Selenocysteine-Mediated Native Chemical Ligation

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C-Terminal peptide thioesters are shown to react efficiently with peptide fragments containing an N-terminal selenocysteine to give selenoproteins. In analogy to the native chemical ligation of thioesters and peptides containing N-terminal cysteines, the selenol presumably attacks the thioester nucleophilically to give a selenoester intermediate that subsequently rearranges to give a native chemical bond. The utility of this procedure was demonstrated by the synthesis of a selenium-containing derivative of bovine pancreatic trypsin inhibitor (BPTI) in which Cys^{38} is replaced by selenocysteine. The artificial selenoprotein folds into a conformation similar to that of wild-type BPTI and inhibits trypsin and chymotrypsin with unaltered affinity.

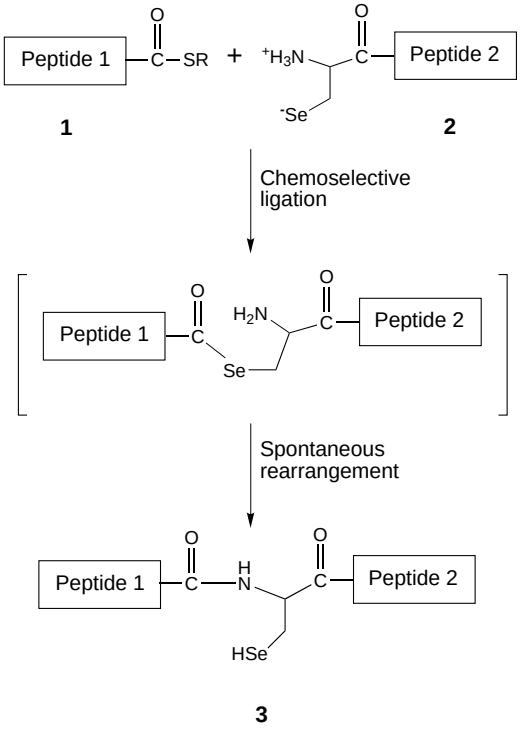
1. Introduction. – Selenium, in the form of selenocysteine (abbreviated Sec or U), is an essential catalytic group in several natural enzymes, including glutathione peroxidase, glycine reductase, thioredoxin reductase, and iodothyronine 5′-deiodinase [1][2]. The properties of this atom also make it useful as a spectroscopic and mechanistic probe [3–5] and as a prosthetic group for creating artificial catalysts with novel hydrolytic and redox activities [4][6–8].

Selenium can be introduced into proteins in a variety of ways. Naturally occurring selenoproteins are prepared by cotranslational insertion of selenocysteine into the growing polypeptide chain *via* the normal biosynthetic machinery of the cell [1][9]. Selenocysteine is encoded by a UGA stop codon in these proteins, which is recognized by a specific selenocysteyl-tRNA, but efficient suppression of the stop codon requires a special translation factor and specific recognition elements within the mRNA. Alternatively, because selenocysteine efficiently charges $tRNA^{Cys}$ in the absence of cysteine, cysteine auxotrophs can be used to create artificial selenoproteins in which all cysteine residues are selectively replaced by selenocysteine [5]. Selenoproteins can also be prepared post-translationally, for example, by chemically converting activated serine residues in existing proteins into selenocysteines [4][7]. If a uniquely reactive serine is available, chemical modification can occur chemoselectively in high yield.

Direct chemical synthesis of selenoproteins is a potentially attractive alternative to these approaches that could make a wider range of proteins available for study. New methods for ligating peptide fragments have made moderately sized proteins readily accessible [10]. The 'native chemical ligation' (NCL) approach of *Kent* and co-workers has proved to be particularly useful in this regard [11]. Coupling is achieved by transthioesterification of a peptide thioester with the N-terminal cysteine of a second peptide, followed by migration of the acyl group from the S- to the N-atom to give a peptide bond. A variety of proteins have been prepared by this method [12].

Given the similarity between sulfur and selenium, it seemed likely that selenocysteine, like cysteine, would undergo efficient reaction with a thioester (*Scheme*).

Scheme. *Native Chemical Ligation of Peptide Thioesters* **1** *with Peptides Containing an N-Terminal Selenocysteine* **2**. In analogy to sulfur, the nucleophilic selenium attacks the thioester **1** to form an intermediate selenoester that rearranges spontaneously to the more stable amide **3**.



Subsequent transfer of the acyl group from the Se- to the N-atom would also be expected to be quite facile [13][47]. Here, we show that such a strategy is indeed viable and apply it to the synthesis of a selenocysteine-containing variant of bovine pancreatic trypsin inhibitor (BPTI).

2. Results. – Condensation of the pentapeptide thioethyl ester LYRAG-SEt (**1a**) [14] and L-selenocysteine (**2a**) served as a convenient model system to assess the feasibility of selenocysteine-mediated peptide ligations. Reactions were carried out as previously described for cysteine-mediated ligations under an inert atmosphere in aqueous buffer containing 6M guanidinium hydrochloride (GdmCl) and 3% thiophenol at pH values between 4.8 and 6. Tris(2-carboxyethyl)phosphine (TCEP) [15][16] was included to reduce L-selenocystine, which was used as the precursor to L-selenocysteine.

Fig. 1 shows a representative ligation reaction monitored by liquid chromatography/mass spectrometry (LC/MS). In the course of the reaction, the starting materials disappeared, and two new compounds formed that eluted with retention times of 27.3 and 34.8 min, respectively. Mass-spectral analysis confirmed that both were derivatives of the expected ligation product, hexapeptide LYRAGU. The slower-moving species had a mass of 837.3 Da, which is in good agreement with the calculated mass of the

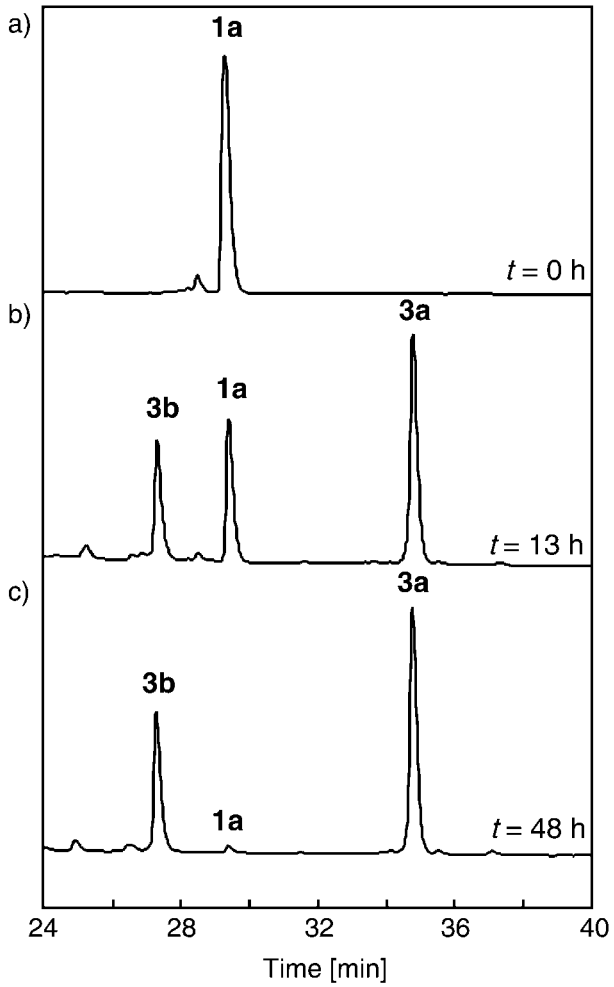


Fig. 1. Analytical-HPLC traces of a model selenocysteine-mediated ligation (pH 5.0). *a*) Thioester LYRAG-SEt (**1a**). *b*) Reaction of **1a** and selenocysteine at $t = 13$ h. The new peaks at 27.3 and 34.8 min were identified by LC/MS to be the mixed selenosulfide LYRAGU(SPh) (**3a**) and the diselenide dimer of LYRAGU (**3b**), respectively. *c*) Same as *b* but at $t = 48$ h, showing essentially complete reaction.

mixed selenosulfide LYRAGU(SPh) **3a** (calc.: 836.9 Da for $C_{35}H_{51}N_9O_8SSe$). The faster-moving compound corresponded to the diselenide dimer of LYRAGU **3b** (calc.: 1455.4 Da for $C_{58}H_{92}N_{18}O_{16}Se_2$; found: 1456.0 Da).

Comparison of the reactions of peptide **1a** with selenocysteine (pH 6) and cysteine (pH 6.5) to give **3** and LYRAGC, respectively, showed that ligation proceeded to *ca.* 50% completion in 4.5 h in both cases. The efficiency of the selenocysteine-mediated ligation is thus comparable to the analogous cysteine-dependent process. In control experiments with alanine in place of selenocysteine or cysteine, no ligation product (LYRAGA) was observed. Furthermore, the importance of having a free selenol was shown by omitting TCEP and thiophenol from the reaction. In the absence of reducing

reagent, no ligation was observed. Together, these results provide strong support for the mechanism postulated in the *Scheme*, involving initial attack of the selenol on the thioester to give a selenoester, followed by acyl migration from the Se- to the N-atom. Interestingly, the same products, **3a** and **3b**, were obtained in reactions containing thiophenol but lacking TCEP. At high concentrations, thiophenol must be sufficiently reducing to generate the selenol nucleophile [17], which can then react further. The rate of ligation did not vary appreciably above pH *ca.* 6, presumably because the selenol is largely deprotonated.

Given these encouraging preliminary results, synthesis of a Se-containing derivative of bovine pancreatic trypsin inhibitor (BPTI) by selenocysteine-mediated native chemical ligation was undertaken. BPTI is a small protein inhibitor of serine proteases, consisting of 58 amino acids and containing three disulfide bonds (Cys^5-Cys^{55}, Cys^{14}-Cys^{38}, Cys^{30}-Cys^{51}; *Fig. 2,a*). It has been extensively studied, especially with respect to its folding pathway [18][19]. BPTI has been previously synthesized by ligating a fragment corresponding to the 37 N-terminal amino acids, activated as a thioester, with the C-terminal 21 amino acids [20]. We adopted an analogous strategy to synthesize Cys38Sec-BPTI, substituting the N-terminal cysteine in the $BPTI^{38–58}$ fragment with selenocysteine (*Fig. 2,b*).

$BPTI^{1–37}$ was prepared as a thioester, **1b**, by conventional Fmoc ([(9*H*-fluoren-9-yl)methoxy]carbonyl) solid-phase peptide synthesis (SPPS) [22] on a PAM resin, followed by direct cleavage of the peptide from the resin with $AlMe_3$ and ethanethiol [23]. The second fragment, Cys38Sec-$BPTI^{38–58}$ (**2b**), was synthesized on a *Wang* resin. The selenocysteine was activated as a pentafluorophenyl ester, and the selenol side chain was protected with a *p*-methoxybenzyl group [24]; cysteine side chains were protected as mixed disulfides [25]. The peptide was cleaved from the resin with a standard cleavage cocktail (reagent B [26]), removing all the protecting groups except those on S- and Se-atoms. It was purified by HPLC and subsequently dissolved in a 1M solution of Me_3SiBr in CF_3COOH (TFA), containing *m*-cresol and thioanisole as scavengers to remove the Se- and S-protecting groups [24]. After purification and lyophilization, it was characterized by electrospray-ionization mass spectrometry (ESI-MS). The mass of the peptide was determined to be 2363.1 Da, which corresponds to the expected value (2363.5 Da) for a partially oxidized species containing a single mixed selenosulfide between Sec^{38} and either Cys^{51} or Cys^{55}.

For ligation of **1b** and **2b**, the lyophilized peptides were weighed into a vial together with TCEP as the reducing agent. Phosphate buffer (100 mM, pH 9.2) containing 6M GdmCl and 3% (*v*/*v*) thiophenol was added under Ar to initiate the reaction. After 20 h, no starting material **2b** (t_R 14 min) remained as judged by HPLC, and a new very broad peak centered around 41 min was evident (*Fig. 3,b*). The product was purified by preparative HPLC (C_8 column). After lyophilization, its mass was determined to be 6563.7 Da by ESI-MS, which is in good agreement with the calculated mass for partially reduced Cys38Sec-BPTI (6562.4 Da, calculated as a mixed selenosulfide $C_{284}N_{436}N_{84}O_{79}S_6Se$). The broadness of the corresponding HPLC peak presumably reflects a complex mixture of peptides with native and nonnative disulfide or mixed selenosulfide bonds.

To fold and oxidize the newly made protein, it was dissolved in buffer containing 6M GdmCl, diluted sixfold in H_2O and gently shaken overnight [20]. The broad peak at

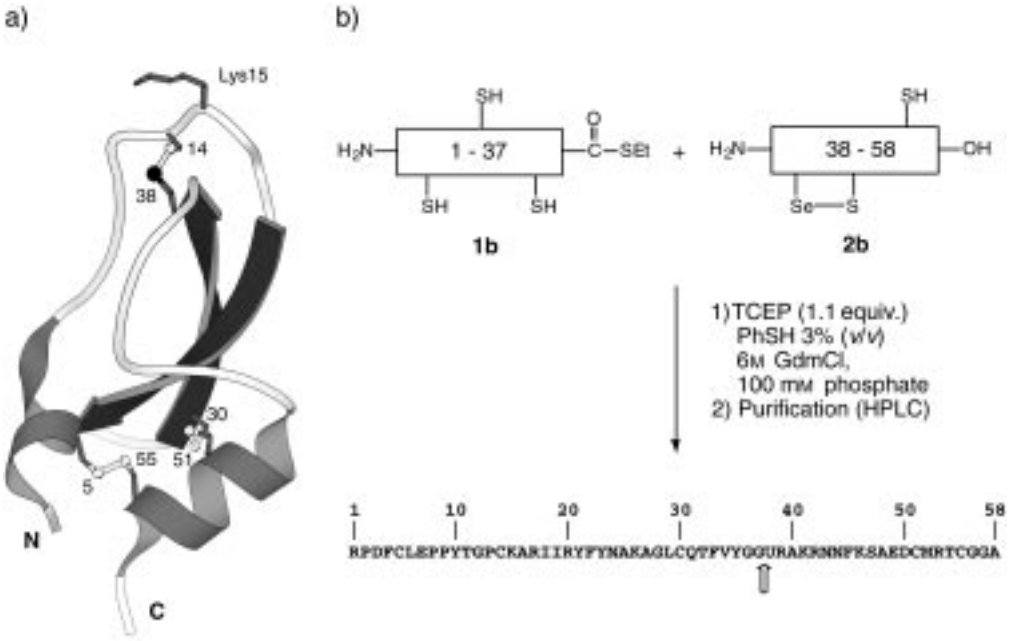


Fig. 2. a) *Three-dimensional structure of BPTI* [21]. Disulfide bonds are shown explicitly. The heteroatom of the side chain of residue 38 is shown in black. Lys^{15} is a key residue group recognized by trypsin. b) *Synthesis of Cys38Sec-BPTI by selenocysteine-mediated native chemical ligations.* The ligation site between residues Gly^{37} and Sec^{38} is indicated by an arrow in the final sequence.

41 min corresponding to the initial ligation product was replaced with a much sharper peak at 19 min. The retention time of the oxidized Cys38Sec-BPTI derivative is the same as for native BPTI. The oxidized protein was isolated by preparative HPLC (*Fig. 3*,*c*) and analyzed by ESI-MS. Its mass (6559.3 Da; see *Fig. 3*,*c*, inset) is in good agreement with the calculated value for fully oxidized Cys38Sec-BPTI ($C_{284}H_{432}N_{84}O_{79}S_6Se$, 6558.3 Da).

Cys38Sec-BPTI was characterized biochemically. Its CD spectrum is essentially identical to that of wild-type BPTI (data not shown). Like the native protein, it stoichiometrically inhibits trypsin [27]. It also inhibits α-chymotrypsin-catalyzed hydrolysis of *N*-succinyl-Gly-Gly-Phe *p*-nitroanilide (SGGPPA) [28] with an apparent dissociation constant of 6.6 nM (*Fig. 4*). Under the same assay conditions, BPTI itself gave an apparent K_i of 7.0 nM. These data suggest that Cys38Sec-BPTI adopts the same overall three-dimensional structure as the native protein (*Fig. 2*,*a*). Introduction of the selenosulfide bond between Cys^{14} and Sec^{38} does not appear to perturb either the folding or inhibitory properties of the protein, despite its proximity to key residues in the protease recognition site (*i.e.*, Lys^{15}; *Fig. 2*).

3. Discussion. – Naturally occurring and artificial selenoproteins catalyze a variety of interesting reactions [1][2]. Selenocysteine-mediated native chemical ligation provides a potentially general method for producing these molecules, which is

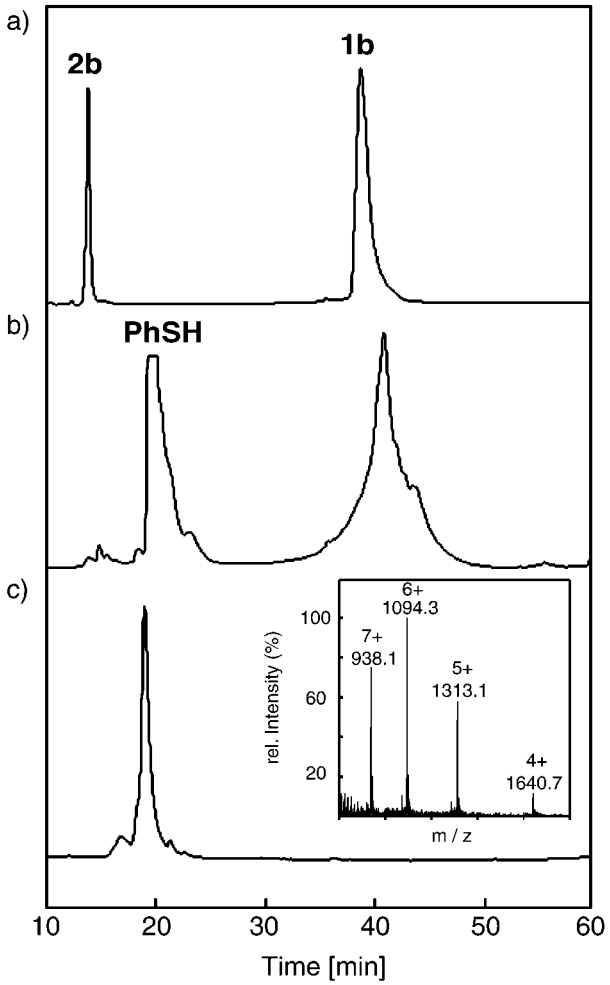


Fig. 3. *Synthesis of Cys38Sec-BPTI by native chemical ligation. a*) Chromatogram of the starting materials, Cys38Sec-BPTI$^{38-58}$ (**2b**, 14 min) and BPTI$^{1-37}$-SEt (**1b**, 39 min). *b*) Reaction mixture at $t = 20$ h. The broad peak at 41 min corresponds to the expected ligation product Cys38Sec-BPTI, as judged by LC-MS. *c*) Cys38Sec-BPTI after oxidation (19 min). The inset shows the ESI-MS of oxidized Cys38Sec-BPTI (observed, 6559.3 Da; calc. average isotope composition for $C_{284}H_{432}N_{84}O_{79}S_6Se$, 6558.3 Da).

complementary to other strategies based on biosynthesis or chemical modification of existing proteins.

Because standard methods for solid-phase peptide synthesis are applied to prepare the individual fragments, and because all operations are carried out *in vitro*, native chemical ligation is quite versatile, allowing selenocysteine to be introduced at any position within a protein simply by choosing an appropriate ligation site. The presence of multiple cysteine residues, as shown in the BPTI example, does not interfere with ligation. A wide range of other non-proteinogenic amino acids should also be compatible with this method. In conjunction with intein technology [29], which can be

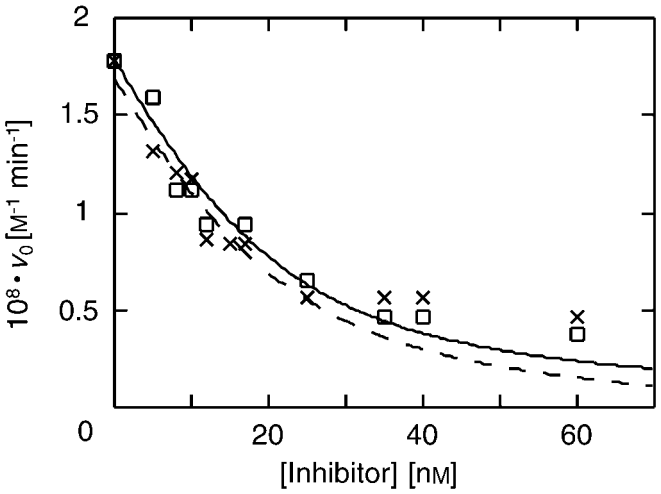


Fig. 4. *Inhibition of bovine α-chymotrypsin by native BPTI* (×) *and by Cys38Sec-BPTI* (□). The data were fitted as described in the *Exper. Part* giving apparent K_i values of 6.6 and 7.0 nM for Cys38Sec-BPTI and the wild-type protein, respectively.

used to produce entire protein domains as activated thioesters, selenoproteins considerably larger than the BPTI derivative described here will be accessible as well.

Although these fragment-condensation reactions must be carried out under reducing conditions and an inert atmosphere, our preliminary results suggest that selenocysteine is at least as effective as cysteine in effecting peptide ligation. Detailed kinetic experiments with different ligation site sequences and with activating agents more reactive than thiophenol may ultimately uncover advantages over cysteine-dependent ligations since a selenol is more acidic (pK_a *ca.* 5.5) [30][31] than a thiol and hence present in its activated form over a wider range of pH. In addition, aminolysis of selenoesters is typically orders of magnitude faster than aminolysis of the corresponding thioesters [13][47], which may prove beneficial for difficult couplings.

Because this technology allows selenium to be incorporated efficiently and site-specifically into proteins, a number of applications is conceivable. The high sensitivity of the ^{77}Se nucleus to its surroundings could be exploited to probe local protein microenvironment systematically by NMR spectroscopy [3][32]. The anomalous scattering of selenium is already used extensively for elucidation of X-ray structures [33][34], circumventing the need for heavy atom derivatives, and synthesis of proteins by selenocysteine-mediated chemical ligation may prove to be an attractive alternative to the biosynthetic incorporation of selenomethionine [35] in some cases. More generally, access to synthetic selenoproteins can be expected to facilitate studies of enzyme mechanism and aid efforts to create new catalysts that capitalize on the reactivity and/or metal-binding properties of the selenol side chain. Incorporation of selenium into proteins is also interesting in the context of folding studies on disulfide-containing proteins since alkaneselenols are known to enhance thiol-disulfide exchange [36]. Results with the Cys38Sec-BPTI derivative obtained here suggest that the Cys^{14}-Sec^{38} selenosulfide is functionally equivalent to the corresponding disulfide in the wild-

type protein, but detailed kinetic experiments will be needed to determine precisely how Sec[38] influences the rate and pathway of the folding process. Replacement of other cysteines in the protein, or incorporation of more than one selenocysteine (allowing formation of very stable non-native diselenides, for example), could also provide valuable insight into these issues [37][38].

Selenocysteine-mediated peptide fragment condensations broaden the scope of the originally reported ligation chemistry to an additional amino acid. Since the selenol side chain can be converted to a variety of anionic, cationic, and neutral functionalities through selective alkylation, proteins with a wide range of properties at the ligation site are accessible. In this regard, selective reduction of the relatively weak C–Se bond to give alanine, another proteinogenic amino acid, at the ligation site should be easier than the reduction of the C–S bond [38]. Selenocysteine-dependent ligations are thus likely to be useful in the more general context of methods to join peptides at a wider variety of sites than has been possible heretofore.

Experimental Part

General. All amino acids were purchased from either *Novabiochem*, *Bachem*, or *Sigma*. Preloaded resins were obtained from *Novabiochem*. Reagents for peptide synthesis were purchased from *Applied Biosystems*. CH_2Cl_2 was dried by distillation over CaH_2 and Et_2O was filtered through a basic alumina plug before use. L-Selenocystine was prepared according to literature procedures [40–42] and converted into *N*-{[(9*H*-fluoren-9-yl)methoxy]carbonyl}-*Se*-(4-methoxybenzyl)selenocysteine pentafluorophenyl ester (Fmoc-Sec-(Mob)-OPfp; **4**) [24][43]. α-Chymotrypsin, trypsin, and BPTI (aprotinin) were purchased from *Calbiochem*.

Reversed-phase HPLC (RP-HPLC) was performed on a *Waters* HPLC system with 220-nm UV detection. Either C_8 (*Macherey-Nagel*, 250 nm × 4.6 mm, 300 Å, 5 μ) or C_{18} (*Macherey-Nagel*, 250 mm × 4.6 mm, 100 Å, 5 μ) columns were used for anal. runs (flow rate 1 ml/min). A C_8 column (*Macherey-Nagel*, 250 mm × 21 mm, 300 Å, 7 μ) was used for prep. RP-HPLC (flow rate 10 ml/min). Peptides were eluted with linear gradients of solvents *A* (H_2O, 0.1% CF_3COOH (TFA)) and *B* (MeCN, 0.05% TFA); gradient 1: 0% *B* for 5 min, then increasing to 65% over 45 min; gradient 2: 15% to 50% *B* over 60 min. Liquid chromatography/mass spectrometry (LC/MS) was performed on a *Spectra System* (*Thermo Separation Products*) HPLC system connected to a diode-array detector (*UV6000LP*, *Thermo Separation Products*) and an ion-trap mass spectrometry detector (*LCQdeca*, *Finnigan*). The same columns and gradients were used as for anal. RP-HPLC. UV Data were collected on a *Perkin-Elmer* UV/VIS spectrophotometer *Lambda 20*. Electrospray ionization mass spectrometry (ESI-MS) was performed on a *Finnigan TSQ7000 Triple-Quad* mass spectrometer. Calculated masses were based on average isotope composition.

Solid-Phase Peptide Synthesis. Peptides were synthesized in stepwise fashion on an *ABI 433A* peptide synthesizer (*Applied Biosystems*) according to 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt)/1-methyl-2-pyrrolidinone (NMP) activation protocols for Fmoc chemistry (*FastMoc*® protocol, *Applied Biosystems*). Activated and protected selenocysteine (**4**) was coupled as in [44]. The following protecting groups were employed: Arg(Pbf), Asn(Trt) or Asn(Tmob), Asp(O*t*Bu), Cys(S*t*Bu) or Cys(Trt), Glu(O*t*Bu), Gln(Trt) or Gln(Tmob), Lys(Boc), Ser(O*t*Bu), Thr(O*t*Bu), Tyr(O*t*Bu). After drying the resin on high vacuum, peptides were cleaved by shaking the beads in TFA in the presence of scavengers (Reagent B [26]) at 25° for 2.5 h. The beads were filtered off, solvents evaporated under reduced pressure, and the residue was triturated in cold Et_2O in a centrifuge tube. After centrifugation at 3000 *g* for 20 min, Et_2O was decanted and the procedure repeated twice. The selenocysteine-containing peptide Cys38Sec-BPTI$^{38–58}$ (**2b**) was deprotected using scavenger mixtures and conditions as described by *Koide et al.* [24]. Peptide thioesters LYRAG-SEt (**1a**) and BPTI$^{1–37}$-SEt (**1b**) were prepared by direct cleavage from resin using $AlMe_3$ and ethanethiol as described in [14][23].

Model Ligations of LYRAG-SEt (**1a**) *with* L-*Selenocysteine* (**2a**). Degassed buffer (1.5 ml, 100 mM phosphate, 6M guanidinium · HCl (GdmCl), 3% thiophenol (*v*/*v*), pH 7.5) was added to a mixture of peptide **1a** (0.93 mg, 1.5 μmol), the diselenide of **2a** (0.28 mg, 0.83 μmol), and TCEP (0.4 mg, 1.4 μmol) under Ar. The mixture was stirred at 25°. Aliquots were periodically removed and analyzed by LC/MS (gradient 1, C_{18}

column). Control experiments without TCEP, without TCEP and thiophenol, and with cysteine or alanine instead of selenocysteine were performed analogously.

Native Chemical Ligation and Folding of Cys38Sec-BPTI. Peptides **1b** and **2b** were ligated as described for **1a** and **2a**. Buffer (100 mM phosphate, 6M GdmCl,PhSH 3% (*v*/*v*), pH 9.2, 18 µl) was added directly to a mixture of **1b** (1.52 mg, 0.3 µmol), **2b** (0.85 mg, 0.3 µmol), and TCEP (0.1 mg, 0.3 µmol) under Ar, and the resulting soln. was stirred at 25° for 20 h. The product was purified by prep. RP-HPLC and lyophilized to give partially oxidized Cys38Sec-BPTI (1.5 mg, 0.2 µmol). This material was folded under oxidizing conditions as described for wild-type BPTI [20]. Briefly, the lyophilized peptide was dissolved in 1.25 ml of buffer (0.6M *Tris*-HCl, 6 mM EDTA, 6M GdmCl, pH 8.7), diluted 6-fold into doubly deionized water, and the resulting soln. was gently shaken in an open flask for 18 h at 25°. The oxidized protein was purified by prep. RP-HPLC (C_8 column, gradient 2: t_R 19 min) and characterized by ESI-MS (observed, 6559.3 Da; calc. average isotope composition for $C_{284}H_{432}N_{84}O_{79}S_6Se$, 6558.3 Da).

Inhibition Assays. Protein concentration was determined spectrophotometrically (trypsin: $\varepsilon(280\text{ nm}) = 37700\ \text{M}^{-1}\text{cm}^{-1}$; chymotrypsin: $\varepsilon(280\text{ nm}) = 49600\ \text{M}^{-1}\text{cm}^{-1}$ [45]; BPTI and Cys38Sec-BPTI: $\varepsilon(280\text{ nm}) = 5400\ \text{M}^{-1}\text{cm}^{-1}$ [26]). Formation of *p*-nitroaniline was monitored at 405 nm. The trypsin-catalyzed hydrolysis of *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPA) was performed in 200 mM triethanolamine · HCl buffer (pH 7.8) at 25° in the presence of 0 to 40 nM inhibitor as previously described [27]; [BAPA] = 38 µM, [trypsin] = 39 nM. A slightly modified literature procedure [27] was used to monitor the inhibition of chymotrypsin: reactions were carried out in *Tris*-HCl buffer (50 mM, 20 mM $CaCl_2$, 0.005% *triton X-100*, pH 8.2) at 25°; [chymotrypsin] = 20 nM, [*N*-succinyl-Gly-Gly-Phe *p*-nitroanilide] = 20 µM, [inhibitor] = 0–60 nM. Apparent K_i values for oxidized Cys38Sec-BPTI and wild-type BPTI were calculated by fitting the initial rate data to the following equation [46]:

$$v = \frac{v_0}{2\alpha\text{E}}\left[\alpha\text{E} - I - K_i + \sqrt{(K_i + \alpha\text{E} - I)^2 + 4K_iI}\right]$$

where v is the initial rate in the presence of inhibitor I (Cys38Sec-BPTI or WT BPTI), v_0 is the initial rate in the absence of inhibitor, αE represents the fractional concentration of functional chymotrypsin binding sites. K_i is the apparent dissociation constant of the complex between enzyme and inhibitor.

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Note added in proof: Another group has recently reported the synthesis of a Sec-containing peptide by native chemical ligation (M. D. Gieselman, L. Xie, A. van der Donk, *Org. Lett.* **2001**, *3*, 1391).

REFERENCES

[1] T. C. Stadtman, *Annu. Rev. Biochem.* **1996**, *65*, 83.
[2] J. Korle, R. Brigelius-Flohé, A. Böck, R. Gartner, O. Meyer, L. Flohé, *Biol. Chem.* **2000**, *381*, 849.
[3] N. P. Luthra, R. C. Costello, J. D. Odom, R. B. Dunlap, *J. Biol. Chem*. **1982**, *257*, 1142.
[4] Z.-P. Wu, D. Hilvert, *J. Am. Chem. Soc*. **1989**, *111*, 4513.
[5] S. Müller, H. Senn, B. Gsell, W. Vetter, C. Baron, A. Böck, *Biochemistry* **1994**, *33*, 3404.
[6] Z.-P. Wu, D. Hilvert, *J. Am. Chem. Soc*. **1990**, *112*, 5647.
[7] L. Ding, Z. Liu, Z. Q. Zhu, G. M. Luo, D. Q. Zhao, J. Z. Ni, *Biochem. J*. **1998**, *332*, 251.
[8] S. Boschi-Muller, S. Muller, A. Van Dorsselaer, A. Böck, G. Branlant, *FEBS Lett*. **1998**, *439*, 241.
[9] A. Böck, K. Forchhammer, J. Heider, W. Leinfelder, G. Sawers, B. Veprek, F. Zinoni, *Mol. Microbiol*. **1991**, *5*, 515.
[10] J. P. Tam, Q. T. Yu, Z. W. Miao, *Biopolymers* **1999**, *51*, 311.
[11] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776.
[12] P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem*. **2000**, *69*, 923.
[13] H. G. Mautner, S.-H. Chu, W. H. H. Günther, *J. Am. Chem. Soc*. **1963**, *85*, 3458.
[14] D. Swinnen, D. Hilvert, *Org. Lett*. **2000**, *2*, 2439.
[15] J. A. Burns, J. C. Butler, J. Moran, G. M. Whitesides, *J. Org. Chem*. **1991**, *56*, 2648.
[16] Z. S. Zhou, A. E. Smith, R. G. Matthews, *Bioorg. Med. Chem. Lett*. **2000**, *10*, 2471.

[17] L. Engman, D. Stern, *J. Org. Chem.* **1994**, *59*, 5179.
[18] J. S. Weissman, P. S. Kim, *Science* **1991**, *253*, 1386.
[19] T. E. Creighton, *Biol. Chem*. **1997**, *378*, 731.
[20] W. Y. Lu, M. A. Starovasnik, S. B. H. Kent, *FEBS Lett*. **1998**, *429*, 31.
[21] S. Parkin, B. Rupp, H. Hope, *Acta Crystallogr.*, *Sect. D* **1996**, *52*, 18.
[22] W. C. Chan, P. D. White, 'Fmoc solid phase peptide synthesis: a practical approach', Oxford University Press, Oxford, 2000.
[23] A. Sewing, D. Hilvert, *Angew. Chem.*, in press.
[24] T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, N. Fujii, *Chem. Pharm. Bull*. **1993**, *41*, 502.
[25] U. Weber, P. Hartter, *Hoppe-Seyler's Z. Physiol. Chem*. **1970**, *351*, 1384.
[26] N. A. Solé, G. Barany, *J. Org. Chem*. **1992**, *57*, 5399.
[27] B. Kassell, *Methods Enzymol*. **1970**, *19*, 844.
[28] M. J. M. Castro, S. Anderson, *Biochemistry* **1996**, *35*, 11435.
[29] C. J. Noren, J. M. Wang, F. B. Perler, *Angew. Chem.*, *Int. Ed*. **2000**, *39*, 451.
[30] K. S. Tan, A. P. Arnold, D. L. Rabenstein, *Can. J. Chem*. **1988**, *66*, 54.
[31] B. Nygård, *Ark. Kemi* **1967**, *27*, 341.
[32] K. L. House, R. B. Dunlap, J. D. Odom, Z.-P. Wu, D. Hilvert, *J. Am. Chem. Soc*. **1992**, *114*, 8573.
[33] I. Uson, G. M. Sheldrick, *Curr. Opin. Struct. Biol*. **1999**, *9*, 643.
[34] D. Besse, N. Budisa, W. Karnbrock, C. Minks, H. J. Musiol, S. Pegoraro, F. Siedler, E. Weyher, L. Moroder, *Biol. Chem*. **1997**, *378*, 211.
[35] W. A. Hendrickson, J. R. Horton, D. M. Lemaster, *EMBO J*. **1990**, *9*, 1665.
[36] R. Singh, G. M. Whitesides, *J. Org. Chem.* **1991**, *56*, 6931.
[37] S. Pegoraro, S. Fiori, S. Rudolph-Bohner, T. X. Watanabe, L. Moroder, *J. Mol. Biol*. **1998**, *284*, 779.
[38] S. Pegoraro, S. Fiori, J. Cramer, S. Rudolph-Bohner, L. Moroder, *Protein Sci*. **1999**, *8*, 1605.
[39] L. Z. Yan, P. E. Dawson, *J. Am. Chem. Soc*. **2001**, *123*, 526.
[40] D. Savithri, C. Leumann, R. Scheffold, *Helv. Chim. Acta* **1996**, *79*, 288.
[41] D. L. Klayman, T. S. Griffin, *J. Am. Chem. Soc*. **1973**, *95*, 197.
[42] E. M. Stocking, J. N. Schwarz, H. Senn, M. Salzmann, L. A. Silks, *J. Chem. Soc.-Perkin Trans. 1* **1997**, *16*, 2443.
[43] L. Kisfaludy, I. Schön, *Synthesis* **1983**, 325.
[44] D. Besse, L. Moroder, *J. Pept. Sci*. **1997**, *3*, 442.
[45] C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, *Protein Sci*. **1995**, *4*, 2411.
[46] J. W. Williams, J. F. Morrison, *Methods Enzymol*. **1979**, *63*, 437.
[47] S.-H. Chu, H.-G. Mautner, *J. Am. Chem. Soc.* **1966**, *31*, 308.

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