Published in final edited form as:

Methods Mol Biol. 2018 ; 1661: 265–283. doi:10.1007/978-1-4939-7258-6_19.

Selenocysteine Mediated Expressed Protein Ligation of SELENOM

Jun Liu, Qingqing Chen, and Sharon Rozovsky

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware, USA

Abstract

A sizeable fraction of the selenoproteome encodes oxidoreductases possessing a thioredoxin fold, a structural motif that is shared among a diverse group of enzymes. In these oxidoreductases, the active site is comprised of a cysteine and a selenocysteine separated by one to two amino acids. In a subset of these selenoproteins, such as human SELENOH, SELENOM, SELENOT, SELENOV, SELENOW, and SELENOF, this redox motif is positioned immediately after the first β -sheet in a short loop, and is essential for interactions with its substrate or partners. Here we describe the preparation of a representative member of this group, SELENOM, by selenocysteine-driven expressed protein ligation. The preparation employs a peptide bond formation between two protein fragments expressed recombinantly in *E. coli*. This method can be employed to prepare other selenoproteins.

Keywords

Selenoproteins; SELENOM; selenoprotein M; expressed protein ligation; selenocysteine-mediated expressed protein ligation

1. Introduction

A large percentage of the selenoproteome is composed of selenoproteins whose catalytic Sec resides in a thioredoxin (Trx) fold. The fundamental Trx fold is composed of a four stranded β -sheets, packed on one side flanked by two α -helices, although variations to this basic unit are common [1]. This fold encompasses several superfamilies of enzymes with functions from detoxification, to the formation and reduction of disulfide bonds in proteins and hydroperoxides [2]. In the majority of enzymes possessing the Trx fold, the redox-active site is typically positioned right before an α helix, capping it (Fig. 1). In contrast, in a subset of selenoproteins, the redox active motif Cys-X-X-Sec (where X stands for any amino acid and Sec stands for selenocysteine) is positioned immediately after the first β -sheet [3]. A conformational switch following the reduction of the intramolecular selenylsulfide bond was proposed to trigger a signaling event or binding of protein partners [4]. In humans, SELENOH, SELENOM, SELENOT, SELENOV, SELENOW, and SELENOF exhibit this common Trx fold and placement of the selenoredox motif [5,6]. This chapter details the

preparation of a representative member of this group, SELENOM, in a manner that can be generalized to other selenoproteins with a minimal Trx fold [7].

Like many selenoproteins, SELENOM (UniProt Q8WWX9) is tied to the management of cellular oxidative stress [8–14]. SELENOM resides in the endoplasmic reticulum [15] and its overexpression governs the expression of antioxidant enzymes like glutathione peroxidase and superoxide dismutase [10]. SELENOM knock-out mice showed increases in weight gain and changes in metabolism [16]. It was proposed to regulate energy metabolism through participation in leptin signaling [17]. The NMR-derived structure of *Mus musculus* SELENOM U48C mutant was reported [4] (Fig. 1a) but the redox properties and precise function of the wild type SELENOM with its CGGU motif remain undetermined.

This chapter presents a versatile chemical approach based on native chemical ligation (NCL) to prepare the wild-type selenium-containing SELENOM [18]. In Sec-driven NCL, a Sec residue mediates the formation of the peptide bond from two respective protein fragments to form the target protein [19–22]. As shown in Figure 2, an N-terminal Sec from one fragment initiates the nucleophilic attack of the C-terminal thioester from the complementary fragment and subsequently undergoes a Se→N acyl shift to generate the native amide bond (Fig. 2). The ligation rate of Sec-mediated NCL is faster and more pH tolerant than Cys-mediated ligation, and the resulting ligation efficiency is often higher than that of Cys-mediated reactions [19,23]. Sec-mediated NCL is an attractive method for preparing selenoproteins because it is possible to achieve high yield, and in addition it is compatible with introducing posttranslational modifications or chemical tags into the protein. However, since the preparation of selenium-containing protein fragments has traditionally relied on chemical synthesis, this method was restricted by limitations on the size of the Sec-containing peptide fragment and its solubility [24–27,21]. The production of SELENOM discussed here is distinguished from previous approaches [28] in that all fragments are prepared by heterologous expression in *E. coli* and rely only on standard protein expression and purification [7]. This reduces cost and simplifies preparation by alleviating the need to synthesize the seleno-containing part of the protein.

To generate the Sec-containing fragment of SELENOM (SELENOM^{CT} (residues 48–145)), a cytosolic maltose binding protein (MBP) is coupled to its N-terminus for enhanced expression and solubility, generating MBP-SELENOM^{CT}. A Tobacco Etch Virus (TEV) protease cleavage site is introduced between the MBP and SELENOM^{CT} so that SELENOM^{CT} can be efficiently released since TEV protease has good tolerance at its P1' position in the recognition site ENLYFQ/P1' (where P1' stands for any canonical amino acid except P) [29]. The Sec residue is incorporated into the fragment by mutating the Sec to Cys and subsequently expressing the protein in *E. coli* grown on defined growth medium supplemented with L-selenocystine [30]. The cysteinyl-tRNA is misloaded with Sec, which becomes incorporated instead of Cys. The thioester-containing fragment of SELENOM is produced by intein technology. The N-terminal fragment of SELENOM (residues 25–47) is fused to the *Saccharomyces cerevisiae* Vacuolar Membrane ATPase (Sce VMA1; 454 residues; 56 kDa) intein [31]. SELENOM is then prepared by ligation of the SELENOM^{CT} to SELENOM^{NT} under native conditions (Fig. 2). Following ligation, SELENOM is purified

to homogeneity and characterized by SDS-PAGE, mass spectrometry, and circular dichroism spectroscopy.

2. Materials

2.1 Gene, plasmids and enzymes for molecular cloning

1. The expression vector pMAL-c5X, designed to produce fusion proteins with MBP, is from New England Biolabs (NEB). To aid purification, a general cloning vector was prepared by introducing a hexahistidine tag to the N-terminus of MBP [32]. In addition, a TEV protease site was inserted at the C-terminus of MBP to facilitate efficient release of the target protein or protein fragment. The VMA intein was introduced following MBP and the TEV cleavage site allowing cloning of the protein of interest fused to MBP, the VMA intein or both. This pMAL-c5X-VMA cloning vector is available through Addgene (plasmid #86590) [7].
2. The gene of *Homo sapiens* SELENOM (accession number: NP_536355.1) optimized for *E. coli* expression can be obtained from Addgene (plasmid #86579). The gene was inserted into a pMAL vector to allow expression of SELENOM U48C fused to hexahistidine-tagged MBP creating the plasmid pMAL-c5X-SELENOM^{U48C}. A TEV protease cleavage site exists between MBP and SELENOM U48C so that SELENOM U48C can be efficiently released from MBP [7].
3. Restriction enzymes SapI and NdeI are from NEB.
4. Q5™ site-directed mutagenesis kit is from NEB.

2.2 Buffers and chemicals for protein expression and purification

1. LB medium: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 2 g/L glucose. Autoclave the medium and allow it to cool to room temperature prior to adding antibiotics.
2. LB agar plate: 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 15 g/L agar, autoclaved. Supplement with 100 µg/mL of ampicillin (Amp) once the solution temperature is below 50 °C but before solidification, and pour into individual petri dishes. Seal the LB agar plate with Parafilm™ after complete solidification, and store at 4 °C for up to one week.
3. Lysis buffer: 50 mM sodium phosphate, 200 mM sodium chloride (NaCl), 2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM benzamidine, pH 7.5.
4. Amylose binding buffer: 50 mM sodium phosphate, 200 mM NaCl, and 2 mM EDTA, pH 7.5.
5. Amylose elution buffer: 50 mM sodium phosphate, 200 mM NaCl, 20 mM maltose, and 2 mM EDTA, pH 7.5.

6. IMAC binding buffer: 50 mM sodium phosphate, 200 mM NaCl, and 10 mM imidazole, pH 7.5.
7. IMAC elution buffer: 50 mM sodium phosphate, 200 mM NaCl, and 1 M imidazole, pH 7.5.
8. Chitin binding buffer: 25 mM 2-(N-morpholino)ethanesulfonic acid (MES), 200 mM NaCl, and 2 mM EDTA, pH 6.5.
9. Chitin cleavage buffer: 25 mM MES, 200 mM NaCl, 75 mM sodium 2-mercaptoethanesulfonate (MESNA), and 2 mM EDTA, pH 6.5.
10. Gel filtration buffer: 50 mM sodium phosphate, 200 mM NaCl, 2 mM EDTA, and 5 mM 1,4-dithio-DL-threitol (DTT), pH 7.5.

2.2 Protein expression and purification

1. Isopropyl 1-thio- β -D-galactopyranoside (IPTG)
2. L-selenocystine (see Note ¹ for preferred vendors of L-selenocystine)
3. Amylose resin and chitin resin from NEB
4. HisTrap™ HP, HiPrep™ 26/10, and HiPrep™ 16/60 Sephacryl™ S-100 HR columns from GE Healthcare Life Sciences

2.3 Expressed selenoprotein ligation

1. Sodium 2-mercaptoethanesulfonate (MESNA)
2. 4-Mercaptophenylacetic acid (MPAA)
2. pH meter with a micro probe

2.4 Mass spectrometry characterization

1. Mass spectrometry autosampler vials (Waters)
2. Trypsin-ultra, mass spectrometry grade (NEB)
3. Iodoacetamide (IAM)
4. Spin desalting columns (Pierce)
5. Buffer for trypsin digestion: 50 mM Tris-hydrochloride, and 20 mM calcium chloride, pH 8.0

2.5 Circular dichroism spectroscopy

1. Circular dichroism spectropolarimeter (such as Jason J-810 spectropolarimeter)
2. 10 mM potassium phosphate buffer, 50 mM sodium sulfate (Na₂SO₄), pH 7.5
3. Superdex™ 75 10/300 GL (GE Healthcare Life Sciences)

¹L-selenocystine is best purchased through Sigma-Aldrich or Acros Organics. In our experience L-selenocystine acquired from other vendors may contain contaminations that impede cell growth and reduce Sec incorporation.

3. Methods

3.1 Molecular cloning of MBP-SELENOM^{NT}-VMA and MBP-SELENOM^{CT}

1. Clone MBP-SELENOM^{NT} gene, which includes MBP, TEV cleavage site and SELENOM residues 25-47, into vector pMAL-c5X-VMA using restriction sites NdeI and SapI to create the vector pMAL-c5X-*SELENOM*^{NT} for the expression of SELENOM^{NT} fragment (Fig. 2). A TEV protease cleavage site assists the efficient release of SELENOM^{NT} fragment from the fusion construct.
2. The expression vector for SELENOM^{CT} is prepared by deleting residues 25-47 from pMAL-c5X-*SELENOM*^{U48C} to generate pMAL-c5X-*SELENOM*^{CT} (SELENOM residues 48-145, beginning with U48 mutated to C48). Mutagenesis can be accomplished with the Q5TM Site-Directed Mutagenesis Kit from NEB.

3.2 Protein expression of MBP-SELENOM^{NT} thioester

1. Transform pMAL-c5X-SELENOM^{NT} into *E. coli* strain BL21(DE3) or BL21. Incubate on ice 30 ng of plasmid DNA with 50 µL of chemically competent BL21(DE3) in a 1.5 mL microcentrifuge tube for 30 min. Heat shock in a water bath at 42 °C for 50 s. Allow the cells to recover on ice for an additional 2-5 min, then add 400 µL of autoclaved LB medium and incubate with shaking at 250 rpm for 1 h at 37 °C. Spin down the cells by centrifugation at 2500 *g* and discard 400 µL of supernatant. Gently resuspend the cell pellet in the remainder of the LB medium and plate cells evenly onto LB agar plates supplemented with 100 µg/mL Amp, and incubate overnight at 37 °C.
2. Begin an overnight culture by inoculating 100 mL of autoclaved LB medium, supplemented with 100 µg/mL Amp, with a few colonies from a freshly transformed plate of MBP-SELENOM^{NT}-VMA. Incubate with shaking at 250 rpm overnight at 37 °C.
3. The next morning, transfer 10 mL of overnight culture to 1 L of fresh autoclaved LB medium supplemented with 100 µg/mL Amp and grow at 37 °C with good aeration. When the optical density (OD) at 600 nm reaches 0.5, the temperature is lowered to 18 °C, and the cells are allowed to shake at the lower temperature for an additional 0.5 h. Protein expression is induced with 0.5 mM of IPTG.
4. The cells are harvested after 18-20 h by centrifugation at 5000 *g* at 4 °C for 15 min. Decant as much supernatant as possible and resuspend the cell paste in the lysis buffer.

3.3 Protein purification of MBP-SELENOM^{NT} thioester

The MBP-SELENOM^{NT} thioester is obtained via two chromatographic steps. First, the MBP-SELENOM^{NT}-VMA fusion protein is obtained by amylose affinity chromatography through MBP binding. Then while immobilized through the chitin-binding domain on the chitin column, the VMA fusion partner is cleaved off using MESNA, releasing the MBP-SELENOM^{NT} fragment in the chitin column flow through while generating the C terminal thioester.

1. Lyse the cells by a high-pressure homogenizer (EmulsiFlex™-C5, Avestin) on ice, operating continuously at 9000 psi.
2. Immediately remove cell debris by a 1 h centrifugation of the cell lysate at 5000 *g* at 4 °C. Collect the supernatant.
3. Carry out amylose purification at 4 °C. Equilibrate the amylose column with 5 column volumes (CV) of amylose binding buffer. Load the supernatant onto the column by gravity.
4. Wash with 5 CV of amylose binding buffer.
5. Elute with 5 CV amylose elution buffer and collect the eluent fusion protein MBP-SELENOM^{NT}-VMA.
6. Equilibrate the chitin column with 4 CV of chitin binding buffer. Load the previous eluent from step 4 onto the chitin column.
7. Wash with 4 CV of chitin binding buffer.
8. Wash with 2 CV of chitin column cleavage buffer, allowing the resin to become fully soaked. Drain the column by gravity until there is about 1 inch of cleavage buffer left above the resin bed, cap the bottom of the column and allow intein cleavage to proceed at 25 °C overnight.
9. Elute with 2 CV of chitin column binding buffer, and collect the fractions that contain MBP-SELENOM^{NT} thioester.
10. Repeat steps 7 and 8 to fully cleave any MBP-SELENOM^{NT}-VMA still bound to the chitin column in order to obtain the highest yield of the thioester fragment (see Note ² regarding the stability of the thioester).
11. Run SDS-PAGE and mass spectrometry to confirm intein cleavage and thioester formation. A typical mass spectrum of the MBP-SELENOM^{NT} with thioester is shown in Fig. 3.
12. Measure protein concentration with an extinction coefficient of 74830 M⁻¹ cm⁻¹; note that excessive thiol reagents may interfere with UV measurements. Concentrate the MBP-SELENOM^{NT} with thioester to 10 mg/mL for later use in expressed selenoprotein ligation of SELENOM.

3.4 Protein expression of MBP-SELENOM^{CT}

The MBP-SELENOM^{CT} fusion protein is obtained via single step purification by amylose affinity chromatography.

1. Transform pMAL-c5X-SELENOM^{CT} into *E. coli* strain BL21(DE3) using the procedure described in Section 3.3.

²Maintain the pH of solutions containing MBP-SELENOM^{NT} thioester or SELENOM^{NT} thioester at or below 6.5, as higher pH accelerates thioester hydrolysis.

2. Begin a small overnight culture by inoculating 10 mL of autoclaved LB medium, supplemented with 100 µg/mL Amp, with a few colonies from a freshly transformed plate of MBP-SELENOM^{CT}. Incubate with shaking at 250 rpm overnight at 37 °C.
3. Collect 1 mL of the above overnight culture and pellet the cells at 2500 *g* for 5 min. Resuspend the pellet in 1 L of modified MDAG growth medium (based on Studier original MDAG [33]) as listed in Table 1 step 1. Grow cells at 37 °C with shaking at 250 rpm until OD at 600 nm exceeds 1.5. This typically takes up to 14 h.
4. Supplement the growth medium as detailed in Table 1 step 2 (see Notes ³ and ⁴ on improving Sec incorporation), continuously shake for an additional 10 min at 37 °C.
5. Lower the temperature to 18 °C, allow cells to equilibrate for an additional 10 min, and induce protein expression by adding 0.5 mM IPTG.
6. Continue protein expression for 18-20 h post-induction at 18 °C, then harvest by centrifugation at 5000 *g* at 4 °C for 15 min. Decant as much of supernatant as possible and resuspend the cell pellet in the lysis buffer.

3.5 Protein purification of MBP-SELENOM^{CT}

1. Lyse the cells by a high-pressure homogenizer on ice, operating continuously at 9000 psi.
2. Immediately remove cell debris by a 1 h centrifugation of the cell lysate at 5000 *g* at 4 °C. Collect the supernatant.
3. Carry out amylose purification at 4 °C. Equilibrate the amylose column with 4 CV of amylose binding buffer. Load the supernatant onto the amylose column, and let it drain by gravity.
4. Wash with 10 CV of amylose binding buffer.
5. Elute with 5 CV amylose elution buffer and collect the sample.
6. Measure MBP-SELENOM^{CT} protein concentration with an extinction coefficient of 79300 M⁻¹ cm⁻¹.
7. Obtain intact mass spectrum of MBP-SELENOM^{CT} to estimate the percentage of Sec incorporation. Take a 0.2 mg/mL sample of MBP-SELENOM^{CT} and incubate with 10 mM DTT at 37 °C for 30 min to reduce the diselenide bond. The protein can then be alkylated by adding excess IAM or analyzed reduced. Inject the sample onto a reversed-phase liquid chromatography (RPLC) column

³Use freshly transformed *E. coli* cells when expressing MBP-SELENOM^{CT} for optimal protein yield and Sec incorporation.

⁴Incorporation of selenium is best carried out by Inductively Coupled Plasma (ICP) - atomic emission spectrometry or ICP - mass spectrometry. However, in simple cases such as proteins with a single amino acid substitution (such as Cys vs. Sec), mass spectrometry provides a good estimation. During acquisition, avoid oversaturating the detector which causes an inaccurate reflection in the respective ion counts of the Cys and Sec-containing species, which in turn leads to errors in deconvolution and misrepresented peak intensities of identified masses.

such as a Waters Xbridge C4 that is appropriate for protein analysis. Perform an acetonitrile elution gradient from 5 – 95% supplemented with 0.1% formic acid. MBP-SELENOM^{CT} elutes close to halfway of the gradient. Sec incorporation is estimated by quantitating the peak intensity of the Sec-containing form over that of the Cys- and Sec-containing protein forms together (See Note ⁴).

With the expression strategy described in Section 3.4, higher than 90% incorporation of Sec is typically achieved for Sec mediated NCL (See Note ⁵ and ⁶ for Sec enrichment). A typical mass spectrum of reduced MBP-SELENOM^{CT} is shown in Fig. 4a.

8. Cleave the MBP-SELENOM^{CT} by TEV protease, purified as previously described [34,32], at 1:5 molar ratio of TEV protease to MBP-SELENOM^{CT} at 25 °C for 16 h in the amylose elution buffer supplemented with 10 mM DTT. A strong reducing reagent such as DTT, and room temperature is required for efficient TEV cleavage of MBP-SELENOM^{CT} (See Fig. 4b and Notes ⁷ and ⁸ on selection of reducing agents). Monitor the progress of TEV cleavage by 16% Tris-Glycine SDS-PAGE.

3.6 Expressed protein ligation and purification of SELENOM

1. Add to the cleavage mixture from Section 3.5 (MBP-SELENOM^{CT} with TEV protease in 50 mM sodium phosphate, 200 mM NaCl, 2 mM EDTA, pH 7.5), the MBP-SELENOM^{NT} thioester obtained in Section 3.3 (MBP-SELENOM^{NT} thioester in 25 mM MES, 200 mM NaCl, 2 mM EDTA, pH 6.5). The molar ratio of SELENOM^{CT} to SELENOM^{NT} thioester is 1:5. Add to the ligation mixture 100 mM MESNA, 50 mM MPAA, and 5 mM DTT. Monitor the reaction progress by the disappearance of SELENOM^{CT} and the appearance of SELENOM using 16% Tris-Glycine SDS-PAGE or Tricine-SDS-PAGE. Ligation is typically over 90% complete within 1 day at 25 °C at pH 7.0 (Fig. 5). However, the optimal pH as seen in Fig. 6 is at 6.5 (Note ⁹).
2. Once 90% of SELENOM^{CT} is ligated to form SELENOM, exchange the reaction buffer into the IMAC binding buffer by desalting column such as HiPrep 26/10 or by dialysis. This step is necessary to remove EDTA and DTT, which are incompatible with IMAC resin at their current concentrations of 2 mM and 10 mM, respectively.

⁵Purification of MBP-SELENOM^{CT} is carried out under non-reducing conditions, because the Sec-containing MBP-SELENOM^{CT} forms an intermolecular diselenide bond faster than the formation of disulfide bond by the Cys-containing variant. Extended wash helps to enrich the fraction of Sec-containing MBP-SELENOM^{CT} due to tighter binding of the dimeric diselenide-containing form to affinity columns over that of the monomeric Cys-containing form.

⁶Excess L-selenocystine in the growth medium can lead to decreased yield due to cellular toxicity. In our experience, 0.1 g L-selenocystine per L growth medium resulted in incorporation of over 90% Sec into target proteins. In the cases where the percent of Sec incorporation is low the concentration of L-selenocystine can be increased to 0.15 g per L growth medium at the cost of about 20-30% decrease in protein yield.

⁷MBP-SELENOM^{CT} forms a diselenide bond that prevents efficient TEV protease cleavage. Therefore, reducing reagents such as DTT or tris(2-carboxyethyl)phosphine (TCEP) is required to break the diselenide bond during TEV protease cleavage. Similarly, the diselenide bond between SELENOM^{CT} must be broken during ligation using reducing agents.

⁸When possible, avoid the use of TCEP and tris(hydroxypropyl)phosphine (THP) which can promote selenium elimination from Sec.

⁹Hydrolysis of the thioester increases with increased pH. Sec-mediated ligation can be carried out at pH lower than 7.0 to stabilize the thioester (Fig. 6).

3. Equilibrate a 5 mL HisTrap™ HP column with 5 CV IMAC binding buffer.
4. Load the protein mixture onto the HisTrap™ HP column and wash with 5 CV IMAC binding buffer. Run the column with a linear gradient starting with the IMAC binding buffer and ending with IMAC elution buffer (corresponding to an increase from 10 mM to 1M imidazole) over 20 CVs of elution buffer.
5. Collect the fractions containing SELENOM, and concentrate using a centrifugal filter unit (Amicon Ultra-15 Centrifugal Filter Units, 3 kDa cutoff) to 5 mg/mL. Then load the sample onto an appropriate size exclusion column suitable for small proteins (<20 kDa) such as Superdex™ 75 10/300 GL or Sephacryl™ S-100, pre-equilibrated with gel filtration buffer. Collect fractions containing SELENOM and verify their purity by visualizing on 16% Tris-Glycine SDS-PAGE.
6. Determine SELENOM protein concentration by UV-Vis spectroscopy with an extinction coefficient of 18450 M⁻¹ cm⁻¹.
7. Obtain intact mass spectrum of purified SELENOM according to Section 3.5.7 in order to further characterize sample purity. A representative spectrum of SELENOM at the final purifications stage is shown in Fig. 7.
8. Co-purified cellular thiols may present an obstacle to downstream applications. See Note ¹⁰ on removing small thiol species from purified SELENOM.

3.7 Tandem mass sequencing of SELENOM

1. Reduce 6 µg of SELENOM in 60 µL of trypsin digest buffer by the addition of 5 mM DTT. Incubate for 5 min at 95 °C to achieve full reduction and denaturation. Allow the sample to cool to room temperature, then immediately add 12 mM IAM and incubate for another 20 min to fully alkylate exposed Sec and Cys residues, as well as the remaining DTT.
2. Add 0.3 µg of the trypsin enzyme into above mixture and digest at 37 °C for 16 h. Employ a 15 µm, 200 Å pore size ZipTip™ to desalt the trypsin digested protein sample using the vendor procedure.
3. The position of the Sec residue is confirmed by sequencing the Sec-containing peptide using a Q Exactive Orbitrap mass spectrometer interfaced with an Ultimate 3000 LC system (or equivalent set up). Load 1 µg of trypsin digested SELENOM onto a reverse-phase LC column such as C18 suitable for small peptide analysis. Perform an acetonitrile gradient supplemented with 0.1% formic acid over a run time of at least 45 minutes to ensure complete elution of all digested fragments. Acquire MS data using a stepped normalized collision

¹⁰An estimated 0.1 – 1 mM of cellular free thiols is usually present in the purified SELENOM protein sample after size exclusion chromatography. It is recommended that this free thiol population be removed to avoid downstream interference with protein activity. Reduce the protein with 10 mM DTT for 2 h at 25 °C or overnight at 4 °C. Then extensively dialyze the protein against 50 mM sodium phosphate, 200 mM NaCl, 2 mM EDTA, pH 7.5, until the free thiol count of the protein is undetectable using 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB assay [35].

energy of 28, 30, 35 eV. A representative sequencing of SELENOM Sec-containing peptide is shown in Fig. 7.

3.8 Circular dichroism spectroscopy of SELENOM

1. Exchange SELENOM buffer into the CD buffer.
2. Record the far-UV spectra using a 1 mm path-length cell for the 190-250 nm region at 20 °C. Take three accumulation scans for baseline, take average of eight accumulation scans for SELENOM.
3. Plot the mean residual ellipticity against wavelength for each sample. A representative CD spectrum for SELENOM prepared by Sec-mediated native chemical ligation is shown in Fig. 7.

Acknowledgments

This project was supported by the Delaware COBRE program, with grants from the National Institute of General Medical Sciences – NIGMS (5 P30 GM110758-02 and 5 P20 GM104316) from the National Institutes of Health. Qingqing Chen acknowledges support by chemical biology training grant award T32GM008550 from the National Institute of General Medical Sciences. This work was primarily supported by the National Science Foundation under Grant No. MCB-1054447 “CAREER: Reactivity of Selenoproteins” and Grant No. MCB-1616178.

References

1. Qi Y, Grishin NV. Structural classification of thioredoxin-like fold proteins. *Proteins: Struct Funct Bioinform.* 2005; 58(2):376–388. DOI: 10.1002/prot.20329
2. Atkinson HJ, Babbitt PC. An atlas of the thioredoxin fold class reveals the complexity of function-enabling adaptations. *PLoS Comput Biol.* 2009; 5(10)doi: 10.1371/journal.pcbi.1000541
3. Aachmann FL, Fomenko DE, Soragni A, Gladyshev VN, Dikiy A. Solution structure of selenoprotein W and NMR analysis of its interaction with 14-3-3 proteins. *J Biol Chem.* 2007; 282(51):37036–37044. DOI: 10.1074/jbc.M705410200 [PubMed: 17928294]
4. Ferguson AD, Labunskyy VM, Fomenko DE, Araç D, Chelliah Y, Amezcua CA, Rizo J, Gladyshev VN, Deisenhofer J. NMR structures of the selenoproteins Sep15 and SelM reveal redox activity of a new thioredoxin-like family. *J Biol Chem.* 2006; 281(6):3536–3543. DOI: 10.1074/jbc.M511386200 [PubMed: 16319061]
5. Hatfield DL, Tsuji PA, Carlson BA, Gladyshev VN. Selenium and selenocysteine: Roles in cancer, health, and development. *Trends Biochem Sci.* 2014; 39(3):112–120. DOI: 10.1016/j.tibs.2013.12.007 [PubMed: 24485058]
6. Labunskyy VM, Hatfield DL, Gladyshev VN. Selenoproteins: Molecular pathways and physiological roles. *Physiol Rev.* 2014; 94(3):739–777. DOI: 10.1152/physrev.00039.2013 [PubMed: 24987004]
7. Liu J, Chen Q, Rozovsky S. Utilizing selenocysteine for expressed protein ligation and bioconjugations. *J Am Chem Soc.* 2017; doi: 10.1021/jacs.6b10991
8. Reeves MA, Bellinger FP, Berry MJ. The neuroprotective functions of selenoprotein m and its role in cytosolic calcium regulation. *Antioxid Redox Signal.* 2010; 12(7):809–818. DOI: 10.1089/ars.2009.2883 [PubMed: 19769485]
9. Hwang DY, Cho JS, Oh JH, Shim SB, Jee SW, Lee SH, Seo SJ, Lee SK, Lee SH, Kim YK. Differentially expressed genes in transgenic mice carrying human mutant presenilin-2 (N141I): correlation of selenoprotein M with Alzheimer’s disease. *Neurochem Res.* 2005; 30(8):1009–1019. DOI: 10.1007/s11064-005-6787-6 [PubMed: 16258850]
10. Hwang DY, Sin JS, Kim MS, Yim SY, Kim YK, Kim CK, Kim BG, Shim SB, Jee SW, Lee SH, Bae CJ, Lee BC, Jang MK, Cho JS, Chae KR. Overexpression of human selenoprotein M differentially regulates the concentrations of antioxidants and H₂O₂, the activity of antioxidant

- enzymes, and the composition of white blood cells in a transgenic rat. *Int J Mol Med*. 2008; 21(2): 169–179. DOI: 10.3892/ijmm.21.2.169 [PubMed: 18204783]
11. Goo JS, Kim YN, Choi KM, Hwang IS, Kim JE, Lee YJ, Kwak MH, Shim SB, Jee SW, Lim CJ, Seong JK, Hwang DY. Proteomic analysis of kidneys from selenoprotein M transgenic rats in response to increased bioavailability of selenium. *Clin Proteomics*. 2013; 10(1):10.doi: 10.1186/1559-0275-10-10 [PubMed: 23937859]
12. Chen P, Wang RR, Ma XJ, Liu Q, Ni JZ. Different Forms of Selenoprotein M Differentially Affect A beta Aggregation and ROS Generation. *Int J Mol Sci*. 2013; 14(3):4385–4399. DOI: 10.3390/ijms14034385 [PubMed: 23439548]
13. Garcia-Triana A, Gomez-Jimenez S, Peregrino-Uriarte AB, Lopez-Zavala A, Gonzalez-Aguilar G, Sotelo-Mundo RR, Valenzuela-Soto EM, Yepiz-Plascencia G. Expression and silencing of selenoprotein M (SelM) from the white shrimp *Litopenaeus vannamei*: effect on peroxidase activity and hydrogen peroxide concentration in gills and hepatopancreas. *Comp Biochem Physiol*. 2010; 155(2):200–204. DOI: 10.1016/j.cbpa.2009.10.037
14. Reeves MA, Berry MJ. Selenoprotein M. *Selenium: Its Molecular Biology and Role in Human Health (Third)*. 2012; doi: 10.1007/978-1-4614-1025-6_15
15. Korotkov KV, Novoselov SV, Hatfield DL, Gladyshev VN. Mammalian selenoprotein in which selenocysteine (Sec) incorporation is supported by a new form of Sec insertion sequence element. *Mol Cell Biol*. 2002; 22(5):1402–1411. DOI: 10.1128/mcb.22.5.1402-1411.2002 [PubMed: 11839807]
16. Pitts MW, Reeves MA, Hashimoto AC, Ogawa A, Kremer P, Seale LA, Berry MJ. Deletion of selenoprotein M leads to obesity without cognitive deficits. *J Biol Chem*. 2013; 288(36):26121–26134. DOI: 10.1074/jbc.M113.471235 [PubMed: 23880772]
17. Ogawa-Wong AN, Berry MJ, Seale LA. Selenium and metabolic disorders: An emphasis on type 2 diabetes risk. *Nutrients*. 2016; 8(2) 10.3390/nu8020080. doi: 10.1126/SCIENCE.1083516
18. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Synthesis of proteins by native chemical ligation. *Science*. 1994; 266(5186):776–779. DOI: 10.1126/science.7973629 [PubMed: 7973629]
19. Hondal RJ, Nilsson BL, Raines RT. Selenocysteine in native chemical ligation and expressed protein ligation. *J Am Chem Soc*. 2001; 123(21):5140–5141. DOI: 10.1021/ja005885t [PubMed: 11457362]
20. Quaderer R, Sewing A, Hilvert D. Selenocysteine-mediated native chemical ligation. *Helv Chim Acta*. 2001; 84(5):1197–1206. DOI: 10.1002/1522-2675(20010516)84:5<1197::aid-hlca1197>3.0.co;2-#
21. Gieselman MD, Xie L, van Der Donk WA. Synthesis of a selenocysteine-containing peptide by native chemical ligation. *Org Lett*. 2001; 3(9):1331–1334. DOI: 10.1021/ol015712o [PubMed: 11348227]
22. Malins LR, Mitchell NJ, Payne RJ. Peptide ligation chemistry at selenol amino acids. *J Pept Sci*. 2014; 20(2):64–77. DOI: 10.1002/psc.2581 [PubMed: 24285588]
23. McGrath NA, Raines RT. Chemoselectivity in chemical biology: acyl transfer reactions with sulfur and selenium. *Acc Chem Res*. 2011; 44(9):752–761. DOI: 10.1021/ar200081s [PubMed: 21639109]
24. Hondal RJ. Incorporation of selenocysteine into proteins using peptide ligation. *Protein Pept Lett*. 2005; 12(8):757–764. DOI: 10.2174/0929866054864319 [PubMed: 16305545]
25. Metanis N, Keinan E, Dawson PE. Synthetic seleno-glutaredoxin 3 analogues are highly reducing oxidoreductases with enhanced catalytic efficiency. *J Am Chem Soc*. 2006; 128(51):16684–16691. DOI: 10.1021/ja0661414 [PubMed: 17177418]
26. Casi G, Roelfes G, Hilvert D. Selenoglutaredoxin as a glutathione peroxidase mimic. *ChemBioChem*. 2008; 9(10):1623–1631. DOI: 10.1002/cbic.200700745 [PubMed: 18548475]
27. Berry SM, Gieselman MD, Nilges MJ, van Der Donk WA, Lu Y. An engineered azurin variant containing a selenocysteine copper ligand. *J Am Chem Soc*. 2002; 124(10):2084–2085. DOI: 10.1021/ja0169163 [PubMed: 11878940]
28. Dery L, Reddy PS, Dery S, Mousa R, Ktorza O, Talhami A, Metanis N. Accessing human selenoproteins through chemical protein synthesis. *Chem Sci*. 2017; doi: 10.1039/c6sc04123j

29. Kapust RB, Tozser J, Copeland TD, Waugh DS. The P1' specificity of tobacco etch virus protease. *Biochem Biophys Res Commun.* 2002; 294(5):949–955. DOI: 10.1016/s0006-291x(02)00574-0 [PubMed: 12074568]
30. Muller S, Senn H, Gsell B, Vetter W, Baron C, Bock A. The formation of diselenide bridges in proteins by incorporation of selenocysteine residues - biosynthesis and characterization of (Se)(2)-thioredoxin. *Biochemistry.* 1994; 33(11):3404–3412. DOI: 10.1021/bi00177a034 [PubMed: 8136378]
31. Chong S, Williams KS, Wotkowicz C, Xu MQ. Modulation of protein splicing of the *Saccharomyces cerevisiae* vacuolar membrane ATPase intein. *J Biol Chem.* 1998; 273(17):10567–10577. DOI: 10.1074/jbc.273.17.10567 [PubMed: 9553117]
32. Liu J, Li F, Rozovsky S. The intrinsically disordered membrane protein selenoprotein S is a reductase in vitro. *Biochemistry.* 2013; 52(18):3051–3061. DOI: 10.1021/bi4001358 [PubMed: 23566202]
33. Studier FW. Protein production by auto-induction in high-density shaking cultures. *Protein Expr Purif.* 2005; 41(1):207–234. DOI: 10.1016/j.pep.2005.01.016 [PubMed: 15915565]
34. Blommel PG, Fox BG. A combined approach to improving large-scale production of tobacco etch virus protease. *Protein Expr Purif.* 2007; 55(1):53–68. DOI: 10.1016/j.pep.2007.04.013 [PubMed: 17543538]
35. Ellman GL. Tissue sulfhydryl groups. *Arch Biochem Biophys.* 1959; 82(1):70–77. DOI: 10.1016/0003-9861(59)90090-6 [PubMed: 13650640]
36. Geoghegan KF, Dixon HB, Rosner PJ, Hoth LR, Lanzetti AJ, Borzilleri KA, Marr ES, Pezzullo LH, Martin LB, LeMotte PK, McColl AS, Kamath AV, Stroh JG. Spontaneous alpha-N-6-phosphogluconoylation of a “His tag” in *Escherichia coli*: the cause of extra mass of 258 or 178 Da in fusion proteins. *Anal Biochem.* 1999; 267(1):169–184. DOI: 10.1006/abio.1998.2990 [PubMed: 9918669]

Fig. 1. Selenoproteins with a minimal thioredoxin fold position their redox motif next to the first β -sheet. **(a)** The structure of *Mus musculus* SELENOM (PDB entry 2A2P) is representative of this class. **(b)** Thioredoxin and related proteins position their redox motif immediately before an α -helix. The structure of human thioredoxin (PDB entry 1ERU) exemplifies this class. The catalytic selenylsulfide bond of SELENOM and disulfide bond of Trx are depicted as spheres. Selenium is in orange and sulfur in yellow.

Fig. 2.

Scheme of Sec mediated expressed protein ligation of SELENOM. *E. coli* bearing the expression vector pMAL-c5X-MBP-SELENOM^{CT} (residues 48-145 of SELENOM) is grown in defined growth medium supplemented with L-selenocystine for the incorporation of Sec into the protein. Following TEV cleavage, the exposed selenolate of SELENOM^{CT} initiates the nucleophilic attack on SELENOM^{NT}-thioester. SELENOM^{NT}-thioester is prepared by intein technology (see Fig. 3). The native peptide bond is formed through the attack of selenolate onto the thioester, followed by a Se→N shift.

Fig. 3. MBP-SELENOM^{NT} thioester characterization by mass spectrometry. **(a)** Scheme demonstrating MBP-SELENOM^{NT} thioester production. MBP-SELENOM^{NT}-VMA fusion is purified by amylose affinity chromatography and then bound to a chitin column through the chitin-binding domain fused at the C-terminal of intein. During cleavage of intein, the thioester form of MBP-SELENOM^{NT} (MBP-SELENOM^{NT}-MES) is formed and eluted from the column with the addition of 75 mM MESNA. **(b)** MBP-SELENOM^{NT} thioester detected by mass spectrometry. The theoretical mass of the MBP-SELENOM^{NT} thioester is 45322 Da, and measured as 45324 Da. The 42809 Da peak arises from a truncation. Inset

shows the M/Z spectrum. Reprinted with permission from Liu J, Chen Q, Rozovsky S (2017) Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations. J Am Chem Soc. doi:10.1021/jacs.6b10991. Copyright 2017 American Chemical Society.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 4. MBP-SELENOM^{CT} purification and the release of SELENOM^{CT} from MBP by TEV protease cleavage. **(a)** Deconvoluted electrospray ionization mass spectrum of reduced MBP-SELENOM^{CT}. The calculated molecular mass of MBP-SELENOM^{CT} is 53674 Da. The 53933 Da form is due to a 6-phosphogluconolactone modification, most likely on the His₆-tag at the N-terminal of MBP [36]. Inset shows the M/Z spectrum. **(b)** MBP-SELENOM^{CT} can be efficiently cleaved by TEV protease in the presence of a reductant (here DTT). Proteins are identified by 16% Tris-Glycine SDS-PAGE under reducing

conditions. MBP-SELENOM^{CT} is incubated with TEV protease at a molar ratio of 5:1 in 50 mM sodium phosphate, 200 mM NaCl, 2 mM EDTA, and 10 mM DTT, pH 7.5. Reprinted with permission from Liu J, Chen Q, Rozovsky S (2017) Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations. J Am Chem Soc. doi:10.1021/jacs.6b10991. Copyright 2017 American Chemical Society.

Fig. 5.

SELENOM ligation as monitored by SDS-PAGE: Lane 1: MBP-SELENOM^{CT}; lane 2: same as lane 1 but after TEV protease cleavage; lane 3: MBP-SELENOM^{NT}-VMA; lane 4: MBP-SELENOM^{NT} thioester; lanes 5-7: the ligation of SELENOM monitored on days 0, 1, and 2. Lane M: molecular mass standards. After TEV protease cleavage, SELENOM^{NT} thioester cannot be detected because of its 2 kDa mass. Reprinted with permission from Liu J, Chen Q, Rozovsky S (2017) Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations. *J Am Chem Soc.* doi:10.1021/jacs.6b10991. Copyright 2017 American Chemical Society.

Fig. 6.

The rate of SELENOM ligation at 25 °C at pH 6 to 8. MBP-SELENOM^{NT} was incubated with TEV cleaved SELENOM^{CT} at a molar ratio of 5:1 in 50 mM sodium phosphate, 200 mM NaCl, and 2 mM EDTA, supplemented with 100 mM MESNA, 50 mM MPAA, and 5 mM DTT. The reaction was monitored for 48 hours. Proteins are identified on a 16% Tricine-SDS-PAGE with reducing agents. The reaction yield is higher than 90% at pH 6 and above as judged by the disappearance of SELENOM^{CT}. Reprinted with permission from Liu J, Chen Q, Rozovsky S (2017) Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations. *J Am Chem Soc.* doi:10.1021/jacs.6b10991. Copyright 2017 American Chemical Society.

Fig. 7. Characterization of SELENOM. **(a)** CD spectrum of SELENOM prepared by Sec-EPL. **(b)** Deconvoluted ESI-MS of intact SELENOM. **(c)** Tandem MS sequencing of the peptide from panel b confirms Sec's presence in SELENOM. Sec and Cys residues were alkylated with iodoacetamide (purple star). Fragment ions that contain Sec are colored red. Adapted with permission from Liu J, Chen Q, Rozovsky S (2017) Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations. *J Am Chem Soc.* doi:10.1021/jacs.6b10991. Copyright 2017 American Chemical Society.

Table 1

Defined growth medium

Component	Volume (mL)
Step 1	
H ₂ O (autoclaved)	926
1 M MgSO ₄	2
1000 X metals *	0.2
Vitamin mix *	1
Ampicillin 100 mg/mL	1
40 % glucose (W/V)	5
50 X M *	20
Step 2	
40 % glucose (W/V)	5
25 % aspartate (W/V)	10
17 amino acids (10 mg/mL) *	20
25 mg/mL methionine	4
Selenocystine	0.1 g

* The recipe is adapted from Studier MDAG medium [33]