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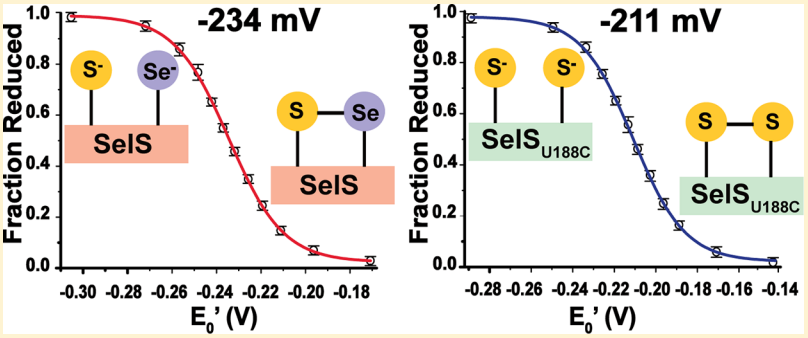
The Intrinsically Disordered Membrane Protein Selenoprotein S Is a Reductase *in Vitro*

Jun Liu, Fei Li, and Sharon Rozovsky*

Department of Chemistry and Biochemistry, University of Delaware, Newark, Delaware 19716, United States

Supporting Information



ABSTRACT: Selenoprotein S (SelS or VIMP) is an intrinsically disordered membrane enzyme that provides protection against reactive oxidative species. SelS is a member of the endoplasmic reticulum-associated protein degradation pathway, but its precise enzymatic function is unknown. Because it contains the rare amino acid selenocysteine, it belongs to the family of selenoproteins, which are typically oxidoreductases. Its exact enzymatic function is key to understanding how the cell regulates the response to oxidative stress and thus influences human health and aging. To identify its enzymatic function, we have isolated the selenocysteine-containing enzyme by relying on the aggregation of forms that do not have this reactive residue. That allows us to establish that SelS is primarily a thioredoxin-dependent reductase. It is capable of reducing hydrogen peroxide but is not an efficient or broad-spectrum peroxidase. Only the selenocysteine-containing enzyme is active. In addition, the reduction potential of SelS was determined to be −234 mV using electrospray ionization mass spectrometry. This value is consistent with SelS being a partner of thioredoxin. On the basis of this information, SelS can directly combat reactive oxygen species but is also likely to participate in a signaling pathway, via a yet unidentified substrate.

Selenoprotein S (SelS) belongs to a family of human enzymes that contains the genetically encoded amino acid selenocysteine (Sec).[1,2] Selenoproteins typically act as oxidoreductases in redox regulation and the management of oxidative species and SelS belong to a eukaryotic protein family that is related to redox stress.[3] Accumulating evidence suggests that it is involved in inflammation and management of oxidative stress.[4,5] It was identified as a member of the endoplasmic reticulum-assisted protein degradation (ERAD) machinery,[6,7] a pathway responsible for transporting misfolded proteins from the ER to the cytoplasm for degradation by the proteasome.[8]

This work focuses on the enzymatic function of human SelS (also known by the alternative names SEPS1, Tanis, VIMP, and SELENOS). SelS is a single-pass transmembrane protein[9] with a short segment in the ER lumen and an extended cytoplasmic region.[10] The cytoplasmic segment contains a disordered segment (residues 123−189 of 189) that includes the Sec at position 188. The Sec forms a selenenylsulfide bond with a nearby Cys (Cys174).[11] SelS was shown to dimerize through a coiled-coil region and contains a valosin-containing protein (VCP, p97) interacting motif.[11,12] It mediates the interactions of the ERAD component Derlin-1 with p97, an AAA ATPase that pulls the protein targets to the cytoplasm, where they are broken down by the proteasome.[13] In addition to p97, SelS also interacts with Derlin-1 and -2, components of the putative ERAD channel;[6] selenoprotein K (SelK), a Sec-containing enzyme with unknown function;[3] VCP accessory protein ubiquitin conjugation factor E4 A (UBE4A), a contributor to multiubiquitin chain extension;[14] UBX domain-containing protein 8 (UBXD8), a sensor for unsaturated fatty acids and a regulator of triglyceride synthesis[15] that together with derlin-1 controls degradation of lipidated apolipoprotein B-100;[16] UBX domain-containing protein 6 (UBXD6, Rep), which tethers p97 to the ER membrane;[17] and kelch-containing protein 2 (KLHDC2, HCLP-1), a regulator of the LZIP transcription factor.[18] As mentioned above, these interactions take place in the cytoplasm, in the context of the ERAD complex. However, the actual function of SelS in the ERAD machinery and its relevance to management of oxidative stress are unknown.

Previously, the cytosolic segment of a SelS U188C mutant (cSelS U188C) was characterized by nuclear magnetic

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resonance spectroscopy and biochemical methods.[11] It was found that the C-terminal domain of SelS is unstructured, classifying it as an intrinsically disordered protein. This is a class of proteins that are rich in charged and polar residues and typically adopt a stable tertiary structure only in the presence of their protein partner.[19] The enzymatic activity of cSelS U188C, however, was not characterized. This information is necessary to tie together not only the role of SelS in the ERAD machinery but also its strong relation to redox stress. Here, we set out to characterize *in vitro* the enzymatic activity of human SelS that contains its native Sec.

Characterization of the Sec-containing form requires incorporation of Sec at position 188. However, the specific incorporation of selenoproteins is typically the bottleneck in their characterization. Selenoproteins are produced *in vivo* by synthesizing Sec on its dedicated tRNA. The Sec codon, which is shared with the opal stop codon, UGA, is recognized by the dual action of a mRNA loop termed the Sec insertion sequence (SECIS) and ancillary proteins.[20] A major challenge for efficient production of selenoproteins is that protein translation is prematurely terminated at the UGA codon because of its misreading as a stop codon. Hence, heterologous expression includes a high ratio of truncated protein. For SelS, the separation of the truncated and full-length Sec-containing forms, which differ by only two amino acids, is complicated by the strong dimerization of truncated and full forms of the protein. To construct a purification strategy that would isolate the full-length, Sec-containing form, we developed an expression system that allows the high-yield preparation of both the full-length and cytoplasmic portions of SelS (cSelS).[21] *Escherichia coli* selenium expression machinery was employed to insert a genetically encoded Sec at the active site. We have purified the full-length cSelS by relying on the tendency of the truncated protein to aggregate in the absence of reducing agents. With a sample enriched with the native Sec-containing protein at hand, we demonstrate that only the Sec-containing enzyme has reductase and peroxidase activities *in vitro*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Chemical Reagents. Enzymes used for molecular biology were acquired from New England Biolabs (Ipswich, MA). The pMHTDelta238 plasmid expressing tobacco etch virus (TEV) protease fused to the cytoplasmic maltose binding protein (cMBP)[22] was purchased from the Protein Structure Initiative Biology Materials Repository.[23] Chromatography media were supplied by GE Healthcare Bio-Sciences Corp. (Pittsburgh, PA) and New England Biolabs. The expression construct of human thioredoxin 1 (hTrx) was generously provided by M. A. Marletta.[24] Human protein disulfide isomerase (hPDI) was a gift from C. Thorpe.[25] The expression construct for human glutaredoxin 1 (hGrx) was a gift from J. J. Mieyal.[26] 15(*S*)-Hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid was from Cayman (Ann Arbor, MI). Bovine insulin was from Cell Application (San Diego, CA). Rat thioredoxin reductase 1 (rTrxR) was from Cayman (recombinant form) or Sigma (purified from rat liver). The pSUABC plasmid was generously provided by E. S. J. Arner from the Karolinska Institutet.[27] All other chemicals and reagents were supplied by Sigma-Aldrich (St. Louis, MO), Acros Organics (Geel, Belgium), and GoldBio (St. Louis, MO). All reagents and solvents were at least analytical grade and were used as supplied.

Cloning. The *Homo sapiens* SelS gene (GenBank entry 45439348) was codon optimized for expression in *E. coli* and the gene synthesized by GeneScript (Piscataway, NJ). The cytoplasmic portion of the gene (residues 52−189, abbreviated as cSelS) was cloned into pMAL-C5X (New England Biolabs) as a fusion with the cytoplasmic maltose binding protein (cMBP). To allow selenocysteine insertion, a nonencoding *E. coli* formate dehydrogenase SECIS element was inserted immediately after the stop codon.[28] A short linker NSSS and a TEV protease cleavage site, ENLYFQS, were used to connect the two proteins. Following cleavage with TEV protease, no non-native residues were retained in cSelS.

Expression and Purification of cSelS. For protein expression of cSelS mutants (cSelS U188C and cSelS U188S), the plasmids were transformed into an *E. coli* BL21(DE3) strain. Cells were grown in LB, supplemented with 0.2% glucose at 37 °C, with good aeration and the relevant antibiotic selection (100 μg/mL). When the optical density (OD) at 600 nm reached 0.5, the temperature was lowered to 18 °C, and the cells were allowed to shake at the lower temperature for an additional 1 h. Protein expression was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) when the OD at 600 nm reached 0.7. The cells were harvested after 18−20 h, and the cell paste (7 g/L) was resuspended in 50 mM sodium phosphate and 200 mM sodium chloride (pH 7.5) (amylose buffer), supplemented with 0.5 mM benziamidine, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 1 mM ethylenediaminetetraacetic acid (EDTA). Cells were lysed using a high-pressure homogenizer (Emulsi-Flex-C5, Avestin, Ottawa, ON) on ice, and all subsequent procedures were conducted at 4 °C. Cell debris was removed by centrifugation at 20000*g* for 1 h. The supernatant was loaded on an amylose column, and the column was washed with the amylose buffer. The cMBP−cSelS fusion protein was eluted using an amylose buffer containing 20 mM maltose. The purity of the eluted cMBP−cSelS fusion protein was ∼90%. Cleavage of the fusion partner cMBP was conducted by adding a hexahistidine-tagged TEV protease to the dialysis bag for 12 h. The TEV protease was added at a molar ratio of 1/10 relative to the fusion protein. Following cleavage, the protein was then loaded on a 5 mL HiTrap SP HP column and eluted with a salt gradient from 200 to 1000 mM sodium chloride, over 20 column volumes. The fractions containing SelS were concentrated to 20 mg/mL, loaded on a Sephacryl S-100 column (GE Healthcare), and eluted at a rate of 0.4 mL/min with 50 mM sodium phosphate and 200 mM sodium chloride (pH 7.5). The column was calibrated using GE Healthcare Bio-Sciences gel filtration protein standards: bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa). The void volume was measured using blue dextran 2000. Protein purity, as determined with a 16% Tris-tricine sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) gel, was higher than 99%. The protein concentration was determined using an extinction coefficient of 14105 M^{-1} cm^{-1} for cSelS U188C and 13980 M^{-1} cm^{-1} for both cSelS U188S and cSelS 188Δ (188Δ indicates that residues 188 and 189 are missing). The successful incorporation of selenium was confirmed by mass spectrometry and inductively coupled plasma (ICP) spectroscopy.

For protein expression of cSelS and cSelS 188Δ, which was generated by mutating UGA to UAA, the plasmid was cotransformed into *E. coli* BL21(DE3), along with the pSUABC plasmid expressing *E. coli* SelA, SelB, and SelC under the

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control of their endogenous promoters.[27] Cells were grown in Studier's MDAG defined medium at 37 °C,[29] with good aeration and the relevant antibiotic selection (100 μg/mL ampicillin with 34 μg/mL chloramphenicol). When the OD at 600 nm reached 2.4, the temperature was lowered to 18 °C, and the cells were allowed to shake at the lower temperature for an additional 1 h. Protein expression was induced with 0.5 mM IPTG. At induction, 5 μM Na_2SeO_3 and 100 μg/mL L-cysteine were added to the media. The remaining steps are identical to those described above. It was possible to distinguish two well-separated peaks on the HiTrap SP HP column, eluting at 400 and 600 mM sodium chloride. The latter contains aggregates and was not included in further purifications. The fraction that eluted at a lower ionic strength was concentrated to 20 mg/mL and further purified on a Sephacryl S-100 column. The dimeric form of the protein was separated from other fractions and assayed for activity. Other fractions also contain full-length protein but at a much lower ratio (<1%) compared to that of the truncated form. The percent of selenium incorporation in samples was measured by ICP spectroscopy. The percent of selenium incorporation in all batches used for activity assays was at least 50%.

Inductively Coupled Plasma (ICP) Spectroscopy. ICP spectrometry (Thermo IRIS Intrepid II XSP Dual View) was used to determine the ratio of sulfur and selenium in protein samples. The instrument was calibrated using a customized reference standard from AccuStandard (New Haven, CT), containing 2 μg/mL elemental sulfur and selenium in 2% nitric acid.

Electrospray Ionization (ESI) Mass Spectrometry. Mass spectra were recorded using a QTOF Ultima instrument (Waters), operating in positive electrospray ionization (+ESI) mode, connected to an LC-20AD instrument (Shimadzu, Kyoto, Japan). Protein samples were separated from small molecules by reverse phase chromatography on a C4 column (Waters XBridge BEH300), using an acetonitrile gradient from 30 to 71.4%, with 0.1% formic acid as the mobile phase, in 25 min, at a flow rate of 0.2 mL/min at room temperature. Data were acquired from m/z 350 to 2500, at a rate of 1 s/scan. The obtained spectra were deconvoluted using maximal entropy in MassLynx (Waters).

Circular Dichroism (CD) Spectroscopy. CD spectra of cSelS were measured by using a J-810 circular dichroism spectropolarimeter (Jasco, Essex, U.K.) that had been calibrated using camphorsulfonic acid for optical rotation and benzene vapor for wavelength. Far-UV spectra were recorded using a 1 mm path-length cell for the 190–250 nm region at 20 °C. Samples for CD spectroscopy were prepared in 10 mM potassium phosphate (pH 7.5) and 50 mM sodium sulfate. Three accumulation scans were collected for baseline, and eight accumulation scans were taken for each sample.

Measurements of cSelS and cSelS U188C Reduction Potential Determined by ESI-MS. A 0.5 mL sample, which contained 0.2 μM fully oxidized protein, was dialyzed in buffer containing 50 mM sodium phosphate (pH 7.0), 1 mM EDTA, and various ratios of glutathione (GSH) and oxidized glutathione (GSSG) at a total concentration of GSH and GSSG of 20 mM. The GSH/GSSG ratio was used to poise the reduction potential of the buffer. To prevent excessive oxidation, the protein solutions were degassed and subsequently flushed with nitrogen. The samples were incubated at 25 °C for 18 h to ensure full equilibration. The equilibrated reaction mixtures were quenched by adding ice-cold 100% (w/v) trichloroacetic acid (TCA) to a final concentration of 20% and were mixed immediately. The quenched reaction mixture was then spun for 10 min at 16110*g*. The supernatant was decanted, and the pellet was washed with 0.25 mL of ice-cold acetone twice and spun at 16110*g* for 10 min at 4 °C after each wash. After the final acetone wash, the pellet was dried by being exposed to air for 10 min and then resuspended in 50 μL of a 15 mM iodoacetamide solution; 10–20 μL of the resuspended sample was used to acquire the mass spectrum. Experiments were repeated three times, using two independent preparations, for each protein. Following deconvolution, the fractional ratio of reduced to oxidized protein was determined from the presence of mass increments of 116 Da (transfer of two acetamide groups). The percentage of reduced and alkylated protein to oxidized protein was determined by integrating the relevant peak area in Excel (Microsoft). Identical results were obtained when *N*-ethylmaleimide (NEM) was used for labeling.

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

$$cSelS_{red} + GSSG \leftrightarrow cSelS_{ox} + 2GSH \qquad \text{(Reaction I)}$$

$$K_{eq} = \frac{[cSelS_{ox}][GSH]^2}{[cSelS_{red}][GSSG]} \qquad (1)$$

The reduction potential of cSelS at pH 7.0 and 25 °C [E_0'(cSelS)] was then calculated from the Nernst equation (eq 2):

$$E_0'(cSelS) = E_0'(GSH/GSSG) - \frac{RT}{nF} \times \ln(K_{eq}) \qquad (2)$$

where E_0'(GSH/GSSG) = −240 mV (pH 7.0 and 25 °C),[30] R is the gas constant (8.315 J K^{-1} mol^{-1}), T is the absolute temperature (298 K), n is the number of electrons transferred in the reaction (here n = 2), and F is the Faraday constant (9.649×10^4 C mol^{-1}). Plots and curve fits were made with Origin (OriginLab Corp., Northampton, MA). The error bars in Figure 2 represent the range of measurements, that is, the highest and lowest values recorded in the series. The measurement error was estimated to be 1 mV by fitting extreme curves to the minimal and maximal fractions of the reduced protein at each point.

Measurements of Reduction Potential by a Gel Shift Assay. The same sample preparation described above for ESI mass spectrometry-based assays was employed for gel shift assays and visualization by SDS–PAGE. Following an 18 h incubation in the respective redox buffer, the protein was precipitated with 100% TCA and dried with acetone. The pellet was dissolved in 50 μL of a 10 mM methyl-PEG24-maleimide [MM(PEG)24] solution in 2× nonreducing Tricine–SDS–PAGE loading buffer. The samples were boiled for 5 min prior to being loaded on 16% Tricine–SDS–PAGE gels. The gels were Coomassie-stained, and the band intensities were visualized and quantified using a FluorChem Q gel imager (ProteinSimple, Santa Clara, CA). The ratio of reduced protein to oxidized protein was used in conjunction with the Nernst equation to calculate the reduction potential.

Insulin Reduction Assay. The insulin reduction assay was performed as previously described by Holmgren.[31] Briefly, the reaction mixture included 100 mM sodium phosphate buffer (pH 7.0), 2 mM EDTA, 0.13 mM bovine insulin, and 0.33 mM dithiothreitol (DTT). The reaction was initiated by addition of

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hTrx, hPDI, cSelS, or cSelS mutants. The reaction's progress was monitored by recording the increase in turbidity at 650 nm versus time. The temperature was kept constant at 25 °C.

Peroxidase Assays. The peroxidase activity was measured via a coupled reaction with rTrxR and hTrx.[32] The reaction mixture contained 100 mM potassium phosphate (pH 7.0), 2 mM EDTA, 150 μM NADPH, 2 nM rTrxR, 5 μM hTrx, and either no enzyme or 5 μM cSelS, cSelS U188C, cSelS U188S, or cSelS 188Δ. Enzymatic oxidation of NADPH was initiated by adding 200 μM H_2O_2 after incubation for 3 min to the sample cuvette and monitoring the consumption of NADPH spectroscopically at 340 nm. The rate of NADPH oxidation for each condition was calculated from three repeats.

cSelS peroxidase activity was also assayed with hGrx and yeast glutathione reductase. The reaction mixture contained 100 mM potassium phosphate (pH 7.0), 2 mM EDTA, 150 μM NADPH, 0.5 mM GSH, 1 milliunit/μL yeast glutathione reductase, 5 μM hGrx, and 5 μM wild-type or mutant cSelS. The reaction was initiated by adding 200 μM H_2O_2, and the change in absorbance at 340 nm was recorded. Glutathione peroxidase (GPx) from human erythrocytes served as a positive control.

Steady-state kinetic analysis was performed using the assay described above. To measure the kinetic parameters of cSelS peroxidase activity, H_2O_2 was added at concentrations of 12.5−1600 μM to a reaction mixture containing 8 nM rTrxR, 5 μM cSelS, 5 μM hTrx, and 150 μM NADPH. For measurements with hTrx as a substrate, hTrx at concentrations of 0.625−20 μM was added to a reaction mixture containing 8 nM rTrxR, 5 μM cSelS, 150 μM NADPH, and 200 μM H_2O_2. Each condition was repeated three times.

To test the substrate specificity of the peroxidase activity of cSelS, a variety of potential peroxide substrates were tested in a similar assay as described above. The soluble H_2O_2, cumene hydroperoxide (COOH), and *tert*-butyl hydroperoxide (tBuOOH) were dissolved in 100 mM potassium phosphate (pH 7.0) and employed at a concentration of 200 μM. To prepare the hydrophobic 15-HETE and 15-HpETE, ethanol was evaporated when the sample was purged with a N_2 stream. The compounds were dissolved in 100 mM potassium phosphate (pH 7.0), and added to the reaction buffer at a concentration of 30 μM. The rate of the initial NADPH oxidation was calculated by monitoring the absorbance at 340 nm. Experiments were repeated three times for each substrate.

Oxidase and Isomerase Assays. The ability of cSelS to act as either an oxidase or isomerase was followed by detecting the recovery of either reduced and denatured or scrambled ribonuclease A (RNase A) activity.[33] The reaction was monitored at 25 °C, by following the hydrolysis of cCMP by newly oxidized and properly folded RNase A at 296 nm.[34] For the oxidase assays, reduced and denatured RNase A was prepared by adding a 100-fold molar excess of DTT to RNase A in 50 mM Tris-HCl (pH 7.5), 6 M guanidine hydrochloride, and 1 mM EDTA. After an overnight incubation at 4 °C, the excess DTT and guanidine were removed with a Nap-5 column, and the buffer was exchanged into oxidase assay buffer consisting of 50 mM HEPES-NaOH (pH 7.0), 150 mM sodium chloride, 75 mM imidazole, 2 mM EDTA, and 0.5% Tween 20. The free thiol count of fully reduced RNase A, determined by thiol titer using Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB),[35] was close to eight thiols per protein, demonstrating that RNase was fully reduced. The assay buffer included 50 mM HEPES-NaOH (pH 7.0), 150 mM sodium chloride, 75 mM imidazole, 2 mM EDTA, 0.5% Tween 20, 4.5 mM cCMP, 4 μM reduced and denatured RNase A, 0.4 mM GSSG, and 1.2 mM GSH as a redox buffer (1/3 [GSSG]/[GSH]). The reaction was initiated by adding 1 μM cSelS. hPDI served as a positive control.

The cSelS isomerase activity was measured using scrambled RNase, prepared as previously described by Thorpe.[25] Briefly, native RNase A was added to 50 mM Tris-HCl (pH 7.5) and 6 M guanidine hydrochloride with an equimolar amount of DTT for 30 h under anaerobic conditions. This solution was then exposed to air and allowed to slowly reoxidize in the dark, at room temperature. The reaction was monitored until <1% free thiol per RNase molecule remained. The buffer was then exchanged with 50 mM HEPES-NaOH (pH 7.0), 150 mM sodium chloride, 75 mM imidazole, 2 mM EDTA, and 0.5% Tween 20. Scrambled RNase was stored in −20 °C until it was used. The assay mixture contained 50 mM HEPES-NaOH (pH 7.0), 150 mM sodium chloride, 75 mM imidazole, 2 mM EDTA, 0.5% Tween 20, 4.5 mM cCMP, 0.4 mM GSSG, and 1.2 mM GSH as a redox buffer and 8 μM scrambled RNase A. The reaction was initiated by adding 1 μM cSelS and monitored at 25 °C. hPDI served as a positive control.

RESULTS

Incorporation of Sec into proteins at a unique position remains an experimental challenge and a barrier for their characterization.[36] To develop preparation strategies for the Sec-containing cSelS, we have first optimized strategies for obtaining high yields and purities for both the full-length protein and the cytoplasmic portion of SelS U188C in *E. coli*.[21] In the remainder of this paper, we focus on the characterization of the cytoplasmic portion of SelS (residues 52−189), abbreviated as cSelS. cSelS was fused to a binding partner, cytoplasmic maltose binding protein (cMBP), and expressed at 18 °C, to aid solubility and folding. Using this strategy, it is possible to prepare >98% pure cSelS U188C (Figure S1A of the Supporting Information) at a yield of 10 mg/L (0.6 μmol). The expected molecular weight was confirmed by ESI mass spectrometry (Figure S1B of the Supporting Information). cSelS U188C dimerized in the presence and absence of reducing agents, even though the transmembrane region was deleted (Figure S2 of the Supporting Information). Hence, the coiled-coil region is sufficient for dimerization, as was previously shown.[11] With the high-yield expression and purification strategy in hand, the production of the native Sec-containing cSelS was undertaken by utilizing *E. coli*'s innate Sec incorporation machinery.[27] Incorporation of Sec into SelS in a genetically encoded fashion required numerous modifications to the expression vector and growth conditions. *E. coli* makes use of an mRNA structure termed the Sec insertion sequence (SECIS). This stem−loop architecture coordinates the recruitment to the ribosome proteins essential for Sec synthesis on its dedicated tRNA. In *E. coli*, those include SelB, an elongation factor; SelC, the Sec tRNA; SelA, a selenocysteine synthase; and SelD, a selenophosphate synthase. Arner and Bock demonstrated that it is possible to introduce Sec into recombinant proteins in *E. coli* by utilizing the SECIS element borrowed from its formate dehydrogenase H and coexpressing SelA, -B, and -C.[27] We have introduced the UGA codon at position 188, as well as *E. coli* formate dehydrogenase H SECIS element past the stop codon of the cMBP−cSelS fusion protein-encoding DNA. The pSUABC vector expressing SelA, -B, and -C under the control of their own natural

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promoter was a gift from E. S. J. Arner. The incorporation of Sec is further augmented by inducing expression at a late exponential phase, in which the level of release factor 2 is decreased.[37] The incorporation ratio of Sec was ~3% of the total expressed protein. We have tested several methods to separate the full-length, selenium-containing protein, cSelS, from the truncated enzyme cSelS 188Δ (188Δ indicates that residues 188 and 189 are missing because of misreading of the Sec codon as a stop codon). The separation of cSelS and cSelS 188Δ is challenging because they are identical for all but two amino acids and form a dimer. To obtain a sample rich in cSelS, we have relied on the tendency of cSelS 188Δ, which has only one cysteine, to aggregate in the absence of reducing agents. We have extracted and purified the protein expressed with a UGA codon using the procedures optimized for the cSelS U188C mutant but deliberately excluded reducing agents. The truncated protein, cSelS 188Δ, aggregated during purification, because of the formation of intermolecular disulfide bonds. The last step relied on size exclusion chromatography to separate the tetramer and higher-order oligomeric forms of SelS from the dimeric Sec-containing form. Subsequently, only Sec-rich dimeric fractions were retained. Using this approach, samples were enriched from a starting ratio of 3% cSelS to ~50%. Figure 1A displays an elution profile from a size exclusion column of a typical purification, in which the first peak that eluted contained tetramer and higher-order aggregates while the peak that eluted last contained the dimeric form of cSelS and cSelS 188Δ (Figure 1B). On average, 50% of the protein in that fraction contained Sec, as detected by inductively coupled plasmon (ICP) spectroscopy. The yield for the selenium-rich fraction was ~0.6 mg/L of growth medium. The resulting protein is pure of contaminations, other than cSelS 188Δ. The CD spectra of cSelS and cSelS U188C are nearly identical (Figure S3 of the Supporting Information). However, as detailed in the following sections, only cSelS has enzymatic activity.

cSelS Reduction Potential. The reduction potential of a given protein, a measurement of its tendency to gain electrons, is an important gauge of its ability to interact with other proteins. The differences in reduction potentials of selenoproteins and their Cys-containing homologues can be substantial; in the case of *E. coli* glutaredoxin 3, the reduction potential of the Cys-containing form was reported to be −194 mV while that of the forms containing Sec in the N-terminal and C-terminal positions of the active site's redox motif are −260 and −275 mV, respectively.[38] The reduction potential of cSelS U188C was previously reported to be −200 mV, as established by gel shift assays using maleimide alkylation.[11] We were interested in measuring the reduction potential of the native selenium-containing cSelS. However, because our samples contained both cSelS and cSelS 188Δ, and the two dimerize, it was not possible to differentiate between different protein forms in the sample and quantify the percent of reduced and oxidized species by gel shift assays or by separating the reduced and oxidized forms chromatographically.[30] Instead, we have opted to determine the redox properties by electrospray ionization mass spectrometry, where the different chemical species can be directly identified. The protein is equilibrated in different buffers whose reduction potential is posed by fixing the ratio between GSH and GSSG. Following an 18 h incubation period to ensure equilibrium had been reached, the protein is denatured with acid and subsequently alkylated using iodoacetamide. Only exposed forms of Cys and Sec, but not

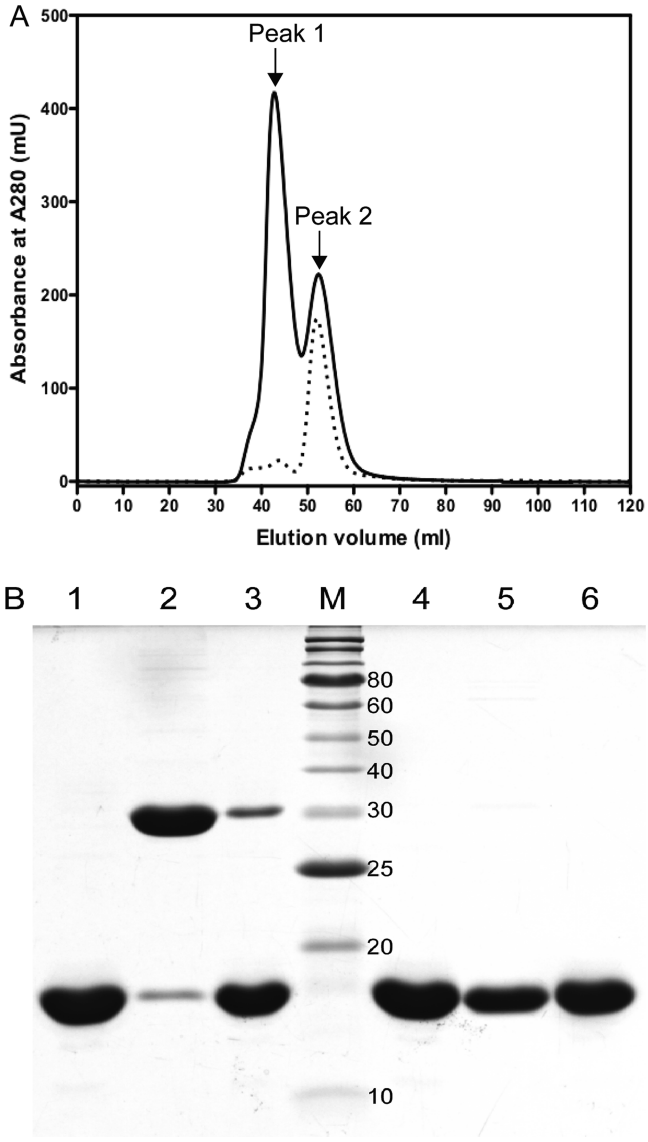

Figure 1. Separation of dimeric forms rich in cSelS from higher-order oligomers rich in cSelS 188Δ by size exclusion chromatography. (A) Elution profiles of cSelS (—) and cSelS U188C (···) from a Sephacryl S-100 column under nonreducing conditions. Peak 2, which eluted last, contains the selenium-containing form of the enzyme, cSelS. (B) SDS−PAGE analysis of cSelS/cSelS 188Δ mixtures following separation by size exclusion chromatography. Lanes 1−3 were run under nonreducing conditions: lane 1, cSelS U188C (control); lane 2, cSelS first peak; lane 3, cSelS second peak; lane M, protein molecular mass standards (the molecular mass in kilodaltons is noted on the right). Lanes 4−6 were run under reducing conditions: lane 4, cSelS U188C (control); lane 5, cSelS first peak; lane 6, cSelS second peak.

those in selenenylsulfide bonds, can be alkylated by iodoacetamide. The fraction of reduced protein acquired a mass shift of 116 Da, because of alkylation of C174 and U188. The oxidized protein is unmodified. Subsequent acquisition of an electrospray ionization mass spectrum allows the identification of the ratio of oxidized to reduced and alkylated cSelS. Figure S4 of the Supporting Information demonstrates that similar ionization efficiencies for the two protein forms were achieved when a mixture of 1/1 oxidized cSelS and reduced and alkylated cSelS was tested. Hence, the mass spectrum reflects their original concentrations in the sample. The identification of

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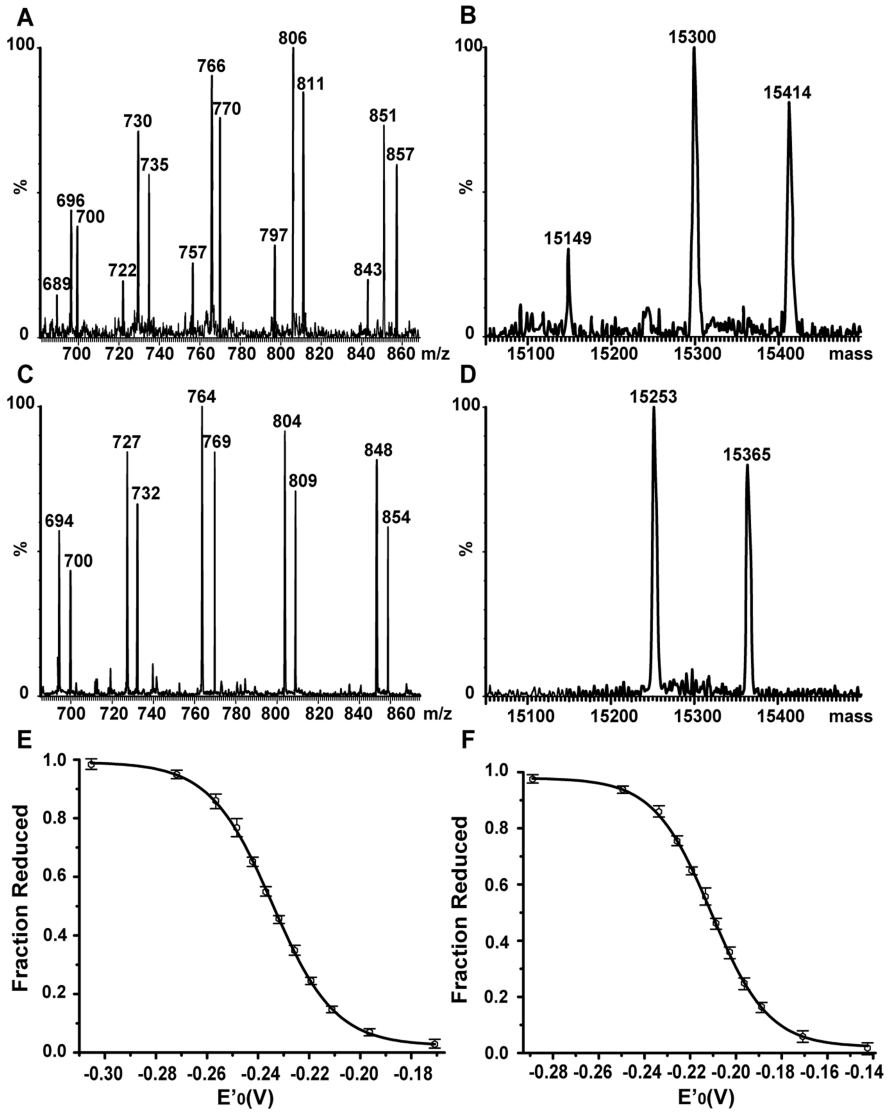

Figure 2. Determination of reduction potentials of cSelS and cSelS U188C in GSH/GSSG redox buffers. The ratio of reduced and oxidized protein was determined by electrospray ionization mass spectrometry. (A) Representative electrospray ionization mass spectrum of cSelS following incubation in a redox buffer poised at −232 mV and subsequent alkylation. The panel shows the charge-state distribution of multiply charged ions $[M + 18H]^{18+}$ to $[M + 22H]^{22+}$ from m/z 680 to 870. (B) Deconvoluted spectrum. Molecular masses: 15149 Da for alkylated cSelS 188Δ, 15300 Da for oxidized cSelS, and 15414 Da for alkylated cSelS. (C) Representative electrospray ionization mass spectrum of cSelS U188C following incubation in a redox buffer poised at −209 mV and subsequent alkylation. The panel shows the charge-state distribution of multiply charged ions $[M + 18H]^{18+}$ to $[M + 22H]^{22+}$ from m/z 685 to 870. (D) Deconvoluted spectrum. Molecular masses: 15253 Da for oxidized cSelS U188C and 15365 Da for alkylated cSelS U188C. Panels E and F display the resulting titration curves for (E) cSelS (reduction potential of −234 ± 1 mV) and (F) cSelS U188C (reduction potential of −211 ± 1 mV). The fraction of reduced protein is plotted against the buffer redox potential poised by the ratio of GSH to GSSG. The error bars represent the range of measurements (that is, highest and lowest values) among three repetitions, using two independent protein preparations.

other forms, such as the alkylated cSelS 188Δ, simplified the interpretation of data.

To rule out the formation of an intermolecular selenenylsulfide bond that may be present due to the existence of cSelS 188Δ, we have isolated the dimeric form of cSelS using size exclusion chromatography and checked by thiol titer and by alkylated iodoacetamide that there are no exposed Cys or Sec residues. This conclusion is further validated by the reproducibility of titrations regardless of a varying percent of cSelS 188Δ in samples.

Using this approach, the reduction potential of cSelS was determined to be −234 ± 1 mV (Figure 2 and Figure S5 of the Supporting Information) and that of cSelS U188C to be −211 ± 1 mV (Figure 2 and Figure S6 of the Supporting Information). The reduction potential of cSelS U188C was also measured by gel shift assays (Figure S7 of the Supporting Information) and found to be identical to that measured by the mass spectrometry-based method. It is within a reasonable margin of error from the value reported by Christensen et al.[11] The truncated form, cSelS 188Δ, which forms an intermolecular disulfide bond, is fully reduced and alkylated at −180 mV. It is easily distinguished by its mass and subsequently can be neglected during analysis. Titration curves shown in Figure 2 represent the average of three measurements using two independent sample preparations. The implications of the

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reduction potential of cSelS are further discussed in the conclusions.

Reductase Activity. To test the potential reductase activity of SelS, we compared two activity assays that are based on reducing the disulfide bond between insulin's chains A and B: by monitoring insulin reduction by NADPH consumption in glutathione reductase coupled assays[39] and by following the process of aggregation of the free A and B chains by turbidity.[31] The former assay relies on GSH as a redox couple, while the latter is compatible with other reducing agents. A reductase activity was detected only in insulin turbidity assays that were conducted with dithiothreitol (DTT) as a reducing agent. GSH is not an efficient electron donor. Interestingly, the reductase activity is present only when Sec is incorporated and is absent for the Cys mutant (Figure 3). When the amount of enzyme

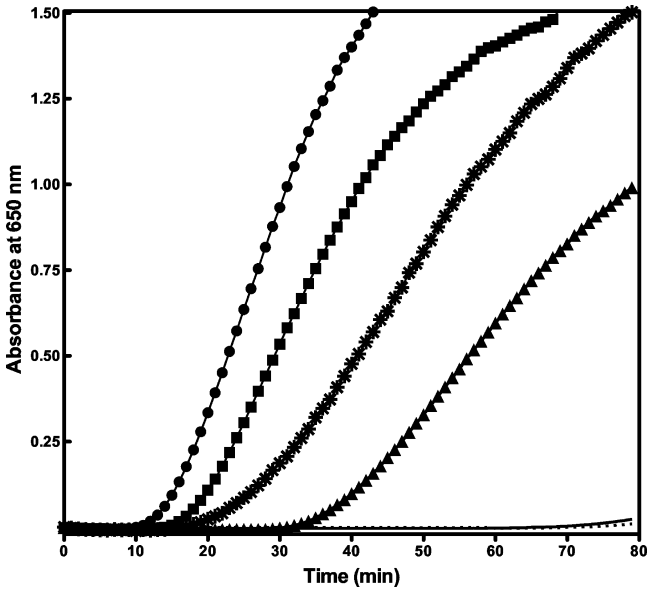

Figure 3. Reductase activity assays. The ability of cSelS to reduce insulin's intermolecular disulfide bond is monitored by recording the increasing turbidity caused by insulin's chain B aggregation. The reaction mixture includes insulin and DTT. Insulin reduction was initiated by adding the protein of interest. The reaction was recorded with 2 μM hTrx (●), 2 μM cSelS (■), 2 μM hPDI (∗), 1 μM cSelS (▲), or 10 μM cSelS U188C (—) or without additional enzymes (···).

was doubled, the interval for the onset of turbidity was cut in half (A_{650} > 0.02) and the reaction rate in the linear region was doubled. cSelS 188Δ causes rapid aggregation in the absence of reducing agents. This form can be isolated only as a tetramer or higher-order oligomers prone to aggregation. The reductase activity did not depend on metals or cofactors. As the insulin turbidity assays cannot be used to extract the catalytic rate, we used hTrx and hPDI to evaluate cSelS efficiency. For comparable concentrations of hTrx, cSelS, and hPDI, the onsets of turbidity were 12, 16, and 20 min, respectively. The reaction rates in the linear region were 0.027, 0.025, and 0.014 ΔA_{650} min^{-1} μM^{-1}, respectively. On the basis of the turbidity assays, the cSelS reductase activity is comparable to that of human thioredoxin 1 (hTrx)[40] but slightly lower. Evidently, cSelS is an effective reductase *in vitro*.

Peroxidase Activity. Other plausible enzymatic functions for SelS are oxidase, isomerase, and peroxidase. cSelS had neither an oxidase nor an isomerase activity for ribonuclease A (RNase A) (Figure S8 of the Supporting Information). This is not surprising, because the active site of SelS is located in the cytosol, while disulfide bond formation and oxidative folding processes take place in the ER. A peroxidase activity is more plausible because several selenoproteins possess a peroxidase activity.[1] Specifically, there are at least two selenoprotein families that specialize in the removal of oxidative species: glutathione peroxidases and selenoproteins with the minimal thioredoxin fold.[41−44] cSelS itself does not efficiently accept electrons from GSH, like the former, nor does it have a known fold. As we show in this section, cSelS is also capable of reducing hydrogen peroxide efficiently, but not hydrophobic peroxide substrates.

We have measured the ability of cSelS to reduce hydrogen peroxide using the rat thioredoxin reductase (rTrxR)/hTrx or the yeast glutathione reductase (yGR)/human glutaredoxin 1 (hGrx) coupled enzymatic assays. cSelS efficiently utilized reducing equivalents from the rTrxR/hTrx system (Figure 4A),

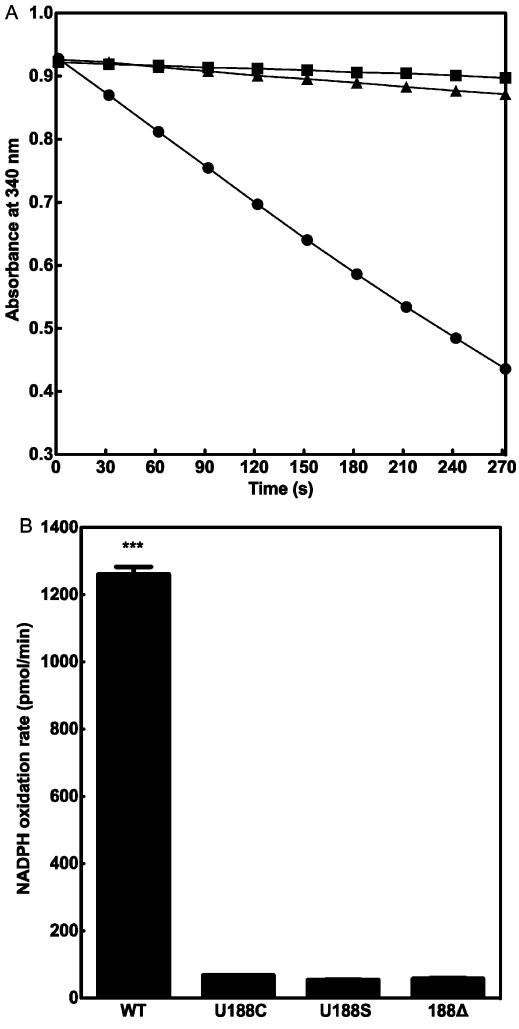

Figure 4. Peroxidase activity assays. (A) cSelS catalyze the reduction of H_2O_2 by coupling with the thioredoxin system. Data for the reaction with 2 nM rTrxR, 5 μM cSelS, and 5 μM hTrx are shown as circles, data for the reaction in the absence of cSelS as squares, and data for the reaction in the absence of hTrx as triangles. (B) The activity of different mutants was compared: 5 μM cSelS (WT), 5 μM cSelS U188C (U188C), 5 μM cSelS U188S (U188S), and 5 μM cSelS 188Δ (188Δ). cSelS peroxidase activity depends on the presence of Sec (***p < 0.001).

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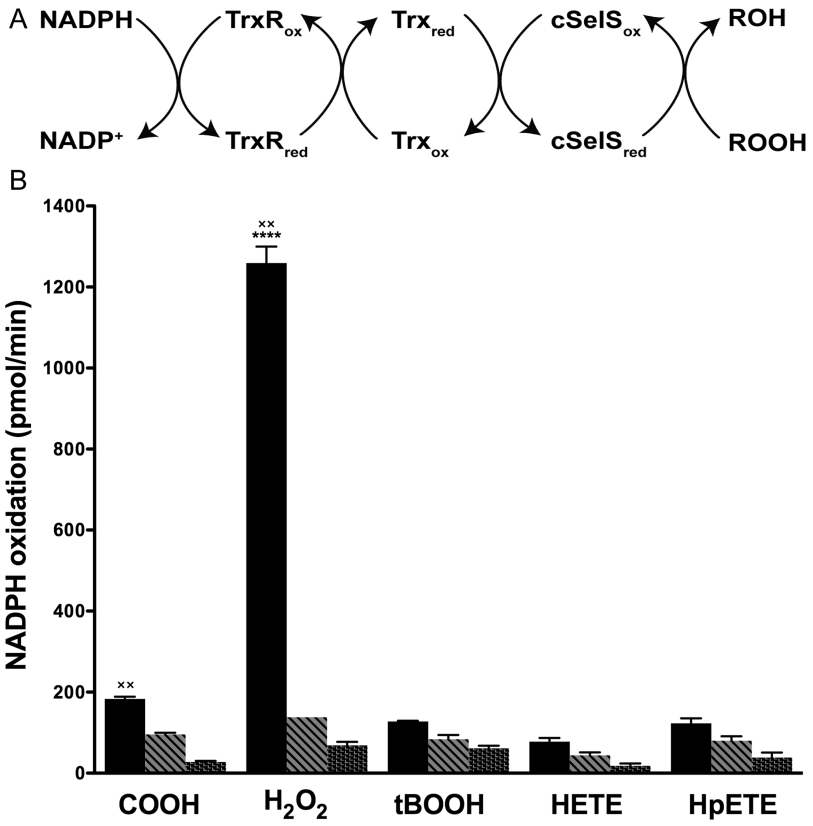

Figure 5. Substrate specificity of cSelS peroxidase activity. (A) Reaction scheme. (B) cSelS substrate specificity. Reaction with cSelS and hTrx (black bars), reaction excluding hTrx (striped bars), and reaction excluding cSelS (dotted bars). Only H_2O_2 and COOH have significant rates compared to the control ($^{××}p < 0.001$; $n = 3$). The affinity for H_2O_2 is the highest among the four substrates ($^{****}p < 0.001$; $n = 3$).

while the yGR/hGrx system is less efficient (Figure S9 of the Supporting Information). While cSelS can accept electrons directly from rTrxR, it is a low-affinity substrate. The peroxidase activity was solely dependent on the presence of Sec (Figure 4B). The specificity of cSelS was tested for hydrogen peroxide (H_2O_2), *tert*-butyl hydroperoxide (tBOOH), cumene hydroperoxide (COOH), 15(*S*)-hydroperoxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (HpETE), and 15(*S*)-hydroxy-6(*E*),8(*Z*),11(*Z*),14(*Z*)-eicosatetraenoic acid (HETE) (Figure 5). Only hydrogen peroxide and cumene hydroperoxide have turnover rates significantly higher than the control ($p < 0.001$). Hence, cSelS is not a broad-specificity peroxidase. Still, the specificity may depend on interactions of cSelS with binding partners, which are likely to influence the structure of the active site.

Next, we measured the steady-state kinetic parameters of the peroxidase activity of cSelS (Figure 6). cSelS exhibits a two-substrate ping-pong mechanism with Michaelis–Menten-type saturable kinetics (Scheme 1).

With H_2O_2 as the substrate, cSelS exhibited a Michaelis constant (K_m) of 58 ± 5 μM, a catalytic constant (k_{cat}) of 0.110 ± 0.003 s^{-1}, and a catalytic efficiency (k_{cat}/K_m) of (2.1 ± 0.8) × 10^3 M^{-1} s^{-1} (Figure 6A). We have also determined the kinetic parameters with hTrx as a substrate: K_m = 1.1 ± 0.1 μM, k_{cat} = 0.098 ± 0.003 s^{-1}, and k_{cat}/K_m = (9 ± 1) × 10^4 M^{-1} s^{-1} (Figure 6B). The catalytic efficiency of cSelS with H_2O_2 as the substrate is lower than that reported for GPx and peroxiredoxin. The k_{cat}/K_m is on the order of 10^7 M^{-1} s^{-1} for the selenium-containing GPx,[45] while that of peroxiredoxins is of the order of 10^4–10^5 M^{-1} s^{-1}.[46] However, k_{cat}/K_m was determined for cSelS in isolation. Because it is an intrinsically disordered enzyme, this value may change in the presence of its binding partner.

DISCUSSION

The experiments establish that human SelS has a reductase as well as peroxidase activities *in vitro*. This is consistent with the observation that most selenoproteins are oxidoreductases. The SelS reductase activity is comparable to that of hTrx. It does not, however, efficiently reduce peroxides compared to GPx or peroxiredoxins and is not able to reduce complex peroxides. Because it is neither an efficient nor a broad-spectrum peroxidase, the SelS peroxidase activity (at least in the absence of its protein partners) should be seen as an extension of SelS's activity as a reductase. This is analogous to the other selenium-containing reductase TrxR.[47] TrxR reduces, in addition to protein disulfides, hydrogen peroxide, selenite, dehydroascorbate, α-lipoic acid, and lipid hydroperoxides.[48] In this way, it provides a broad antioxidative capacity even though its peroxidase activity is lower than that of SelS. Its catalytic efficiency (k_{cat}/K_m) with H_2O_2 as the substrate is 6.7 × 10^2 M^{-1} s^{-1} compared to cSelS's value of 2.1 × 10^3 M^{-1} s^{-1}.[49] Furthermore, while the SelS peroxidase efficiency is not on par with that of GPx or peroxiredoxins, it is possible that because it is a selenoprotein it is more resistant to damage by H_2O_2 and hence able to eliminate reactive oxygen species (ROS) even under oxidative stress. Overall, it is yet unclear what role the peroxidase activity of SelS plays *in vivo*.

Our assays show that the selenenylsulfide bond is preferentially reduced by hTrx and not by hGrx. Surprisingly, the SelS reduction potential, −234 mV, is similar to that of its

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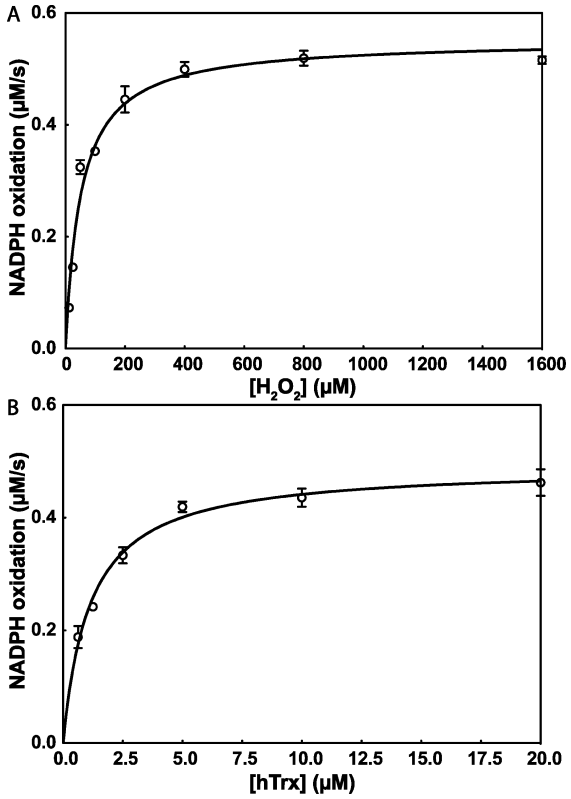

Figure 6. Kinetic parameters of cSelS peroxidase activity. (A) With H_2O_2 as the substrate, K_m = 52 ± 5 μM, k_{cat} = 0.110 ± 0.003 s^{-1}, and k_{cat}/K_m = (2.1 ± 0.8) × 10^3 M^{-1} s^{-1}. (B) With hTrx as the substrate, K_m = 1.1 ± 0.1 μM, k_{cat} = 0.098 ± 0.003 s^{-1}, and k_{cat}/K_m = (9 ± 1) × 10^4 M^{-1} s^{-1}. Means ± the standard deviation of three independent experiments are shown.

Scheme 1

$$cSelS_{ox} + hTrx_{red} \leftrightarrow cSelS_{red} + hTrx_{ox}$$
$$cSelS_{red} + H_2O_2 \leftrightarrow cSelS_{ox} + H_2O$$

partner, hTrx, whose reduction potential is −230 mV.[50] Typically, redox pairs have a difference in reduction potential driving the reduction of one by the other. This does not appear to be the case for SelS and hTrx, but the abundance of hTrx in the cytoplasm is significantly higher than that of SelS; its steady-state reduction potential is −280 mV.[51] SelS is most likely to encounter the reduced hTrx and subsequently be reduced by it.

The rare amino acid Sec is essential for enzymatic activity; both the reductase and peroxidase activities require the presence of Sec and are not present when Sec is substituted with Cys. Why the selenium is critical for activity is not easy to explain. The enzymatic activity of only a subset of selenoproteins was characterized,[2] and the unique role of Sec in catalysis still awaits a unified description.[52,53] In the case of SelS, the differences do not stem from a large change in reduction potentials; the reduction potentials of cSelS and cSelS U188C are −234 ± 1 and −211 ± 1 mV, respectively. A difference in reduction potential of a similar magnitude, between 20 and 25 mV, was recorded by our group for substitution of Sec to Cys at the C-termini of proteins.[54] Both forms are efficiently reduced by hTrx. Differences in pK_a may be a potential explanation for the reliance on Sec, but the pK_a is easily fine-tuned by the protein microenvironment and can be shifted as needed to introduce activity for cSelS U188C. Certainly, other members of the SelS/SelK eukaryotic family do not rely on Sec.[3,55] Perhaps a better explanation would invoke the formation of a particularly short-lived selenenic acid (Se-OH) or seleninic acid (SeO_2^-) as part of the mechanism. Indeed, the mechanism of peroxidase activity appears to be related to atypical 2-cysteine peroxiredoxin where the resolving cysteine is in the same subunit and in which a sulfenic acid (S-OH) intermediate was identified.[56] However, this is beyond the scope of the data presented here.

The finding that SelS is primarily a reductase suggests future directions in delineating its biological mechanism of action. SelS is a member of the ERAD machinery and presumably may take part in unfolding and transporting target proteins. However, SelS, whose active site is in the cytoplasm, is unlikely to remove disulfide bonds in ER-residing soluble misfolded proteins as several ER-resident reductases are involved in that process prior to the transport in the channel.[57,58] Similarly, management of disulfide bonds in misfolded transmembrane proteins is not well understood, but SelS does not appear to be a critical component of the machinery.[59] A unique feature of SelS is that it is an intrinsically disordered enzyme, a class that relies on order to disorder transition or conformational selection to restrict the conformational space upon binding of the substrate.[60] Most intrinsically disordered proteins take part in signaling or regulation. This suggests that SelS's physiological partner(s) is a signaling protein, possibly a member of the unfolded protein response (UPR),[61,62] a pathway responsible for combating oxidative stress. The ERAD path is linked to the UPR response via amplification loops in the cytoplasm.[63] Many of the proteins in the path are also single-pass transmembrane proteins, raising the possibility that they interact with a membrane-bound reductase, such as SelS. Indeed, several selenoproteins act as stress sensors in regulatory pathways.[64,65] One notable binding partner of SelS that was also implicated in signaling is selenoprotein K (SelK), a single-pass transmembrane enzyme with unknown function.[3,66] SelK does not contain a conventional seleno-redox motif in which the Sec is coupled with a Cys, Ser, or Thr in its proximity, and the reactive Sec may be able to interact with the active site of SelS. We are currently testing whether SelK binding influences the structure and enzymatic activity of SelS.

In summary, here we describe the enzymatic function and reduction potential of SelS. We demonstrate that it is enzymatically active in the absence of its binding partner and that the Sec is critical to cSelS function. However, why only the Sec-containing form is enzymatically active remains an open question.

■ ASSOCIATED CONTENT

Supporting Information

Expression, purification, and characterization of cSelS U188C, *m*/*z* spectra of reduction potential measurements, gel shift assays, oxidase and isomerase activity, and peroxidase assays with hGrx. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Address: 136 Brown Laboratory, Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716.

Phone: (302) 831-7028. Fax: (302) 831-6335. E-mail: rozovsky@udel.edu.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SelS: selenoprotein S; SelK, selenoprotein K; Trx, thioredoxin; TrxR, thioredoxin reductase; Grx, glutaredoxin; ERAD, ER-assisted protein degradation; TEV, tobacco etch virus.

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