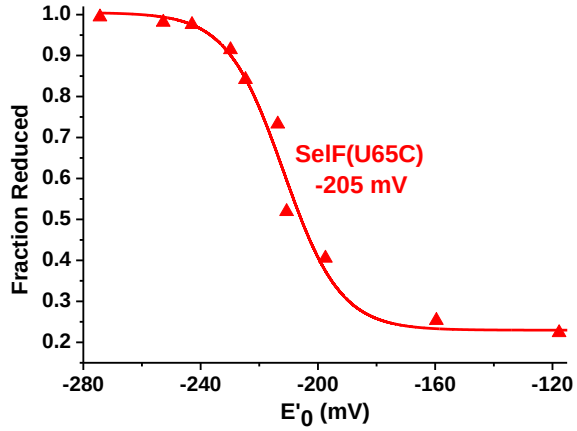


Figure S54. Determination of reduction potentials of SelF(U65C) in GSH/GSSG redox buffers and its reduction potential of −205 mV.

10.2 Measurements of SelF(Q74A) reduction potential determined by analytic HPLC

The fully oxidized protein SelF(Q74A) (20 μg) was dissolved in a buffer (0.5 mL, 100 mM NH_4HCO_3, 1 mM EDTA, pH 7.0) with different ratios of GSH/GSSG[24, 25] ((a) 10 mM GSH/0.01 mM GSSG; (b) 20 mM GSH/0.1 mM GSSG; (c) 20 mM GSH/0.2 mM GSSG; (d) 10 mM GSH/0.1 mM GSSG; (e) 10 mM GSH/0.15 mM GSSG; (f) 10 mM GSH/0.2 mM GSSG; (g) 10 mM GSH/0.3 mM GSSG; (h) 10 mM GSH/0.5 mM GSSG; (i) 10 mM GSH/0.9 mM GSSG; (j) 10 mM GSH/2 mM GSSG; (k) 10 mM GSH/10 mM GSSG; (l) 5 mM GSH/10 mM GSSG, respectively. The GSH/GSSG ratio was used to poise the reduction potential of the buffer. The protein solutions were degassed to prevent excessive oxidation. The samples were incubated at 25 °C for 15 h to ensure full equilibration. The equilibrated reaction mixtures were quenched by adding 10 μL of 20% TFA were mixed immediately. The quenched reaction mixture was then spun for 10 min at 8000 rpm. The supernatant was injected into analytical HPLC (C4 column) using a gradient of 30-55% MeCN (with 0.1% TFA) in 30 min. The percentage of reduced and oxidized protein was determined by integrating the relevant peak area.

The reaction of SelF(Q74A) with the GSSG/GSH redox pair (Reaction II) and the corresponding equilibrium constant (K_{eq}) are given in Reaction II:

$$\mathrm{SelF(Q74A)_{red}} + 4\mathrm{GSSG} \longleftrightarrow \mathrm{SelF(Q74A)_{ox}} + 8\mathrm{GSH} \quad \text{(Reaction II)}$$

$$K_{eq} = {[\mathrm{SelF(Q74A)}_{ox}][GSH]^8}\Big/{[\mathrm{SelF(Q74A)}_{red}][GSSG]^4} \quad \text{(Eq.3)}$$

The reduction potential of SelF(Q74A) at pH 7.0 and 25°C [E_0'(SelF(Q74A))] was then calculated from the Nernst equation (Eq.4):

$$E_0'(SelF(Q74A)) = E_0'(GSH/GSSG) - \frac{RT}{nF} \times \ln(k_{eq}) \quad \text{(Eq.4)}$$

Where E_0'(GSH/GSSG) = −240 mV (pH 7.0 and 25 °C), R is the gas constant (8.315 $J \cdot K^{-1} \cdot mol^{-1}$), T is the absolute temperature (298 K), n is the number of electrons transferred in the reaction (n = 8), and F is the Faraday constant (9.649×10^4 $C \cdot mol^{-1}$). Plots and curve fits were made with Origin.

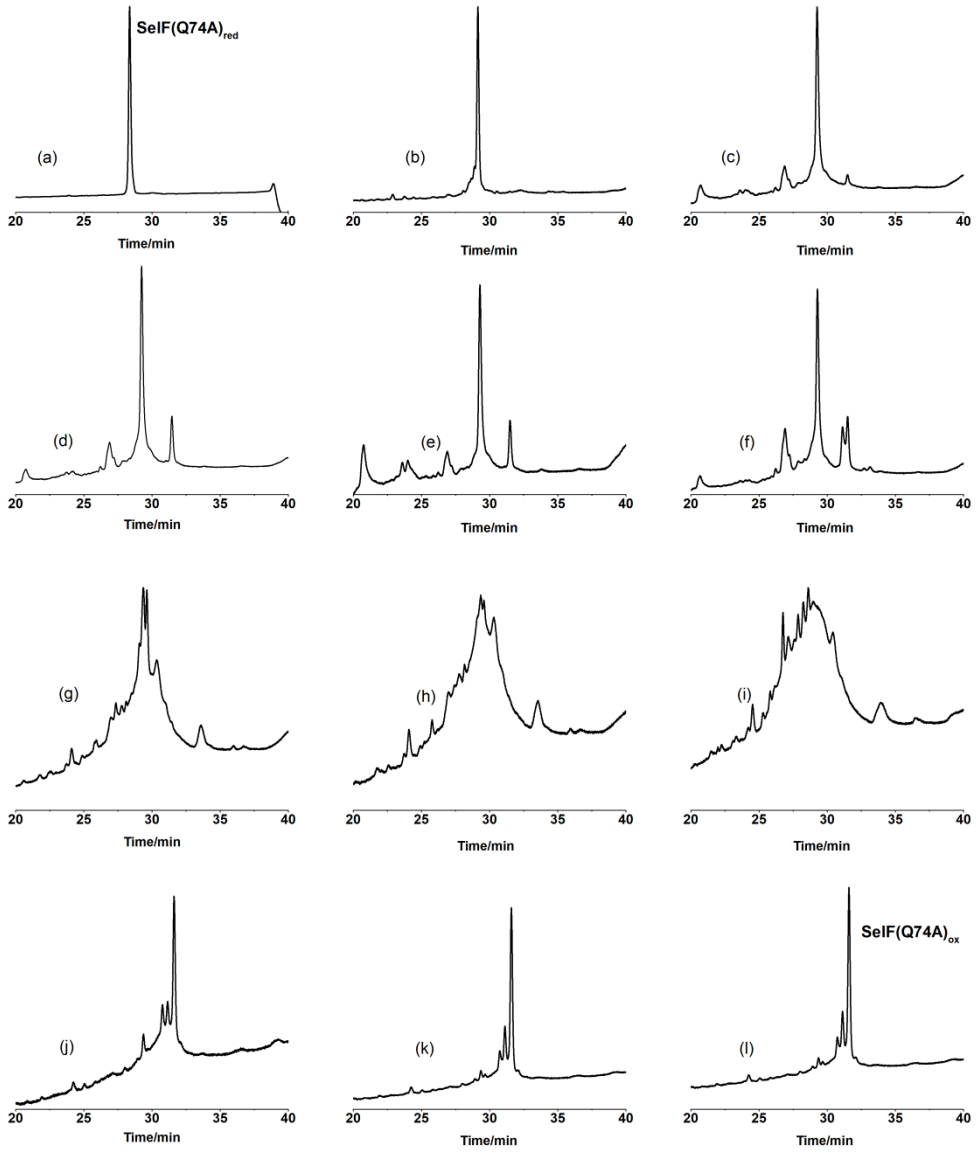


Figure S55. Analytical HPLC traces of the SelF(Q74A) following incubation in various redox buffers. Each panel shows the reduced and oxidized protein. The calculated redox potential of SelF(Q74A) under each condition: (a) -284 mV, (b) -269 mV, (c) -260 mV, (d) -249 mV, (e) -241 mV, (f) -233 mV, (g) -226 mV, (h) -219 mV, (i) -210 mV, (j) -197 mV, (k) -174 mV, (l) -157 mV, respectively.

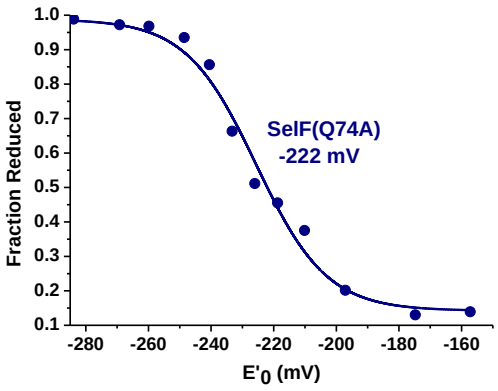


Figure S56. Determination of reduction potentials of SelF(Q74A) in GSH/GSSG redox buffers and its reduction potential of −222 mV.

11 Turbidimetric assay of insulin disulfide reduction

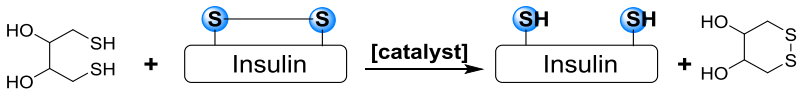


Figure S57. Protein catalysed reduction of insulin by DTT.

The follow stock solutions were prepared freshly before use in the dissolving buffer (0.1 M Na_2HPO_4, 0.1 M NH_4HCO_3, 2 mM EDTA, pH 7.0): Bovine insulin, 1.5 mg/mL; DTT, 100 mM; Catalyst protein of SelF(Q74A) (**8**), SelF(63–134)(Q74A) (**9**), SelF(U65C/Q74A) (**10**) or SelF(U65C) (**11**), 10 mM. For the assay, 300 µL of insulin and 120 µL of catalyst protein were mixed in a cuvette, and the dissolving buffer was added to afford a total volume of 600 µL. The reaction was initiated by the addition of DTT (2 µL), after thorough mixing, the absorbance at 650 nm was measured every 0.5 min. Generally, no further mixing of the cuvettes was done during the assay over 80 minutes. In all experiments, the nonenzymatic reduction of insulin by DTT was recorded in a control cuvette without catalyst protein.[24, 26]

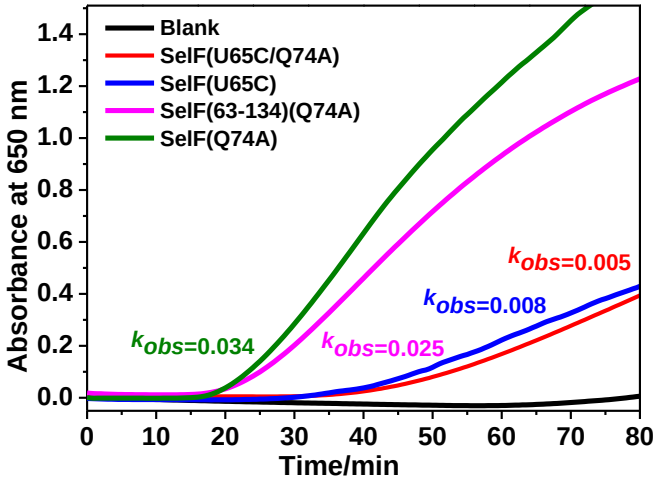


Figure S58. Spectroscopic analysis of the reductase activity assays. The ability of SelF proteins to reduce insulin's intermolecular disulfide bond is monitored by recording the increasing turbidity caused by insulin's chain B aggregation. The reaction mixture includes insulin and DTT. The reaction was recorded with 2 µM of SelF(Q74A), SelF(63–134)(Q74A), SelF(U65C/Q74A), SelF(U65C) and without additional enzymes.

To probe the effect of the concentration on the reductase activity, the above experiments were repeated and compared in the presence of 1, 2 and 4 µM SelF.

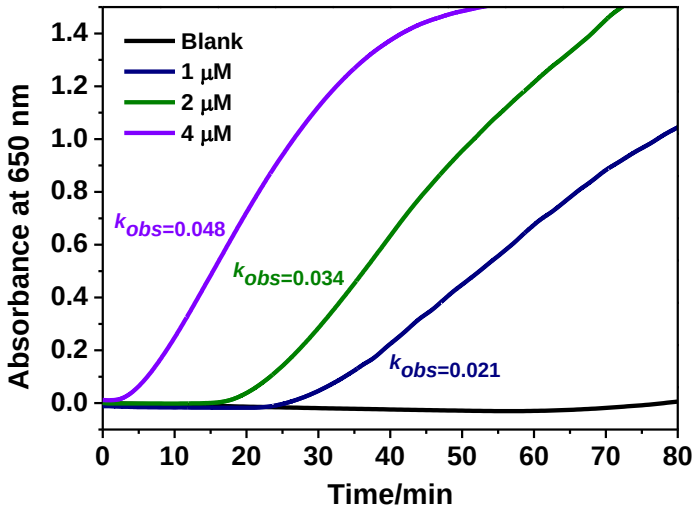


Figure S59. Spectroscopic analysis of the reductase activity assays with different concentrations of SelF(Q74A). The reaction mixture includes insulin and DTT. The reaction was recorded with 4 µM, 2 µM and 1 µM SelF(Q74A), and without additional enzymes.

12 Disulfide isomerase activity assays

12.1 Preparation of scrambled RNase A

The following procedure was modified from literature.[27] Scrambled RNase A was prepared by incubating RNase A with an equimolar amount of DTT (2 equiv) for 36 h under anaerobic conditions in 100 mM Tris, 0.3 mM EDTA and 6 M Gdn·HCl, pH 8.0. This solution was then opened to air and stirred in the dark at room temperature until fewer than 0.1 thiol per RNase A molecule remained and the concentration of sulfhydryl groups was determined using DTNB. The protein was exchanged into a solution of 50 mM NH_4HCO_3, 1 mM EDTA, pH 7.5 (Amicon® Ultra-0.5 concentrator, 15 mL, 3 K MWCO, 11000 rpm, 4 °C, 5 times). Scrambled RNase A was stored at –20 °C until it was used. Analytical HPLC (C4 column) trace of reduced and misfolded RNase A with a gradient of 20-55% MeCN (with 0.1% TFA) in 30 min.

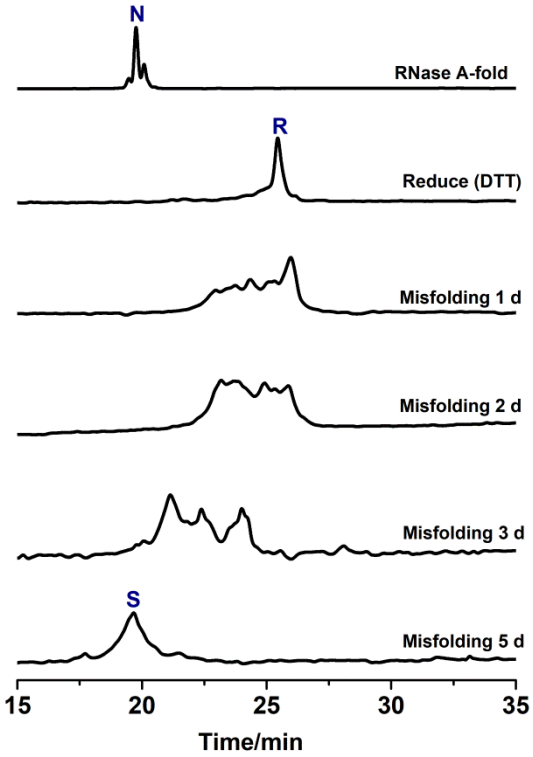


Figure S60. Analytical HPLC traces of the reduced and misfolded RNase A (the folded RNase A was marked N; reduced RNase A was marked R; scrambled RNase A was marked S).

12.2 Refolding of scrambled RNase A assays with SelF(Q74A) or SelF(U65C)

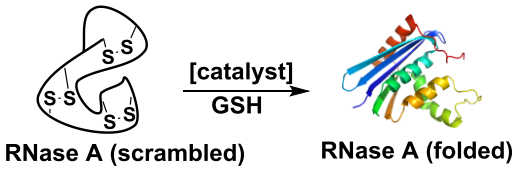


Figure S61. Protein catalysed refolding of scrambled RNase A.

The following procedure was modified from literature.[28] The buffer solution (0.1 M NH_4HCO_3, 1 mM EDTA, pH 7.5) deoxygenated by bubbling nitrogen gas for 0.5 h was used for preparation of all mixture solution. The concentration of scrambled RNase A (S) solution was determined by UV absorbance at 280 nm using the molar extinction coefficient (ε_{280} = 8600 $M^{-1}\cdot cm^{-1}$), and the concentration was adjusted to 75 μM with the buffer solution at pH 7.5. The resulting scrambled RNase A solution (400 μL) was mixed with SelF(Q74A), SelF(U65C) (50 μL of 60 μM stock solution) or no catalyst protein (blank), and a buffer solution (0.1 M NH_4HCO_3, 1 mM EDTA, pH 7.5) was added to give a final volume of 500 μL. The SS-reshuffling reaction was then initiated by the addition of 4 μL GSH (100 mM). The sample solution was incubated in 25.0 °C for 2 h, respectively, and analysed by HPLC (C4 column) with a gradient of 20–45% MeCN (with 0.1% TFA) in 50 min.

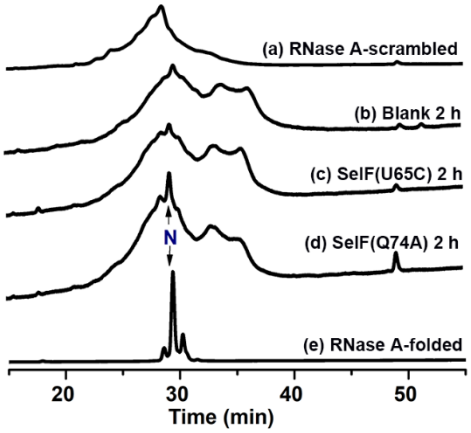


Figure S62. Isomerase activity assay via the refolding of the scrambled RNase A as followed by analytical HPLC. [RNase A] = 60 μM; [GSH] = 1 mM; [catalyst] = 7.5 μM.

References

1 L. Dery, P. S. Reddy, S. Dery, R. Mousa, O. Ktorza, A. Talhami and N. Metanis, *Chem. Sci.*, 2017, **8**, 1922–1926.

2 N. J. Mitchell, J. Sayers, S. S. Kulkarni, D. Clayton, A. M. Goldys, J. Ripoll-Rozada, P. J. Barbosa Pereira, B. Chan, L. Radom and R. J. Payne, *Chem*, 2017, **2**, 703–715.

3 B. Eckenroth, K. Harris, A. A. Turanov, V. N. Gladyshev, R. T. Raines and R. J. Hondal, *Biochemistry*, 2006, **45**, 5158–5170.

4 N. Metanis, E. Keinan and P. E. Dawson, *J. Am. Chem. Soc.*, 2006, **128**, 16684–16691.

5 G. Casi, G. Roelfes and D. Hilvert, *ChemBioChem*, 2008, **9**, 1623–1631.

6 R. J. Hondal, B. L. Nilsson and R. T. Raines, *J. Am. Chem. Soc.*, 2001, **123**, 5140–5141.

7 K. Medini, P. W. R. Harris, A. Menorca, K. Hards, G. M. Cook and M. A. Brimble, *Chem. Sci.*, 2016, **7**, 2005–2010.

8 R. Quaderer, A. Sewing and D. Hilvert, *Helv. Chim. Acta*, 2001, **84**, 1197–1206.

9 N. Metanis and D. Hilvert, *Angew. Chem., Int. Ed.*, 2012, **51**, 5585–5588.

10 N. Metanis and D. Hilvert, *Chem. Sci.*, 2015, **6**, 322–325.

11 S. M. Berry, M. D. Gieselman, M. J. Nilges, W. A. van der Donk and Y. Lu, *J. Am. Chem. Soc.* , 2002, **124**, 2084–2085.

12 Z. Zhao, D. Shimon and N. Metanis, *J. Am. Chem. Soc.*, 2021, **143**, 12817–12824.

13 Z. Zhao and N. Metanis, *Angew. Chem., Int. Ed.*, 2019, **58**, 14610–14614.

14 T. Takei, T. Ando, T. Takao, Y. Ohnishi, G. Kurisu, M. Iwaoka and H. Hojo, *Chem. Commun.*, 2020, **56**, 14239–14242.

15 M. P. Malakhov, M. R. Mattern, O. A. Malakhova, M. Drinker, S. D. Weeks and T. R. Butt, *J. Struct. Funct. Genomics*, 2004, **5**, 75–86.

16 A. L. Adams, B. Cowper, R. E. Morgan, B. Premdjee, S. Caddick and D. Macmillan, *Angew. Chem., Int. Ed.*, 2013, **52**, 13062–13066.

17 D. T. Flood, J. C. J. Hintzen, M. J. Bird, P. A. Cistrone, J. S. Chen and P. E. Dawson, *Angew. Chem., Int. Ed.*, 2018, **57**, 11634–11639.

18 L. A. Wessjohann, A. Schneider, G. N. Kaluđerović and W. Brandt, *Mol. Diversity*, 2013, **17**, 537–545.

19 K. A. Jenny, E. J. Ste.Marie, G. Mose, E. L. Ruggles and R. J. Hondal, *J. Pept. Sci.*, 2019, **25**, e3209.

20 J. L. Lauer, C. G. Fields and G. B. Fields, *Lett. Pept. Sci.*, 1995, **1**, 197–205.

21 P. Liao and C. He, *Front. Chem.*, 2021, **9**, 735149.

22 Q. Wan and S. J. Danishefsky, *Angew. Chem., Int. Ed.*, 2007, **46**, 9248–9252.

23 S. K. Maity, M. Jbara, S. Laps and A. Brik, *Angew. Chem., Int. Ed.*, 2016, **55**, 8108–8112.

24 J. Liu, F. Li and S. Rozovsky, *Biochemistry*, 2013, **52**, 3051–3061.

25 A. D. Ferguson, V. M. Labunskyy, D. E. Fomenko, D. Araç, Y. Chelliah, C. A. Amezcua, J. Rizo, V. N. Gladyshev and J. Deisenhofer, *J. Biol. Chem.*, 2006, **281**, 3536–3543.

26 A. Holmgren, *J. Biol. Chem.*, 1979, **254**, 9627–9632.

27 P. C. Rancy and C. Thorpe, *Biochemistry*, 2008, **47**, 12047–12056.

28 K. Arai, H. Ueno, Y. Asano, G. Chakrabarty, S. Shimodaira, G. Mugesh and M. Iwaoka, *ChemBiochem*, 2018, **19**, 207–211.