Supporting Information

Selenocysteine in Native Chemical Ligation and Expressed Protein Ligation

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General Methods. Reagents and solvents were obtained from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO). Solvents were dried and distilled by using standard procedures.[1] Reactions were monitored by thin-layer chromatography using Whatman TLC plates (AL SIL G/UV) with visualization by illumination with ultraviolet light or staining with I_2.

Instruments. NMR spectra were obtained with Bruker AC-300 and Varian UNITY-500 spectrometers. Mass spectra were obtained with Perkin Elmer Sciex API 365 electrospray ionization (ESI) and Bruker Biflex III matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) instruments at the University of Wisconsin Biotechnology Center. Ultraviolet and visible absorbance was recorded with a Cary Model 3 UV/VIS spectrophotometer from Varian equipped with a Varian temperature controller.

Synthesis of Disodium Diselenide. A 1 M solution of Na_2Se_2 was prepared by the procedure of Klayman and Griffin.[2] Elemental selenium (4.5 g, 56 mmol) was added to water (25 mL) in a stoppered three-necked flask with magnetic stirring. Sodium borohydride (4.5 g, 119 mmol) dissolved in water (25 mL) was added dropwise to the slurry of elemental selenium. The flask

(1) Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon: Oxford, 1988.

was chilled in an ice bath to prevent boiling. After all of the sodium borohydride had been added and the solution had become colorless, additional elemental selenium (4.5 g, 56 mmol) was added to the solution. The solution was then reddish brown, which is characteristic of disodium diselenide. The flask was then stoppered and flushed with Ar(g).

Synthesis of Selenocystine. Selenocystine was synthesized essentially by the procedure of Tanaka and Soda.[3] β-Chloro-L-alanine (5.0 g, 31 mmol) was dissolved in water (40 mL), and the pH of the resulting solution was adjusted to 9.0. The resulting solution was then added dropwise over 30–60 min to the solution of Na_2Se_2 through one of the septa in the three-necked flask. The mixture was stirred under Ar(g) at 37 °C for 12–16 h. The solution was then acidified with concentrated HCl until vigorous reaction stops, and hydroxylamine (0.33 g, 9.7 mmol) was added to reduce remaining elemental selenium. Additional concentrated HCl was added until there was no more vigorous reaction with the solution. The resulting solution was flushed for at least 1 h with Ar(g). (The hydrogen selewnide exhaust was trapped with a saturated aqueous solution of lead acetate.) The solution was then filtered, and the pH of the yellow filtrate was adjusted to 6–6.5. The concentration of selenocystine was high, and a yellow precipitate formed immediately. The precipitate was dissolved in the smallest volume possible of 2 N HCl. The resulting dark yellow solution was filtered, and the pH of the filtrate was increased to 6–6.5 by the addition of 10 N NaOH. Selenocystine was isolated as a bright yellow crystalline solid in 60% yield (4.1 g from 5.0 g of β-chloro-L-alanine).

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(2) Klayman, D. L.; Griffin, T. S. *J. Am. Chem. Soc*. **1973,** *95*, 197–199.
(3) Tanaka, H.; Soda, K. *Methods Enzymol*. **1987,** *143*, 240–243.

Reaction of Cysteine and Selenocysteine with $AcGlySCH_2C(O)NHCH_3$. $AcGlySCH_2C(O)NHCH_3$ was synthesized by the procedure of Nilsson et al.[4] Cystine (121 mg, 0.50 mmol) or selenocystine (83.5 mg, 0.25 mmol) was dissolved in 5 mL of 0.2 N HCl to form a yellow solution. The disulfide or diselenide bond was reduced in situ by addition under Ar(g) of 5 mL of 0.10 M Tris–HCl buffer (pH 9.0) containing tris-(2-carboxyethyl)phosphine (TCEP; 0.10 M, 0.50 mmol). The reaction was inititiated by the addition of 10 mL of acetonitrile containing $AcGlySCH_2C(O)NHCH_3$ (0.10 g, 0.5 mmol). The pH of the resulting solution was adjusted to 8.0 with a few drops of 10 N NaOH. The reaction was stirred under Ar(g) for 1 h at room temperature. Two control reactions were also performed: selenocystine and $AcGlySCH_2C(O)NHCH_3$ were mixed in the absence of TCEP, and $AcGlySCH_2C(O)NHCH_3$ and TCEP were mixed in the absence of selenocystine or cystine. After 1 h of reaction, aliquots were removed and analyzed by mass spectrometry. Analysis of the reaction with selenocysteine showed that the major products were $(AcGlySecOH)_2$ ($[M + H]^+$ *m*/*z* calcd for $C_{14}H_{23}N_4O_8Se_2$, 533; found 531) and the mixed thioselenide between AcGlySecOH and $HSCH_2C(O)NHCH_3$ ($[M + H]^+$ *m*/*z* calcd for $C_{10}H_{18}N_3O_5SeS$, 371; found 369). In the reaction with cystine, the product was predominantly AcGlyCysOH ($[M + H]^+$ *m*/*z* calcd for $C_7H_{13}N_2O_4S$, 221; found 219) plus a small quantity of $(AcGlyCysOH)_2$ ($[M + H]^+$ *m*/*z* calcd for $C_{14},H_{23}N_4O_8S_2$, 439; found 439). Mass spectrometric analysis showed that selenocysteine and $AcGlySCH_2C(O)NHCH_3$ did not react under these conditions in the absence of TCEP.

Synthesis of $AcGlySC_6H_4$-*p*-NO_2 (Thioester 1). AcGlyOH (2.6 g, 22 mmol) and *p*-nitrobenzenethiol (3.4 g, 22 mmol) were dissolved in dimethylformamide (60 mL).

(4) Nilsson B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000,** *2*, 1939–1941.

N,*N*′-Dicyclohexylcarbodiimide (4.6 g, 22 mmol) was added, and the resulting solution was stirred for 12 h. The *N*,*N*′-dicyclohexylurea precipitate was removed by filtration, and the solvent was removed under reduced pressure to yield $AcGlySC_6H_4$-*p*-NO_2 as a tan solid in 72% crude yield. $AcGlySC_6H_4$-*p*-NO_2 was used without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.28 (d, 2 H, *J* = 9 Hz), 7.63 (d, 2 H, *J* = 9 Hz), 6.28 (bs, 1 H), 4.36 (d, 2 H, *J* = 6 Hz), 2.14 (s, 3 H) ppm.

Reaction of Cysteine and Selenocysteine with $AcGlySC_6H_4$-*p*-NO_2. Reactions (0.60 mL) were performed at (23 ± 2) °C in 0.10 M buffer (pH 5.0: sodium acetate, pH 5.5: sodium succinate, pH 6.0 and 6.5: MES–NaOH, pH 7.0 and 7.5: HEPES–NaOH, pH 8.0: potassium phosphate) containing $AcGlySC_6H_4$-*p*-NO_2 (25 mM), TCEP (0.10 mM), and cystine (20 μM–0.50 mM) or selenocystine (1.0–20 μM). The pK_a of HSC_6H_4-*p*-NO_2 is 4.77 in 40% v/v ethanol.[5] This value is likely to be lower in aqueous solution, such that the *p*-nitrothiophenol product is virtually completely ionized in all of our assays. In aqueous solution, *p*-nitrothiophenolate was found to have ε = 11,230 $M^{-1}cm^{-1}$ at 410 nm. Beer's Law ($A = \varepsilon lc$) was used to determine the initial rate at each pH and concentration of cysteine or selenocysteine. A plot of reactant concentration versus initial rate was a straight line. The slope of this line was taken as the pseudo-first order rate constant for the reaction. The rate of background hydrolysis was subtracted at each pH. Only the background rate was observed either in the absence of TCEP or in the absence of cystine or selenocystine.

(5) Danehy, J. P.; Parameswaran, K. N. *J. Chem. Eng. Data*, **1968,** *13*, 386–389.

Synthesis of Sec(PMB)OH. A *p*-methoxybenzyl (PMB) group was used to protect the selenium, following the examples of Koide et al.[6] and Besse & Moroder.[7] The following procedure was adapted from that of Koide et al.[6] Their procedure used an excess of *p*-methoxybenzylchloride in the reaction under highly basic conditions. In our hands, this procedure always resulted in a dibenzylated product in which the nitrogen as well as the selenium of selenocysteine was alkylated with a PMB group. To produce a product that was alkylated only at selenium, the following protocol was used. Selenocystine (1.8 g, 5.3 mmol) was dissolved in 0.5 N NaOH (5 mL). $NaBH_4$ (1.7 g, 43 mmol) dissolved in water (10 mL) was added dropwise and with stirring to the solution of selenocystine in a 100-mL roundbottom flask. The flask was chilled to prevent boiling. After the vigorous reaction had subsided (the solution turns from yellow to colorless), glacial acetic acid was added dropwise until the pH was near 6.0. *p*-Methoxybenzylchloride (1.44 mL, 10.6 mmol) was then added dropwise to the solution. The reaction proceeded quickly and was complete in 30 min. White crystals of Se-benzylated selenocysteine were apparent along with some selenocystine, which appeared as yellow crystals. The solution was acidified with concentrated HCl to complete the formation of the white precipitate, which was purified by crystallization from hot water. Sec(PMB)OH–HCl was isolated as a white solid in 72% yield (2.5 g from 1.8 g of selenocystine).

Synthesis of Fmoc-Sec(PMB)OH. Fmoc-Sec(PMB)OH was synthesized by a procedure adapted from that of Koide et al.[6] Sec(PMB)OH–HCl (1.2 g, 3.7 mmol) was dissolved in water (10 mL) to make a slurry. Triethylamine (TEA; 0.52 mL, 3.7 mmol) was added to the slurry in a

(6) Koide, T.; Itoh, H.; Otaka, A.; Yasui, H.; Kuroda, M.; Esaki, N.; Soda, K.; Fujii, N. *Chem. Pharm. Bull*. **1993,** *41*, 502–506.
(7) Besse, D.; Moroder, L. *J. Peptide Sci*. **1997,** *3*, 442–453.

100-mL roundbottom flask. Fmoc-*O*-succinimide (where "Fmoc" refers to 9-fluorenylmethoxycarbonyl; 1.25 g, 3.7 mmol) dissolved in acetonitrile (10 mL) was added to the solution, and another equivalent of TEA was then added. Additional acetonitrile was added until all of the solutes were dissolved completely. The resulting solution was stirred at room temperature for 1 h, and the reaction progress was monitored by TLC. In methylene chloride, Fmoc-Sec(PMB)OH did not migrate, but impurities from the reaction, especially dibenzofulvene, had high mobility. After 1 h, the reaction mixture was acidified with 1 N HCl (5 mL) and then extracted with ethyl acetate. The organic layer was washed (3×) with 1 N HCl. The resulting, combined aqueous phases were then extracted (3×) with ethyl acetate. The ethyl acetate extracts were combined and dried over $MgSO_4$(s). After filtering, the organic layer was concentrated under reduced pressure to produce a yellow oil. This oil was dissolved in methylene chloride (10–20 mL) and purified by chromatography on a column of silica gel (20 cm × 20 cm^2). The impurities in the reaction, primarily dibenzofulvene, were eluted when the column was washed extensively with CH_2Cl_2. Fmoc-Sec(PMB)OH was eluted with methanol/CH_2Cl_2 (1:4). The solvent was removed under reduced pressure. Fmoc-Sec(PMB)OH was isolated as a slightly yellow crystalline solid in 53% yield (1.0 g of Fmoc-Sec(PMB)OH from 1.2 g of Sec(PMB)OH–HCl). ^{1}H NMR data of Fmoc-Sec(PMB)OH were similar to those reported previously.[6]

Solid-Phase Synthesis of a Peptide Containing Selenocysteine. A methylbenzhydrylamine polystryrene resin functionalized with a 4-hydroxymethylphenoxy acid labile linker that had been loaded with the C-terminal amino acid was used for all syntheses. Monomers were coupled by using cycles of *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethylunonium

hexafluorophosphate/diisopropylamine (HBTU/DIEA) activation of the carboxylic acid group, followed by piperidine deprotection of the Fmoc group. Syntheses were done on a 25-μmol scale with a 3-fold excess of each amino acid monomer using an Applied Biosystems Model 432A synthesizer. The sequence of the wild-type RNase A peptide used in the ligation reactions was CEGNPYVPVHFDASV (which corresponds to residues 110–124) and the sequence of the selenocysteine variant was UEGNPYVPVHFDASV, where "U" refers to selenocysteine.

Deprotection of a Peptide Containing Selenocysteine. Deprotection of the wild-type peptide (which does not contain selenocysteine) was achieved by using a cleavage cocktail containing trifluoroacetic acid/ethanedithiol/H_2O (95:2.5:2.5) for 3 h at room temperature. When this cocktail was used for the selenocysteine-containing peptide, only partial removal of the PMB group was achieved. The PMB group was removed successfully, however, using conditions reported by Koide et al.[6] The cleavage cocktail (1.0 mL of cocktail per g of resin) for the selenium-containing peptide contained m-cresol/thioanisole/trifluoroacetic acid/trimethylsilyl trifluoromethanesulfonate (50:120:690:194). After purification by HPLC, the intact peptide was observed, along with some dehydrated peptide. Dehydration most likely occurred during deprotection.

Semisynthesis of a Protein Containing Selenocysteine. To incorporate a selenocysteine residue into RNase A, a protein was produced in which residues 1–109 of RNase A were fused to the *Mxe* GyrA intein and a chitin-binding domain. Plasmid pTXB1-RNase (which was a kind gift from New England BioLabs) was transformed into ER2566 *E. coli* cells. LB medium (0.10 L) containing ampicillin (0.10 mg/mL) was inoculated with a single colony and grown for 8 h at

37 °C. A 2-L flask that contained 1 L of LB medium containing ampicillin (0.10 mg/mL) was then inoculated with 10 mL of the 8-h culture, and grown at 37 °C until OD = 0.6 at 600 nm. A total of 6 L of culture were grown in this way. The flasks were then cooled on ice for 10 min, and expression was induced by the addition of IPTG (to 0.5 mM). The flasks were shaken at room temperature for 5 h. Cells were harvested by centrifugation, and the wet cell pellet was frozen for storage. Frozen cells were thawed and homogenized in 50 mM Tris–HCl buffer (pH 8.5) containing NaCl (0.50 M), and then lysed by sonication. Lysed cells were subjected to centrifugation at $6000 \times g$ for 30 min. The supernatant was passed over a 30-mL column of chitin resin (New England BioLabs) that had been equilibrated with the same buffer. After loading, the column was washed with 5 column volumes of buffer to elute non-specific proteins. Cleavage of RNase A(1–109) was initiated by equilibrating the column in cleavage buffer, which was 50 mM Tris–HCl buffer (pH 7.4) containing NaCl (0.10 M) and *N*-methylmercaptoacetamide (NMA, 50 mM). We have found NMA to be better than β-mercaptoethanol, dithiothreitol, or 2-mercaptoethanesulfonic acid for the cleavage of target protein–intein fusions (R. J. H. and R. T. R., unpublished results). The chitin resin was incubated with the cleavage buffer for 2 h at room temperature. Fractions containing the thioester-tagged RNase A(1–109) were then collected. The protein solution was concentrated with a Centriplus 3000 apparatus (Amicon) to a volume of 5 mL. For ligation reactions with the peptide UEGNPYVPVHFDASV, 5 mL of 20 mM Tris–HCl buffer (pH 7.0[8]) containing guanidine hydrochloride (7 M) was added to the protein solution containing RNase A(1–109). The solution

(8) At low pH, the nucleophilic attack of selenocysteine on a thioester is much faster than that of cysteine (Figure 1). Ligation reactions with cysteine-containing peptides are typically performed at pH 8.0. We used a lower pH for ligation reactions with selenocysteine-containing peptides. For example, we prepared C110U RNase A at pH 7.0. This lower pH suppressed β-elimination of the selenol group from selenocysteine but was high enough to allow for the intramolecular attack of nitrogen on the intermediate selenoester to form an amide (Scheme 1).

was then added to the lyophilized peptide (15 mg, 10 μmol) and the resulting solution was zwhich was 50 mM Tris–HCl buffer (pH 7.8) containing NaCl (0.10 M), reduced glutathione (GSH; 1 mM), and oxidized glutathione (GSSG; 0.2 mM). The protein solution was dialyzed for an additional 4 h against fresh refolding buffer and then overnight against 50 mM sodium acetate buffer (pH 5.0).

Intact RNase A has a much higher affinity for guanidine 3′-diphosphate (GDP) than does RNase A(1–109). Hence, affinity chromatography with GDP~Sepharose resin (Sigma) was used to purify the intact protein. A 5-mL column of GDP~Sepharose equilibrated with 10 mM sodium acetate buffer (pH 5.0) was loaded with the protein solution and then washed with 50 mL of the same buffer. The bound protein was then eluted with buffer containing NaCl (1.0 M). Fractions containing active RNase A were dialyzed against 50 mM sodium acetate buffer (pH 5.0) overnight at 4 °C. Approximately 1 mg of pure C110U RNase A can be prepared in this manner from 6 L of culture.[9]

(9) The yield of C110U RNase A was limited by the recovery of sufficient amounts of thioester-tagged RNase A(1–109). The cleavage of RNase A with an alanine residue at the junction with the intein results in poor yields in recovery of thioester-tagged proteins (Evans, T. C., Jr.; Benner, J.; Xu, M. -Q. *Protein Sci.* **1998,** *7,* 2256–2264). We do not believe that the low yield of C110U RNase A was attributable to selenocysteine because the yields of RNase A variants produced by expressed protein ligation with cysteine are similar (R. J. H. and R. T. R., unpublished results).