[7] Semisynthesis of Proteins Containing Selenocysteine

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Introduction

Selenocysteine (Sec or U) is often referred to as the 21st amino acid.[1] Yet, unlike other nonstandard amino acid residues (such as hydroxyproline), selenocysteine is not created by posttranslational modification. Instead, selenocysteine shares many features with the 20 common amino acids. Like those, selenocysteine (1) has its own codon, (2) has its own unique tRNA molecule, and (3) is incorporated into proteins cotranslationally.[2]

Currently there are more than 20 known mammalian proteins that contain selenocysteine.[3,4] Detailed structural and functional information about most of these proteins is lacking because of their low natural abundance and the absence of a method to produce selenium-containing proteins by recombinant DNA

[1] A. Bock, K. Forchhammer, J. Heider, W. Leinfelder, G. Sawers, B. Veprek, and F. Zinoni, *Mol. Microbiol.* **5,** 515 (1991).
[2] D. Hatfield and A. Diamond, *Trends Genet.* **9,** 69 (1993).
[3] A. Lescure, D. Gautheret, P. Carbon, and A. Krol, *J. Biol. Chem.* **274,** 38147 (1999).
[4] D. Behne and A. Kyriakopoulos, *Annu. Rev. Nutr.* **21,** 453 (2001).

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0076-6879/02 $35.00

technology. There is one report of formate dehydrogenase being overproduced in *Escherichia coli*[5] and a description of the production of rat thioredoxin reductase (TR) in *E. coli*.[6] The gene for tRNA^{Sec} also had to be overexpressed to achieve formate dehydrogenase production. Moreover, formate dehydrogenase could be produced because it is native to *E. coli* and the elaborate machinery required for selenocysteine insertion is present in its gene. To produce TR, a gene fusion was created that placed a bacterial selenocysteine insertion sequence (SECIS) element immediately downstream of the UGA stop codon. There, the SECIS element allowed decoding of the UGA codon as one for selenocysteine. This strategy is possible only because the UGA codon in TR is proximal to the 3′ end of its mRNA. This strategy is not viable for other selenocysteine-containing proteins in which the UGA codon is distal from the 3′ end. Selenium incorporation into TR was low, even with the overexpression of several accessory genes.

The limitations to the biosynthesis of selenium-containing proteins are severe. Chemical modification has been used to overcome these limitations in one instance. Specifically, the active-site serine residue of subtilisin was converted to selenocysteine by activation with phenylmethylsulfonyl fluoride (PMSF) and reaction with hydrogen selenide.[7] This approach is not general, however, as it relies on the especially high reactivity of the active-site serine residue in subtilisin.

A semisynthetic approach for incorporating selenocysteine residues into proteins has become evident to us. Our approach makes use of "native chemical ligation."[8,9] Native chemical ligation allows for two peptide chains to be joined chemoselectively through the use of a C-terminal thioester on one peptide and an N-terminal cysteine residue on the other peptide (Fig. 1). Several proteins have been synthesized using this method.[10] A technique related to native chemical ligation is called "expressed protein ligation."[11] This technique makes use of an engineered intein. Inteins are a type of mobile genetic element at the protein level.[12] *In vivo,* inteins catalyze their own excision from a larger precursor. The flanking regions of this precursor are referred to as exteins.[13] (Inteins also have a homing endonuclease activity that allows for the insertion of their DNA into the host.) The protein splicing ability of inteins is a powerful tool for protein engineering. When a target

[5] G. T. Chen, M. J. Axley, J. Hacia, and M. Inouye, *Mol. Microbiol.* **6,** 781 (1992).

[6] E. S. Arner, H. Sarioglu, F. Lottspeich, A. Holmgren, and A. Bock, *J. Mol. Biol.* **292,** 1003 (1999).

[7] Z.-P. Wu and D. Hilvert, *J. Am. Chem. Soc.* **111,** 4513 (1989).

[8] T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang, and H. Lau, *Liebig's Ann. Chem.* **583,** 129 (1953).

[9] P. E. Dawson, T. W. Muir, I. Clark-Lewis, and S. B. Kent, *Science* **266,** 776 (1994).

[10] P. E. Dawson and S. B. H. Kent, *Annu. Rev. Biochem.* **69,** 923 (2000).

[11] T. W. Muir, D. Sondhi, and P. A. Cole, *Proc. Natl. Acad. Sci. U.S.A.* **95,** 6705 (1998).

[12] A. A. Cooper and T. H. Stevens, *Trends Biochem. Sci.* **9,** 351 (1995).

[13] F. B. Perler, E. O. Davis, G. E. Dean, F. S. Gimble, W. E. Jack, N. Neff, C. J. Noren, J. Thorner, and M. Belfort, *Nucleic Acids Res.* **22,** 1125 (1994).

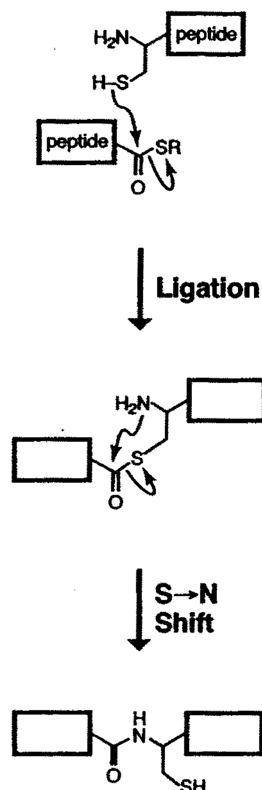

FIG. 1. Scheme for native chemical ligation, which is the chemoselective ligation of peptide segments via a C-terminal thioester and an N-terminal cysteine residue.

protein is fused to an intein, the intein catalyzes the formation of a thioester at the C terminus of the target protein (Fig. 2). This thioester exists in equilibrium with the amide starting material. Addition of exogenous thiol drives the equilibrium toward the thioester, resulting in cleavage of the target protein from the intein. The cleaved target protein now has a thioester moiety at its C terminus. The reactivity of this thioester can be used to ligate an exogenous peptide containing an N-terminal cysteine to the target protein (Fig. 2).

Strategies for Synthesis of Proteins Containing Selenocysteine

The methods we describe herein for the synthesis of selenocysteine-containing proteins are variations of the peptide ligation method first described by Wieland *et al.*[8] and later developed by Kent and co-workers.[9] This method makes use of

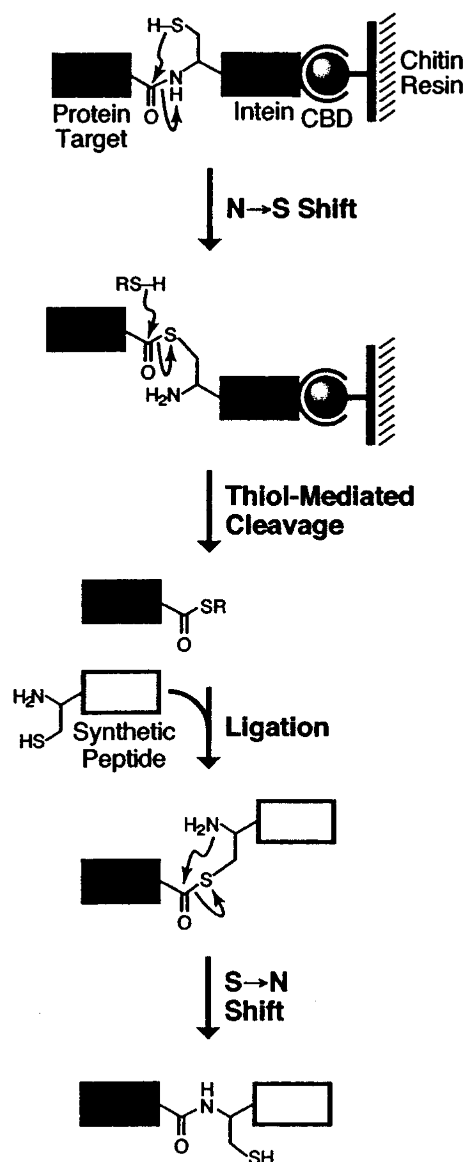

FIG. 2. Scheme for expressed protein ligation, which is the chemoselective ligation of a protein segment with a C-terminal thioester and a peptide segment with an N-terminal cysteine (or, here, selenocysteine) residue. CBD, Chitin-binding domain.

TABLE I
STRATEGIES FOR SEMISYNTHESIS OF PROTEINS CONTAINING SELENIUM BY CHEMICAL LIGATION OF PEPTIDE/PROTEIN FRAGMENTS

Strategy	Fragment: N Terminal	Fragment: C Terminal	Selenocysteine participates in ligation reaction?	Target selenoprotein
1	$H_2N\cdots Sec\cdots C(O)SR$	$Cys\cdots C(O)OH$	No	Selenoprotein W
2	$H_2N\cdots C(O)SR$	$Cys\cdots Sec\cdots C(O)OH$	No	Thioredoxin reductase
3	$H_2N\cdots C(O)SR$	$Sec\cdots C(O)OH$	Yes	C110U RNase A
4	$H_2N\cdots C(O)SR$	SecOH	Yes	Sec125 RNase A

peptide ligation through a C-terminal thioester and an N-terminal cysteine residue (Fig. 1). Using this general method, we envision four distinct strategies for introducing selenocysteine into a protein, as summarized in Table I.

1. A peptide that contains an embedded selenocysteine residue and C-terminal thioester could be ligated to a peptide that contains an N-terminal cysteine residue. This strategy could be applied to the semisynthesis of selenoprotein W, which has a single selenocysteine residue proximal to its N terminus.[14]
2. A selenocysteine residue could be embedded within a peptide that has an N-terminal cysteine residue such that the selenocysteine does not participate directly in the ligation reaction. This peptide could then be ligated to a thioester fragment. This strategy is viable for TR, which has a single selenocysteine residue proximal to its C terminus.
3. A selenocysteine residue could participate directly in the ligation reaction by using selenocysteine rather than cysteine as the N-terminal residue in the C-terminal fragment. In the example described elsewhere[15] and below, we show that selenocysteine can indeed replace cysteine in the ligation reaction to create a semisynthetic RNase A molecule containing a selenocysteine residue.
4. Selenocysteine itself could be used to cleave a target protein–intein fusion. The result is the target protein with an extra C-terminal selenocysteine residue. This approach is useful for adding a nucleophile with orthologous reactivity to a protein. Below, we show how to add a selenocysteine residue to the C terminus of RNase A.

[14] S. C. Vendeland, M. A. Beilstein, C. L. Chen, O. N. Jensen, E. Barofsky, and P. D. Whanger, *J. Biol. Chem.* **268,** 103 (1993).
[15] R. J. Hondal, B. L. Nilsson, and R. T. Raines, *J. Am. Chem. Soc.* **123,** 5140 (2001).

Embedding selenocysteine within a peptide (so as to effect strategies 1 and 2) is not described explicitly herein, but is a straightforward extension of the methods described below.

Preparation of Selenocysteine for Peptide Synthesis

Synthesis of Disodium Diselenide

A 1 *M* solution of Na_2Se_2 is prepared by the procedure of Klayman and Griffin.[16] Elemental selenium (4.5 g; 56 mmol) is 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) is added dropwise to the slurry of elemental selenium. The flask may be chilled in an ice bath to prevent boiling. After all of the sodium borohydride is added and the solution has become colorless, additional elemental selenium (4.5 g; 56 mmol) is added to the solution. The solution should be reddish brown, which is characteristic of disodium diselenide. The flask is then stoppered and flushed with Ar(g) or N_2(g).

Synthesis of Selenocystine

The following procedure is essentially the same as that described by Tanaka and Soda.[17] β-Chloro-L-alanine (5.0 g; 31 mmol) is dissolved in water (40 ml), and the pH of the resulting solution is adjusted to pH 9.0. The resulting solution is 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 is stirred under Ar(g) or N_2(g) at 37° for 12–16 hr. The solution is then acidified with concentrated HCl until vigorous reaction stops, and hydroxylamine (0.33 g; 9.7 mmol) is added to reduce remaining elemental selenium. Additional concentrated HCl is added until there is no more vigorous reaction with the solution. The resulting solution is flushed for at least 1 hr with Ar(g). (The hydrogen selenide exhaust can be trapped with a saturated aqueous solution of lead acetate.) The solution is then filtered, and the pH of the yellow filtrate is adjusted to pH 6–6.5. If the concentration of selenocystine is high, then a yellow precipitate forms immediately. If the concentration is low, then the solution is cooled to 4° overnight and yellow crystals of selenocystine are collected. The yellow crystals of selenocystine may contain some black material. Selenocystine can be recrystallized by dissolving the crystals in the smallest volume possible of 2 *N* HCl. This dark yellow solution is filtered to remove the black material. The pH of the yellow filtrate is then increased to pH 6–6.5 by the addition of 10 *N* NaOH. Selenocystine is isolated as a bright yellow crystalline solid in 60% yield (4.1 g from 5.0 g of β-chloro-L-alanine).

[16] D. L. Klayman and T. S. Griffin, *J. Am. Chem. Soc.* **95,** 197 (1973).
[17] H. Tanaka and K. Soda, *Methods Enzymol.* **143,** 240 (1987).

Synthesis of Sec(PMB)OH

A *p*-methoxybenzyl (PMB) group is used to protect the selenium, according to the examples of Koide *et al.*[18] and Besse and Moroder.[19] The following procedure was adapted from Koide *et al.*[18] Their procedure uses an excess of *p*-methoxybenzyl chloride in the reaction under highly basic conditions. In our hands, this procedure always results in a dibenzylated product in which the nitrogen as well as the selenium of selenocysteine are alkylated with a PMB group. To produce a product that is alkylated only at selenium, the following protocol is used. Selenocystine (1.8 g; 5.3 mmol) is dissolved in 0.5 *N* NaOH (5 ml). $NaBH_4$ (1.7 g; 43 mmol) dissolved in water (10 ml) is added dropwise and with stirring to the solution of selenocystine in a 100-ml round-bottom flask. The flask can be chilled to prevent boiling. After the vigorous reaction has subsided (the solution turns from yellow to colorless), glacial acetic acid is added dropwise until the pH is near 6.0. *p*-Methoxybenzyl chloride (1.44 ml; 10.64 mmol) is then added dropwise to the solution. The reaction proceeds quickly and is complete in 30 min. White crystals of selenium-benzylated selenocysteine are apparent along with some reoxidized selenocystine, which appears as yellow crystals. The solution is acidified with concentrated HCl to complete the formation of the white precipitate, which is purified by crystallization from hot water. Sec(PMB)OH–HCl is isolated as a white solid in 72% yield (2.5 g from 1.8 g of selenocystine).

Synthesis of Fmoc-Sec(PMB)OH

The procedure is from Koide *et al.*[18] but is again modified. Sec(PMB)OH–HCl (1.2 g; 3.7 mmol) is dissolved in water (10 ml) to make a slurry. Triethylamine (TEA; 0.27 ml; 3.7 mmol) is added to the slurry in a 100-ml round-bottom flask. Fmoc-*O*-succinimide (where "Fmoc" refers to 9-fluorenylmethoxycarbonyl; 1.25 g; 3.7 mmol), dissolved in acetonitrile (10 ml), is added to the solution and another equivalent of TEA is then added. Additional acetonitrile should be added until all of the solutes are dissolved completely. The resulting solution is stirred at room temperature for 1 hr, and reaction progress is monitored by thin-layer chromatography on silica plates. In dichloromethane, Fmoc-Sec(PMB)OH will not migrate, but impurities from the reaction, especially dibenzofulvene, will have high mobility. After 1 hr, the reaction mixture is acidified with 1 *N* HCl (5 ml) and then extracted with ethyl acetate. The organic layer is washed (three times) with 1 *N* HCl. The resulting, combined aqueous phases are then extracted (three times) with ethyl acetate. The ethyl acetate extracts are combined and dried over

[18] T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, and N. Fujii, *Chem. Pharm. Bull.* **41,** 502 (1993).

[19] D. Besse and L. Moroder, *J. Peptide Sci.* **3,** 442 (1997).

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                                      2
1                                     0
K E T A A A K F E R Q H M D S S T S A A
S S S N Y C N Q M M K S R N L T K D R C
K P V N T F V H E S L A D V Q A V C S Q
K N V A C K N G Q T N C Y Q S Y S T M S
I T D C R E T G S S K Y P N C A Y K T T
Q A N K H I I V A C E G N P Y V P V H F
D A S V
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      2
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FIG. 3. Primary sequence of RNase A. To produce C110U RNase A, residues 1–109 are synthesized as a fusion protein to the *Mxe* GyrA intein, as shown in Fig. 2. RNase A(1–109) is cleaved from the fused intein and then ligated to a synthetic peptide (underlined) with selenocysteine replacing cysteine (boldface).

$MgSO_4$(s). After filtering, the organic layer is concentrated under vacuum to produce a yellow oil. This oil is dissolved in dichloromethane (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, are eluted when the column is washed extensively with CH_2Cl_2, and Fmoc-Sec(PMB)OH is eluted with methanol–CH_2Cl_2 (1 : 4, v/v). The solvent is removed under vacuum. Fmoc-Sec(PMB)OH is 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].

Solid-Phase Synthesis of 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 is used for all syntheses. 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, are used to couple monomers. Syntheses are done on a 25-μmol scale with a 3-fold excess of each amino acid monomer, using an Applied Biosystems (Foster City, CA) model 432A synthesizer.

As a model protein to demonstrate the efficacy of our methods, we chose ribonuclease A (RNase A; EC 3.1.27.5), which has been the object of much seminal work in protein chemistry and enzymology.[20] RNase A has 124 residues, including 8 cysteine residues that form 4 disulfide bonds in the native enzyme (Fig. 3). Of these eight cysteine residues, Cys-110 is closest to the C terminus. A semisynthetic

[20] R. T. Raines, *Chem. Rev.* **98**, 1045 (1998).

RNase A has already been constructed by expressed protein ligation to form the peptide bond between Ala-109 and Cys-110.[21] The sequence of the wild-type RNase A peptide used in our ligation reactions is CEGNPYVPVHFDASV (which corresponds to residues 110–124) and the sequence of the selenocysteine variant is UEGNPYVPVHFDASV, where "U" refers to selenocysteine (Fig. 3).

Deprotection of Peptides Containing Selenocysteine

Deprotection of the wild-type peptide (which does not contain selenocysteine) is achieved by using a cleavage cocktail containing trifluoroacetic acid–ethanedithiol–H_2O (95 : 2.5 : 2.5, v/v/v) for 3 hr at room temperature. When this cocktail is used for the selenocysteine-containing peptide, only partial removal of the PMB group is achieved. The PMB group is removed successfully, however, using conditions reported by Koide *et al.*[18] The cleavage cocktail for the selenium-containing peptide contains *m*-cresol–thioanisole–trifluoroacetic acid–trimethylsilyl trifluoromethane sulfonate (50 : 120 : 690 : 194, v/v/v/v). After purification by high-performance liquid chromatography (HPLC), the intact peptide is observed, along with some dehydrated peptide (Fig. 4). Dehydration most likely occurs during deprotection.

Semisynthesis of a Protein Containing Selenocysteine

One way to incorporate selenocysteine into a protein is to ligate a protein fragment with a C-terminal thioester to a peptide that contains an N-terminal selenocysteine residue (strategy 3 in Table I).[15] To incorporate a selenocysteine residue into RNase A, a protein is produced in which residues 1–109 of RNase A are fused to the *Mxe* GyrA intein and a chitin-binding domain.[21] Plasmid pTXB1-RNase (a kind gift from New England BioLabs, Beverly, MA) is transformed into ER2566 *E. coli* cells. Luria–Bertani (LB) medium (0.10 liter) containing ampicillin (0.10 mg/ml) is inoculated with a single colony and grown for 8 hr at 37°. Six 2-liter flasks that each contain 1 liter of LB medium containing ampicillin (0.10 mg/ml) are then inoculated with 10 ml of the 8-hr culture, and grown at 37° until the A_{600} equals 0.6. The flasks are then cooled on ice for 10 min, and expression is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, to 0.5 mM). The flasks are shaken at room temperature for 5 hr. Cells are harvested by centrifugation, and the wet cell pellet is frozen for storage. Frozen cells are thawed and homogenized in 50 mM Tris-HCl buffer, pH 8.5, containing NaCl (0.50 M), and then lysed by sonication. Lysed cells are subjected to centrifugation at 6000g for 30 min at 4°. The supernatant is passed over a 30-ml column of chitin resin

[21] T. C. Evans, Jr., J. Benner, and M.-Q. Xu, *Protein Sci.* **7**, 2256 (1998).

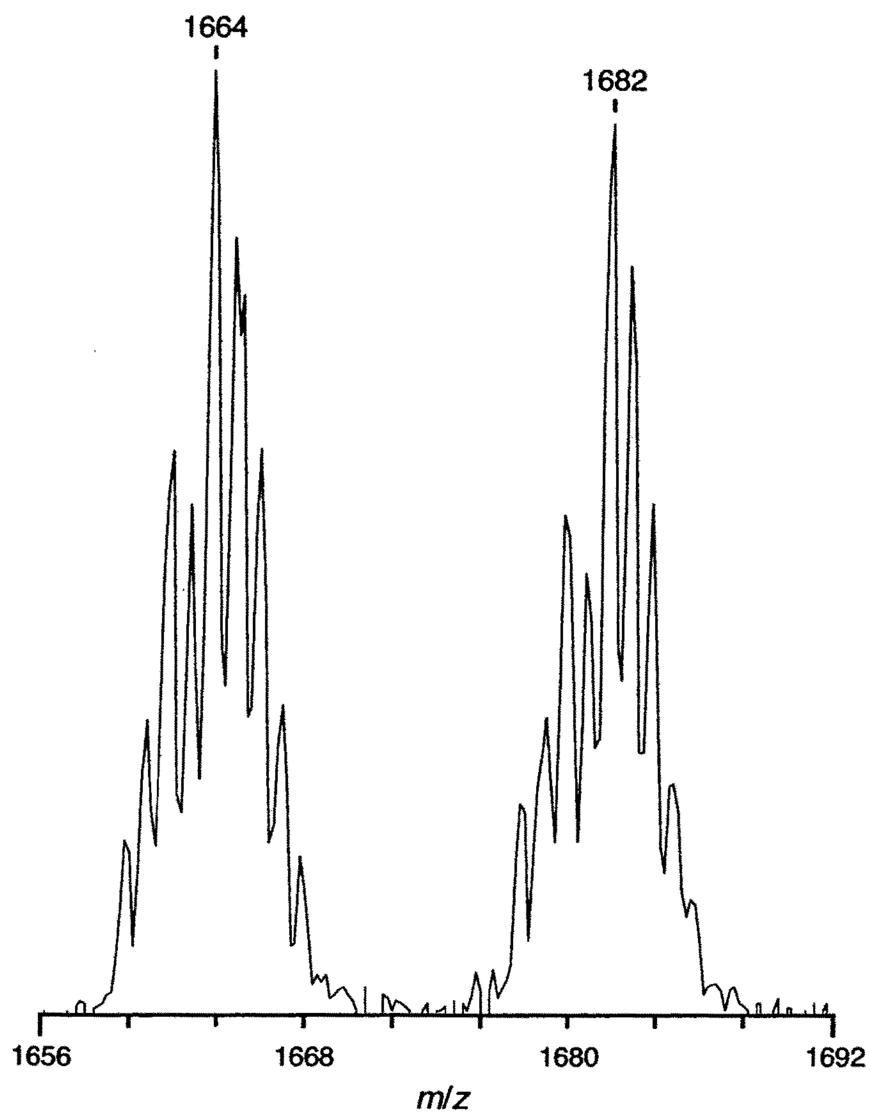

FIG. 4. Electrospray-ionization (ESI) mass spectrum of the deprotected selenocysteine-containing peptide corresponding to residues 110–124 of RNase A ($m/z = 1682$). The dehydrated peptide is also present ($m/z = 1664$).

(New England BioLabs) that had been equilibrated with the same buffer. After loading, the column is washed with 5 column volumes of buffer to elute non-specific proteins. Cleavage of RNase A(1–109) is initiated by equilibrating the column in cleavage buffer, which is 50 m*M* Tris-HCl buffer, pH 7.4, containing NaCl (0.10 *M*) and *N*-methylmercaptoacetamide (NMA; 50 m*M*) as the cleavage reagent. We have found NMA to be better than 2-mercaptoethanol, dithiothreitol (DTT), or 2-mercaptoethanesulfonic acid for the cleavage of target protein–intein fusions (data not shown). The chitin resin is incubated with the cleavage buffer for 2 hr at room temperature. Fractions containing the thioester-tagged RNase A(1–109) are then collected. The protein solution is concentrated with a Centriplus 3000 apparatus (Amicon, Danvers, MA) to a volume of 5 ml. For ligation reactions with the peptide UEGNPYVPVHFDASV, 5 ml of 20 m*M* Tris-HCl buffer, pH 7.0, containing guanidine hydrochloride (7 *M*) is added to the protein solution containing RNase A(1–109). The solution is then added to the lyophilized peptide (15 mg; 10 μmol) and the resulting solution is incubated overnight at room temperature. The protein is unfolded by adding 10 ml of 20 m*M* Tris-HCl buffer, pH 8.0, containing guanidine hydrochloride (7 *M*). Dithiothreitol is added (to 0.10 *M*) to reduce any disulfide or selenosulfide bonds, and the resulting solution is incubated for 1 hr at room temperature. This solution is then dialyzed overnight at 4° against folding buffer, which is 50 m*M* Tris-HCl buffer, pH 7.8, containing NaCl (0.10 *M*), reduced glutathione (GSH; 1 m*M*), and oxidized glutathione (GSSG; 0.2 m*M*). The protein solution is dialyzed for an additional 4 hr against fresh refolding buffer and then overnight against 50 m*M* 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 using GDP–Sepharose resin (Sigma, St. Louis, MO) is used to purify the intact protein. A 5-ml column of GDP–Sepharose equilibrated with 10 m*M* sodium acetate buffer, pH 5.0, is loaded with the protein solution and then washed with 50 ml of the same buffer. The bound protein is then eluted with buffer containing NaCl (1 *M*). Fractions containing active RNase A are dialyzed against 50 m*M* sodium acetate buffer, pH 5.0, overnight at 4°. Approximately 1 mg of pure C110U RNase A can be prepared in this manner from 6 liters of culture.

pH of Ligation Reactions

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

Characterization of Proteins Containing Selenocysteine

The atomic mass of selenium is 47 amu greater than that of sulfur. Hence, mass spectrometry is a useful tool for demonstrating the replacement of sulfur by selenium. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectral analysis (Fig. 5) shows that the mass of folded wild-type RNase A is 13,820 Da (13,812 Da predicted) and that of folded C110U RNase A is 13,865 Da (13,820 Da predicted). Not only are the experimental values in excellent agreement with the predicted values, but also the mass difference of 45 amu is close to that expected for the replacement of sulfur by selenium.

A critical measure of the successful ligation and folding of a selenium-containing protein is the demonstration of function. For C110U RNase A, that demonstration involves measuring ribonucleolytic activity. The k_{cat}/K_m for the cleavage of 6-FAM~dArU(dA)$_2$~6-TAMRA[22] is $(1.13 \pm 0.06) \times 10^7\ M^{-1}\ sec^{-1}$ for wild-type RNase A and $(1.1 \pm 0.1) \times 10^7\ M^{-1}\ sec^{-1}$ for C110U RNase A. The similarity of these values indicates that selenium can replace sulfur with minimal perturbation to function and (presumably) structure.

An important problem to consider when using selenocysteine in ligation reactions is the redox state of the selenium in the folded protein. RNase A contains eight cysteine residues that are oxidized to form four disulfide bonds in the native enzyme. When replacing cysteine with selenocysteine in RNase A, misfolding could result in the irreversible formation of an improper selenosulfide bond. Selenosulfide bonds are more difficult to reduce than disulfide bonds,[23] and thus could limit the yield of properly folded protein. Indeed, the overall yield of C110U RNase A is low. Yet, the main difficulty encountered is the recovery of sufficient amounts of thioester-tagged RNase A(1–109). As noted by Evans *et al.*[21] 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. The low yield of C110U RNase A may not be attributable to selenocysteine because the yields of other variants of RNase A produced by expressed protein ligation are similar (R. J. Hondal and R. T. Raines, unpublished results, 2000).

Use of Selenocysteine as Cleavage Reagent

Another way to incorporate a selenocysteine residue into a protein is to use selenocysteine itself to cleave the thioester produced by intein fusion (strategy 4 in Table I). A 30-ml solution of 0.10 *M* selenocysteine is prepared as follows.

[22] B. R. Kelemen, T. A. Klink, M. A. Behlke, S. R. Eubanks, P. A. Leland, and R. T. Raines, *Nucleic Acids Res.* **18,** 3696 (1999).

[23] D. Besse, F. Siedler, T. Diercks, H. Kessler, and L. Moroder, *Angew. Chem. Int. Ed. Engl.* **36,** 883 (1997).

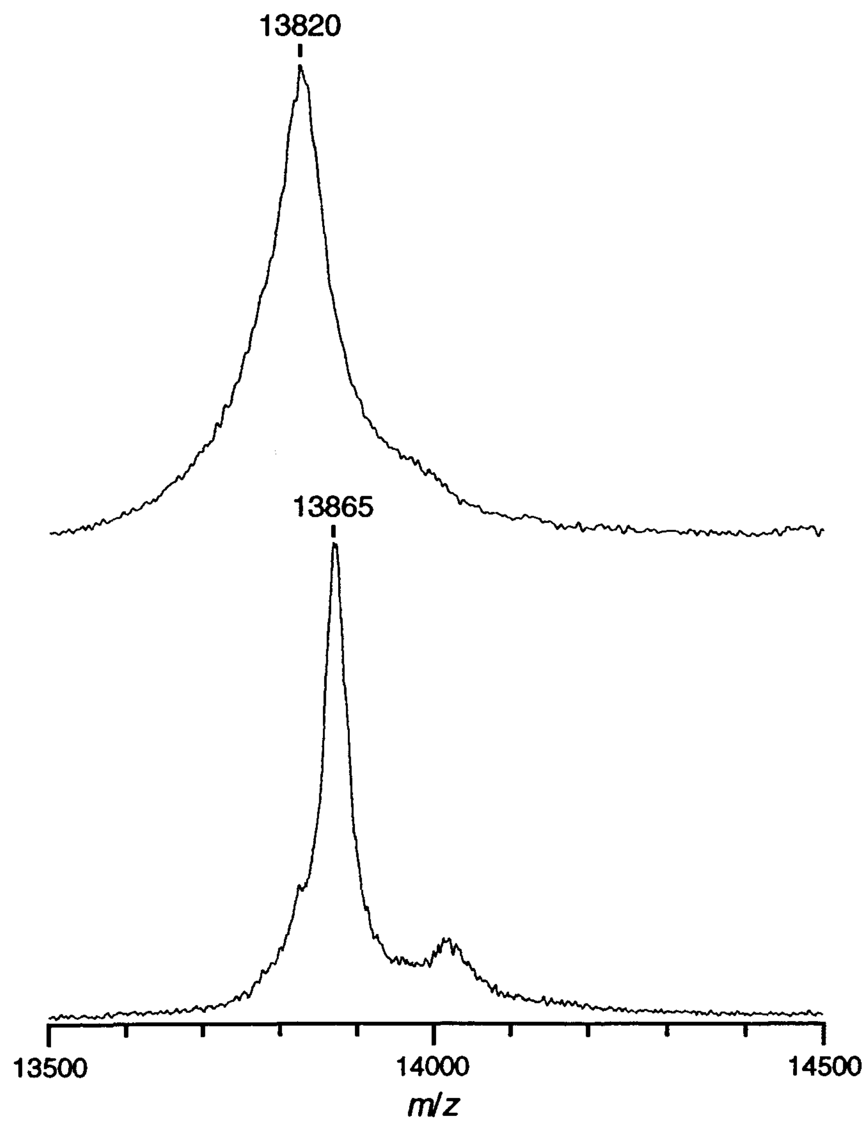

FIG. 5. MALDI-TOF mass spectra of semisynthetic wild-type RNase A (*top*; m/z = 13,820) and C110U RNase A (*bottom*; m/z = 13,865).

A 25-ml solution of 0.2 *N* NaOH is flushed with Ar(g) for 1 hr. Selenocystine (507 mg) is dissolved in this solution. Water (5 ml) is flushed with Ar(g) for 1 hr, and tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 430 mg; 1.5 mmol) is dissolved in the water. The two aqueous solutions are mixed, and the pH of the resulting solution is adjusted to pH 6.0 by the addition of 10 *N* NaOH. This 0.10 *M* solution of selenocysteine is then applied to a target protein–intein fusion that has been purified by affinity chromatography with a chitin resin. To do so, the buffer from the chitin resin is drained until the resin is nearly dry. The resin is transferred to the solution of selenocysteine and incubated overnight at room temperature. The slurry of resin is filtered, and the protein in the filtrate has one selenocysteine residue at its C terminus. (*Note:* The selenocysteine residue may be in the form of a mixed diselenide with free selenocysteine.) Using this method, we have prepared RNase A containing an additional residue, Sec-125, at its C terminus (R. J. Hondal and R. T. Raines, unpublished results, 2001).

Summary

The methods described herein can be used to incorporate one or more selenocysteine residues into a protein. These methods enable detailed structure–function analyses of proteins containing selenocysteine. In addition, the methods provide a means to incorporate a selenol into a protein.

Acknowledgments

We thank Dr. U. Arnold, B. L. Nilsson, and C. L. Jenkins for advice and assistance. R.J.H. was supported by Postdoctoral Fellowship GM20180 (NIH). This work was supported by Grant GM44783 (NIH).