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Site-specific incorporation of selenocysteine using an expanded genetic code and palladium-mediated chemical deprotection

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Abstract

Selenoproteins containing the 21st amino acid selenocysteine (Sec) exist in all three kingdoms of life and play essential roles in human health and development. The distinct low pKa, high reactivity and redox property of Sec also afford unique routes to protein modification and engineering. However, natural Sec incorporation requires idiosyncratic translational machineries that are dedicated to Sec and species-dependent, which makes it challenging to recombinantly prepare selenoproteins with high Sec specificity. As a consequence, the function of half of human selenoproteins remain unclear, and Sec-based protein manipulation has been greatly hampered. Here we report a new general method enabling the site-specific incorporation of Sec into proteins in *E. coli*. An orthogonal tRNA^{Pyl}-ASecRS was evolved to specifically incorporate Se-allyl selenocysteine (ASec) in response to the amber codon, and the incorporated ASec was converted to Sec in high efficiency through palladium-mediated cleavage under mild conditions compatible with proteins and cells. This approach completely obviates the natural Sec-dedicated factors, thus allowing various selenoproteins, regardless of Sec position and species source, to be prepared with high Sec specificity and enzyme activity, as shown by the preparation of human thioredoxin and glutathione peroxidase 1. Sec-selective labeling in the presence of Cys was also demonstrated on the surface of live *E. coli* cells. The tRNA^{Pyl}-ASecRS pair was further used in mammalian cells to incorporate ASec, which was converted into Sec by palladium catalyst *in cellulo*. This robust and versatile method should greatly facilitate the study of diverse natural selenoproteins and the engineering of proteins in general via site-specific introduction of Sec.

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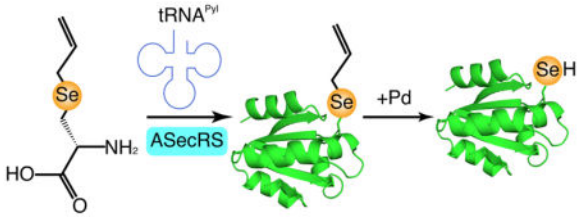
Supporting Information. The Supporting Information is available free of charge on the ACS Publications website. It contains experimental details for chemical synthesis, library construction, synthetase screening, ASec incorporation and deprotection, protein expression, purification and characterization, Sec labeling, enzyme assay, and mass spectrometry. Figures S1–S14 are also included.

Graphical Abstract



INTRODUCTION

Selenoproteins are a special class of proteins containing the 21st amino acid, selenocysteine (Sec), which closely resembles Cys with sulfur replaced by selenium. Sec has unique properties including a low pKa, high reactivity, and resistance to excessive oxidation, rendering selenoproteins distinct biological functions. Selenoproteins are found in all three domains of life, and there are 25 selenoprotein-encoding genes in human genome. Implicated in antioxidant defense and various diseases such as cancer and aging, selenoproteins are important for human health and development.[1–3] Nonetheless, the function of at least half of the human selenoproteins remains unclear. Meanwhile, the unique properties of Sec also provide attractive avenues for various applications, such as selective protein modification and bio-conjugation, chemical mutagenesis (to Ala, Ser, and dehydroalanine), formation of novel bonds (selenylsulfide Se-S, diselenide Se-Se, or selenoether Se-C bonds), facilitation of challenging native chemical ligation, promotion of protein oxidative folding, and enhancement of enzyme activity[4–19]. However, detailed investigation of native selenoproteins and exploitation of Sec for general proteins have been hampered by the difficulty of procuring selenoproteins with high Sec specificity.

Natural incorporation of Sec into proteins involves multiple dedicated factors using a complicated mechanism distinct from that for the 20 common amino acids (Fig. 1a).[20–24] First, Sec has no cognate aminoacyl-tRNA synthetase and is biosynthesized on the $tRNA^{Sec}$. $tRNA^{Sec}$ is initially charged with serine by SerRS to form Ser-$tRNA^{Sec}$. The charged serine is then converted into Sec by selenocysteine synthase (SelA) in bacteria, while in archaea and eukaryotes serine on the $tRNA^{Sec}$ is first converted into *o*-phosphoserine by a kinase (PSTK) and then into Sec by SepCysS synthase. Second, the resulting Sec-$tRNA^{Sec}$ requires a Sec-specific elongation factor, SelB in bacteria and eEFSec in archaea and eukaryotes, to enter the ribosome A site, where it decodes the opal stop codon UGA as Sec translationally. Third, the reassignment of the target UGA codon from a stop signal to Sec is specified by the Sec-insertion sequence (SECIS), which forms a stem-loop structure and is recognized by SelB/eEFSec. SECIS is located immediately next to the UGA codon within the selenoprotein coding region in bacteria, but is situated in the 3'-untranslated region many nucleotides downstream of the UGA codon in mRNAs of archaea and eukaryotes. This complex and species-dependent mechanism for Sec incorporation makes it challenging to express selenoproteins recombinantly.

Chemical and biosynthetic methods have been developed to prepare Sec-containing proteins yet with various limitations. Solid phase peptide synthesis and native chemical ligation

provide complete control over the number and placement of Sec in a protein sequence,[4, 7, 25–27] but it becomes challenging when synthesizing and refolding large proteins. The natural Sec incorporation machinery of *E. coli* has been exploited to produce selenoproteins,[28–31] but the species differences in SECIS sequence and location often introduce unwanted amino acid changes when an archaeal or eukaryotic selenoprotein is heterologously expressed in *E. coli*. To circumvent the need for SelB and SECIS element in bacteria, efforts have been focused on engineering the *E. coli* $tRNA^{Sec}$ or $tRNA^{Sec}$ and EF-Tu together[32–33], allowing the resultant $tRNA^{Sec}$ to be recognized by EF-Tu directly. These efforts successfully enabled the expression of both bacterial and mammalian selenoproteins in *E. coli* without additional mutations.[32–37] Nonetheless, this approach still relies on SelA to convert serine to Sec on the mutant $tRNA^{Sec}$, and mutation of $tRNA^{Sec}$ often decreases the conversion efficiency thus resulting in misincorporation of serine. The engineered $tRNA^{Sec}$ can be used in bacteria only.

Expansion of the genetic code using an orthogonal tRNA/synthetase pair[38–39] has the potential to site-specifically incorporate Sec while completely obviating Sec-dedicated factors. Recently, an orthogonal $tRNA^{Leu}$/CmnRS pair[40] was employed to incorporate a photocaged Sec analog into a model protein EGFP in yeast, and the Sec analog was converted into Sec through photolysis.[41] Unfortunately, photolysis to generate Sec results in significant contamination of dehydroalanine (>15%).[41] The photocaged Sec analog is markedly bulkier than Sec in size, which may not fit well in buried Sec sites in native selenoproteins due to potential interference with protein folding. In fact, no native selenoprotein has been prepared with this approach.[41] The $tRNA^{Leu}$/CmnRS pair cannot be used in the workhorse *E. coli* or other bacteria either. In addition, photolysis using UV light will prevent its effective application in animal tissues and intact animals.

Exploiting genetic code expansion and chemical deprotection, we report here a facile and general method for site-specific incorporation of Sec into proteins in high specificity. We evolved an orthogonal $tRNA^{Pyl}$/ASecRS pair to genetically incorporate unnatural amino acid ASec, a Sec analog with a small allyl protecting group, without needing any Sec-dedicated factors. Following incorporation, ASec was converted into the native Sec in high efficiency by a facile palladium-mediated cleavage under mild biocompatible conditions (Fig. 1b). Using this approach, we generated both artificial and human native selenoproteins from *E. coli*, obtaining high enzyme activity owing to the exceptional specificity of Sec incorporation. We also labeled Sec-incorporated proteins selectively in the presence of Cys on live *E. coli* cell surface, and demonstrated the incorporation of ASec and conversion into Sec in proteins in mammalian cells. This method will be compatible with various cell types and facilitate the production, engineering and research of artificial and native selenoproteins.

RESULTS

A chemical protection-deprotection strategy for site-specific incorporation of Sec into proteins

Sec is not stable in its free form due to its high reactivity, and directly feeding cells with Sec may result in misincorporation with endogenous Cys at respective sites because of the structural similarity between Cys and Sec.[42] Inspired by recent progress on chemically

decaging cysteine from the allyl, acetamidomethyl or thiazolidine protecting groups using palladium catalysts under mild conditions,[43–47] we envisioned that an orthorgonal tRNA/synthetase pair could be evolved to genetically incorporate Se-allyl selenocysteine (ASec) in response to the amber stop codon and that the allyl group could be chemically removed by palladium to generate Sec (Fig. 1b). We decided to incorporate ASec because the allyl group will distinguish ASec from Cys structurally, potentially allowing a synthetase to be evolved to recognize ASec specifically. Meanwhile, the allyl group is relatively small in size, which will mitigate potential interference to protein folding when ASec is incorporated at native Sec sites. In addition, since the allyl group can be readily removed from the thiol of Cys under mild conditions compatible with proteins, we reasoned that the C-Se bond in ASec would be similarly cleaved by palladium to generate Sec in proteins.

Chemical synthesis of ASec

We initially tried to synthesize ASec starting from the unprotected or fully protected L-selenocysteine following the published procedures, yet the yield was less than 10% or the final product had low quality, respectively (Supplementary results). We then developed a semi-protected strategy based on a method for the synthesis of *N*-Boc-*p*-methoxy benzyl-L-cysteine (Scheme 1).[48] Specifically, the *N*-Boc-L-selenocystine was reduced by NaBH4 in ethanol. Treatment of the resulting *N*-Boc-L-selenocysteine with allyl bromide afforded the *N*!Boc!*Se*!allyl!L!selenocysteine, which was deprotected *via* 4 M HCl/dioxane, affording the targeted ASec HCl salt as a white solid with high purity (74% yield, over 3 steps).

Genetic incorporation of ASec into proteins

To genetically encode ASec in *E. coli*, we evolved a pyrrolysyl-tRNA synthetase (PylRS) mutant specific for it. A *Methanosarcina mazei* PylRS (*Mm*PylRS) mutant, C348W/W417S, was reported to incorporate S-allyl cysteine (ACys).[44] However, when testing this mutant using the EGFP reporter with a TAG stop codon at the permissive site 182,[49] we didn't detect green fluorescence, suggesting that the *Mm*PylRS C348W/W417S mutant was inefficient in charging ACys. We then transplanted the mutations to the *Methanosarcina barkeri* PylRS (*Mb*PylRS), and the corresponding *Mb*PylRS(C313W/W382S) showed faint yet ACys-dependent green fluorescence (Fig. S1). To improve the incorporation efficiency, we generated a focused *Mb*PylRS library by randomizing residues C313 and W382, and selected the library for ACys specificity using methods previously described.[50] All identified hits converged into a single mutant (C313W/W382T), which was 3-fold more efficient than *Mb*PylRS(C313W/W382S) in incorporating ACys, as demonstrated by fluorescence, SDS-PAGE, and Western analysis of the expressed EGFP proteins (Fig. S1–S2). On the basis of this *Mb*PylRS(C313W/W382T) mutant we further randomized active site residues L270, Y271, and L274, but all selected mutants, despite changes on the DNA level, had the same amino acid sequence as *Mb*PylRS(C313W/W382T) (Supplementary results).

Considering the structural similarity between ACys and ASec, we attempted using the evolved ACys-specific synthetase to incorporate ASec into EGFP in *E. coli*. However, the initial trial of the *Mb*PylRS(C313W/W382S) or *Mb*PylRS(C313W/W382T) synthetase did not produce any ASec-containing proteins, and *E. coli* cells grew considerably slower when ASec was added in the media. We reasoned that the observed toxicity could be due to allyl

selenide resulting from the cleavage of ASec by cysteine-S-conjugate beta-lyase (β-lyase) *in vivo*.[51] To alleviate the toxicity, we explored ASec incorporation into EGFP (182TAG) in the presence of various additives, including L-Cys, DL-propargylglycine, and 2-(aminooxy)acetic acid, which inhibit β-lyase or selenium toxicity (Fig. S3). We found that L-Cys could rescue the toxicity of ASec: the yield of cells grown in the presence of Cys and ASec was significantly higher than ASec alone (Fig. S4). Western analysis showed that full-length EGFP could now be expressed in the presence of ASec accompanied by L-Cys only (Fig. S3). This L-Cys enabling effect of ASec incorporation was corroborated by expressing another protein thioredoxin (Trx) (see below and Fig. S5). The *Mb*PylRS(C313W/W382S) was also able to incorporate ASec after adding L-Cys, but the *Mb*PylRS(C313W/W382T) was 2–3 times more efficient than *Mb*PylRS(C313W/W382S) for incorporating ASec (Fig. 2a). We thus named this *Mb*PylRS bearing mutations of C313W/W382T as ASecRS for clarity and used it in all subsequent experiments.

To determine ASec incorporation fidelity and site flexibility, we expressed human Trx gene containing an amber stop codon TAG at either Cys32 or Cys73 site and a C-terminal Hisx6 tag, together with the $tRNA^{Pyl}$/ASecRS pair. Full-length Trx was obtained in good yield (3 mg/L) in the presence of 0.5 mM ASec and 0.8 mM L-Cys, but was undetectable on SDS-PAGE and Western blot in their absence (Fig. 2b and S5). Analysis of the expressed Trx by ESI-MS confirmed incorporation of ASec into Trx. An observed peak at 12786 Da corresponds to Trx lacking the initiator Met and containing ASec at site 32 (expected $[M\text{-}Met+H]^+$ = 12787 Da). The observed peak at 12746 Da corresponds to this protein with the allyl group cleaved from ASec (expected $[M\text{-}Met\text{-}allyl+H]^+$ = 12747 Da) (Fig. 2c). Similar ESI-MS results were also obtained from Trx with ASec incorporated at site 73 (Fig. S6a). No peaks were observed corresponding to Trx containing a natural amino acid at the TAG site, indicating the incorporation of ASec into Trx proteins in high specificity.

Conversion of ASec into Sec in proteins

To fully convert the incorporated ASec into Sec, we explored using palladium to cleave the allyl protection group on ASec in proteins. To facilitate the detection of allyl group cleavage, we generated a Ub-GyrA fusion construct that bears a TAG codon at the first Cys position of *Mycobacterium xenopi* GyrA intein.[52] The regeneration of a Cys residue at this site would result in an active GyrA intein, which would cleave Ub from Ub-GyrA for ready detection by SDS-PAGE or Western blot (Fig S7a). We first incorporated ACys at the TAG site, and the intein activity was blocked by ACys, as shown by no cleavage of Ub from Ub-GyrA (Fig S7b). For Pd catalyst selection, [PdCl(allyl)]2 is stable and compatible for use *in vivo*,[53] and this Pd^{2+} compound can be readily reduced to Pd(0) upon nucleophilic attack.[53–54] Pd(TPPTS)4 is a water soluble Pd(0) species, suitable for our needs for deprotection under native or physiological conditions.[55] We also include other Pd^{2+} species for comparison. The results showed that only Pd(0) could cleave the allyl group from ACys incorporated in protein Ub-GyrA, with Pd(TPPTS)4 providing the highest efficiency (>80%) (Fig. S7c, S8). The preference of Pd(0) for allyl removal was also observed for ASec incorporated into proteins: both Pd(TPPTS)4 and [PdCl(allyl)]2 were able to cleave ASec incorporated in the MBP-Z protein (Fig. S9).

We next treated the ASec incorporated Trx with Pd(TPPTS)4, and analyzed the treated protein sample with ESI-MS (Fig. 2d). A peak at 12746 Da was observed, corresponding to the conversion of ASec into Sec in > 93% efficiency (expected $[M\text{-Met-allyl+H}]^+$ = 12747 Da). In some cases, we observed that a small amount of ASec incorporated Trx already had the ASec converted into Sec after purification and without Pd treatment, while ACys incorporated Trx retained its ACys intact (Fig. 2C, S10–11). This difference could be due to the fact that the C-Se bond is weaker than the C-S bond and thus may be more prone to cleavage when incubation with nickel or cobalt during affinity purification.[56] These results indicate that the incorporated ASec could be converted into Sec in high efficiency under mild conditions compatible with proteins, and our method is flexible with Sec incorporation site.

Preparation of native human selenoprotein GPx1

Our method uses the TAG amber codon to specify the Sec incorporation site and obviates the aid of Sec-dedicated SelB, SelA and SECIS elements. This straightforward incorporation mechanism similar to that for canonical amino acids should allow us to incorporate Sec at any desired site of proteins, which will be especially useful for recombinantly expressing mammalian selenoproteins that have internal Sec residues in *E. coli*. To further demonstrate the application of our method in terms of preparation and characterization of native selenoproteins, we attempted the production of the human selenoprotein glutathione peroxidase 1 (GPx1). GPx1 is a major antioxidant enzyme to combat reactive oxygen species and has cardiovascular protective function.[57–58] It has an essential Sec49 residue at the active site, substitution of which with Cys results in 1000-fold activity loss and with Ser leads to inactivation. Currently there is no human GPx1 with Sec incorporated available commercially.

Using the orthogonal $tRNA^{Pyl}$/ASecRS pair, we expressed GPx1 with ASec incorporated at site 49 in *E. coli* in the yield of 3 mg/L (Fig. 3a, 3b). The purified GPx1 intact protein was analyzed by ESI-MS (Fig. 3c). The observed peak at 21671 Da corresponds to GPx1 containing ASec49 (expected $[M+H]^+$ = 21671 Da), and the peak at 21540 Da corresponds to GPx1 lacking the initiator Met and containing ASec49 (expected $[M\text{-Met+H}]^+$ = 21539 Da). No peaks corresponding to GPx1 containing any natural amino acids at site 49 were observed. In addition, we also analyzed GPx1 using trypsin digestion followed by tandem MS (Fig. 3d). The precursor ion for the ASec-containing peptide was identified in high accuracy (expected M/Z = 888.46, observed 888.47), and a series of b and y fragment ions of this peptide clearly indicate that ASec was unambiguously incorporated at site 49 specified by the amber codon. Moreover, the isotopic pattern of the ASec-containing peptide was also analyzed, which was consistent with the expected isotopic distribution of selenium (Fig. S12), further confirming ASec incorporation. Altogether these data indicated that ASec was incorporated into GPx1 at the amber codon specified position in high fidelity without contamination of other amino acids.

After palladium treatment of GPx1, ASec was almost fully converted into Sec as shown by ESI-MS analysis (Fig. 3e). The observed peak at 21630 Da corresponds to GPx1 containing Sec49 (expected $[M+H]^+$ = 21630 Da), and the peak at 21498 Da corresponds to GPx1

lacking the initiator Met and containing Sec49 (expected $[M\text{-}Met+H]^+$ = 21498 Da). No peak corresponding to ASec-GPx1 was detected. The peroxidase activity of resultant GPx1 was measured using a glutathione/glutathione reduc-tase coupled assay, and a high activity of 25 Units (Fig. 3f) was obtained (1 unit was defined as per μmol of NADPH oxidized per min per mg of enzyme). The enzymatic activity measured compares favorably with what have been reported in the literature (Table S2).[30, 32, 36] These results indicate that our method could produce native selenoprotein with high Sec incorporation specificity and enzyme activity.

Selective labeling of Sec on live *E. coli* cell surface

Protein labeling through cysteine thiol alkylation is frequently used because of the high reaction rate and readily available reagents, but it lacks site specificity when multiple cysteine exists.[59] For Sec, one of the most appealing applications is selective labeling in the presence of all natural amino acids including Cys, which obviates the mutation of endogenous Cys residues and affords the shortest linkage placing the resultant modification close to the protein backbone. Due to the large pKa difference between Cys (pKa 8.3) and Sec (pKa 5.2), it is possible to poise the pH to selectively label Sec only with Cys residues unaffected on a single selenoprotein *in vitro*.[5, 60] Since our method can genetically incorporate ASec into proteins in live cells, we explored if Sec could be converted from ASec in live cells and if the resultant Sec could be selectively labeled on the surface of live cells.

We incorporated ASec into an outer membrane protein eCPX (enhanced circularly permuted outer membrane protein OmpX) in *E. coli* to display the ASec residue on the cell surface,[61] treated the cells with Pd(TPPTS)4 to generate Sec, and then incubated cells with a membrane impermeable, Cys-reactive dye Alexa Fluor™ 488 C5 maleimide (Fig. 4a). At pH 7.4, control cells without ASec incorporation were all fluorescently labeled through the dye reacting with endogenous Cys at the cell surface; as expected, the labeling was abolished when pH was decreased to 5.0. In contrast, cells with ASec incorporation into eCPX followed by Pd deprotection were clearly labeled by the dye at pH 5.0, indicating the selectively labeling of Sec on live *E. coli* cell surface in the presence of excess thiols (Fig. 4b).

Protein crosslinking and labeling via the terminal alkene of ASec

Besides the seleno group, ASec also affords a biorthogonal alkene functional group. The side chain length and size of ASec are comparable to those of canonical amino acids, which will minimize potential perturbation to proteins. Labeling or reaction through this terminal alkene will also be valuable for maintaining the modification close to the protein backbone. We therefore explored biorthogonal usage of this alkene group as well.

Photocatalytic thiolene reaction has been widely employed for protein labeling and bio-conjugations.[62] To the best our knowledge, it has not be applied for protein-protein crosslinking. Here we genetically encoded ASec into the 103rd position of the *E. coli* glutathione transferase (GST), and mutated the proximal Lys107 at the dimer interface into Cys. After expressing ASec-containing GST in *E. coli* cells, the cells were illuminated with

302 nm UV light. Western blot analysis of cell lysate showed that GST dimerization was strongly induced after 30 mins (Fig. 5a). Through photo-controlled thiolene reaction, genetically encoded ASec thus may provide a valuable tool for protein crosslinking.

In addition to the thiolene reaction, a terminal alkene can also be utilized through inverse electron demand Diels–Alder reaction (IEDDA).[63–65] Since ASec can selectively introduce a terminal alkene onto the surface of a protein, we tested whether ASec would be compatible with IEDDA for protein labeling. We site specifically incorporated ASec into the MBP-Z fusion protein, and treated the purified protein with or without Cy3 tetrazine derivative. The MBP-Z protein was fluorescently labeled by the Cy3 dye, and the labeling was stable and resistant to reduction by DTT (Fig. 5b). These results demonstrate that the genetically incorporated ASec could be used as a biorthogonal reactive handle for labeling through IEDDA.

Genetic incorporation of ASec into proteins in mammalian cells

Sec and Sec-derived Uaas have not been incorporated into proteins in mammalian cells using engineered tRNA/synthetase pairs. As the $tRNA^{Pyl}$/ASecRS was derived from the $tRNA^{Pyl}$/PylRS, which is orthogonal in prokaryotic and eukaryotic cells,[50, 66] we tested the incorporation of ASec into proteins in mammalian cells using the $tRNA^{Pyl}$/ASecRS pair. We transfected HeLa-EGFP-182TAG reporter cells[49] with plasmid pMP-3xPyltRNA-ASecRS, which expresses ASecRS and 3 copies of $tRNA^{Pyl}$ genes. Suppression of the 182TAG codon of the genome-integrated EGFP gene in the reporter cells would produce full-length EGFP rendering cells fluorescent. Using fluorescence confocal microscopy, we observed strong green fluorescence from cells cultured in the presence of ASec only (Fig. 6a). FACS analysis of cells confirmed ASec-dependent cellular fluorescence, and showed that more cells became green fluorescent with ASec incubation time (Fig. 6b). ASec could be used in the final concentration of up to 0.2 mM, and co-adding 3.2 mM of Cys further ameliorated cellular toxicity of ASec. The successful incorporation of ASec into EGFP in HeLa cells was also demonstrated by Western blot analysis of reporter cell lysates with an anti-GFP antibody (Fig. 6c): Full-length EGFP was produced only when ASec was added to the growth media.

We next applied [PdCl(allyl)]2 to these cells for *in vivo* deprotection of ASec. [PdCl(allyl)]2 has been shown as an effective Pd cleavage reagent with little cellular toxicity.[53, 67] Similarly, we found that the EGFP expression level was not affected by [PdCl(allyl)]2 treatment of the HeLa-EGFP-182TAG reporter cells (Fig. 6c). To investigate the cleavage of ASec into Sec in mammalian cells, we utilized Sec's unique property of selective labeling at pH 5 due to its lower pKa and high nucleophilicity.[5, 41] ASec-EGFP expressing HeLa cells were treated with [PdCl(allyl)]2, and EGFP was trapped by an EGFP specific antibody and then labeled with a Cy3 maleimide through Se-Michael addition at pH 5. The samples were separated by SDS-PAGE and imaged for Cy3 fluorescence. As a negative control, Uaa *p*-azido-L-phenylalanine (AzF) was incorporated at the same site into EGFP by transfecting the HeLa-EGFP-182TAG reporter cells with plasmid pIRE-Azi3 and incubating with 1 mM of Azi,[68] and the sample was similarly treated. As shown in Fig. 6d, Cy3 fluorescence was

detected only on the EGFP with ASec incorporated and treated with [PdCl(allyl)]2, suggesting the successful deprotection of ASec into Sec in mammalian cells.

DISCUSSION

We have developed a new general method to prepare selenoproteins with Sec site-selectively introduced in high specificity. Through genetic incorporation of the allyl protected ASec in response to the amber TAG codon followed by facile palladium-mediated cleavage to generate Sec, our method obviates Sec-dedicated factors completely and thus generally enables the expression in *E. coli* of various selenoproteins regardless of the Sec position and source species. In comparison with existing methods using either $tRNA^{Sec}$ mutants compatible with the common EF-Tu or a photocaged Sec analog, our method affords high specificity for Sec incorporation, as shown in the preparation of the native selenoprotein GPx1, and enables ASec/Sec incorporation into proteins in mammalian cells.

To achieve protein translation in high fidelity, our approach features a “chemical protection-deprotection” strategy to solve the challenge of distinguishing Sec from the structural similar Cys. Nature solves this challenge by biosynthesizing Sec on the tRNA without evolving an aminoacyl-tRNA synthetase specific for Sec. Our chemical protection-deprotection strategy represents a facile alternative that we expect can be generally useful for the incorporation of various intractable amino acids due to their structural, physical, chemical, or biological properties. For instance, we recently devised a similar strategy to genetically incorporate phosphotyrosine, which is cell-impermeable and chemically labile inside cells.[69] In addition, in solid phase peptide synthesis and Sec-mediated multiple steps of challenging protein ligations, Sec needs to be protected and regenerated using *p*-methoxybenzyl, 2,2'-dithiobis(5-nitropyridine), or selenazolidine groups.[70–73] The facile conversion of ASec to Sec shown here may provide an attractive alternative route to such applications.

Another obstacle we overcame in this work is circumventing the toxicity of the target Uaa. It has been generally considered infeasible to genetically encode Uaas that are toxic to cells.[74] By devising a strategy to inhibit the toxicity, we showed here the successful incorporation of the toxic ASec, which may guide future efforts in incorporating other toxic yet useful Uaas. As a starting point, we first demonstrated that it is feasible to incorporate a Sec-derived Uaa into proteins in mammalian cells followed by *in cellulo* deprotection to generate Sec. Therefore, our method has the potential to be compatible for use in both prokaryotic and eukaryotic cells. We expect that the use of $tRNA^{Pyl}$/ASecRS in eukaryotic cells will serve as a valuable tool for studying and charactering the native selenoproteins with unknown biological functions.

We further demonstrated the selective labeling of the incorporated Sec in the presence of Cys on live *E. coli* cell surface. With this applicability, there is no need to mutate endogenous Cys residues of the protein to be labeled, which is especially useful for proteins containing essential Cys residues or disulfide bonds. A unique advantage of labeling through the seleno group of Sec is that it results in the shortest possible linkage (similar to Cys) placing the label closer to the protein backbone, which is valuable in applications such as the attachment of biophysical probes (e.g., spin labels for electron paramagnetic resonance and

fluorescent labels for Förster resonance energy transfer) for measurements in high accuracy. In addition, no catalyst is needed for sele-no-mediated labeling, and many probes compatible for thiol and seleno labeling are readily available commercially. On the other hand, for applications in which linkage length is more appropriate on the scale of other canonical amino acids such as Met and Lys, the site-specifically incorporated ASec also affords a bioorthogonal alkene handle for selective protein labeling and bio-conjugations through metathesis,[75] thiolene reaction,[76] or oxidative heck coupling.[77] We have demonstrated the use of the terminal alkene of ASec for photo-induced GST dimerization through thiol-ene reaction and for site specific protein labeling through IEDDA reaction.

In summary, the generality and robustness of this method to prepare native selenoproteins would enable the study of diverse selenoproteins currently elusive to procure; facile introduction of Sec into non-Sec-containing proteins in high specificity would dramatically expand the scope and versatility of chemical biology means for general protein research and exploitation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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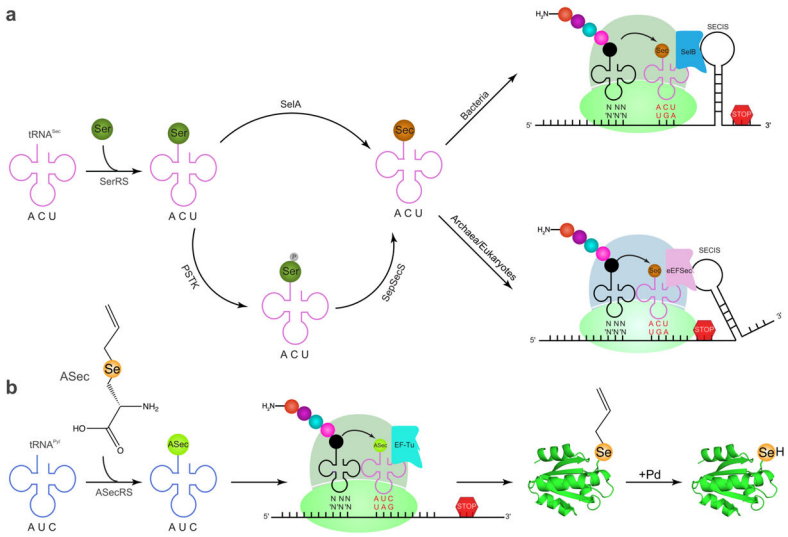


Figure 1.

Site-specific incorporation of Sec into proteins. (a) Natural translation machinery for Sec incorporation uses Sec-dedicated genetic factors and is species dependent. (b) Our method for Sec incorporation obviates all Sec-dedicated factors and is generally compatible with selenoproteins.

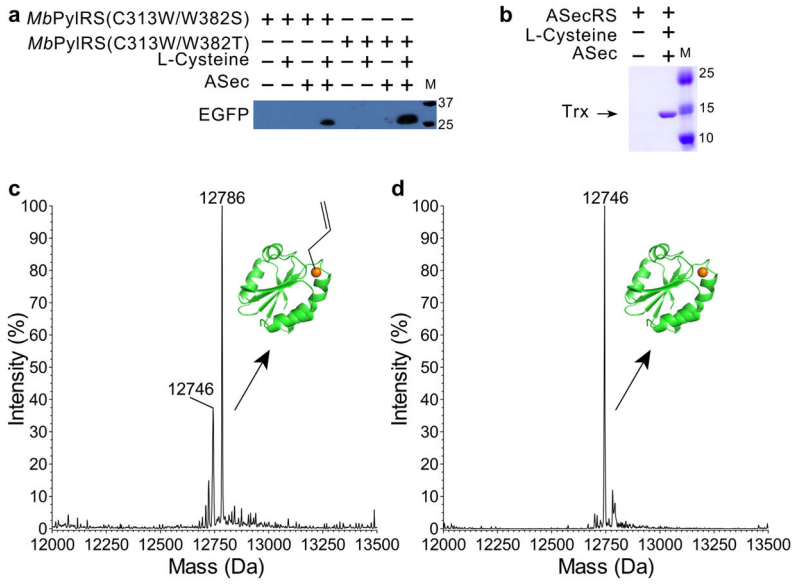


Figure 2.

Site-specific incorporation of ASec into proteins and conversion to Sec through Pd-mediated cleavage. (a) Western analysis of incorporation of ASec into EGFP in *E. coli.* *Mb*PylRS(C313W/W382T) was 3 times more efficient than *Mb*PylRS(C313W/W382S). (b) SDS-PAGE analysis of affinity purified Trx with ASec incorporated. (c) ESI-MS spectrum of ASec-incorporated Trx, confirming the incorporation of ASec at site 32. (d) ESI-MS spectrum of Trx after Pd-mediated cleavage confirming the conversion of ASec into Sec.

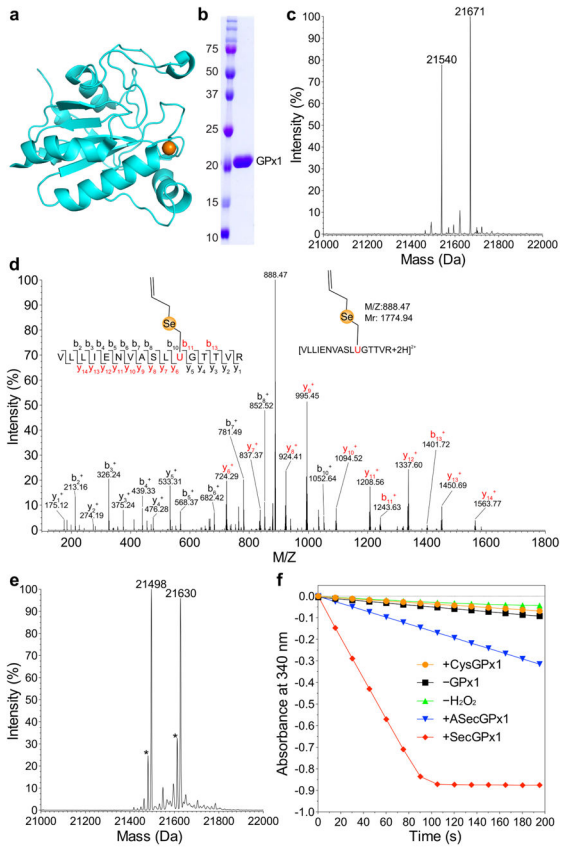


Figure 3.

Preparation of human native selenoprotein GPx1 with high Sec specificity and enzyme activity. (a) Structure of human GPx1 (PDB code:2F8A) with an orange sphere showing the position of Sec49. (b) SDS-PAGE analysis of purified GPx1 with ASec incorporated. (c) ESI-MS spectrum of intact GPx1 confirming ASec incorporation in high fidelity. (d) Tandem MS spectrum of trypsin digested GPx1 confirming ASec incorporation at site 49 specified by the amber codon. U represents ASec in the peptide sequence. The fragment ions that contain ASec are denoted in red color. (e) ESI-MS spectrum of GPx1 after Pd-mediated cleavage of ASec, confirming conversion of ASec to Sec. Peaks denoted by a star have mass corresponding to elimination of H2O from the two main peaks. (f) GPx1 glutathione peroxidase activity measurements showing high activity for the GPx1(Sec49) prepared using our method. The activity was monitored by the absorbance decrease at 340 nm that reflects the consumption of NADPH, the cofactor of glutathione reductase.

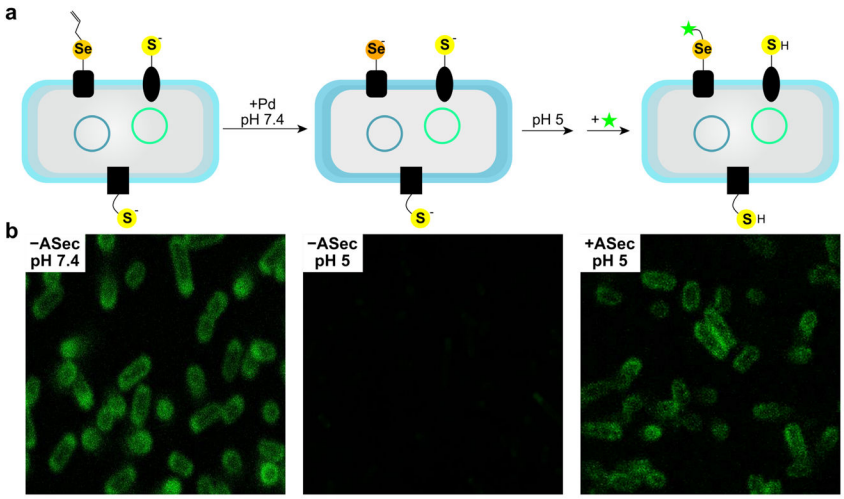


Figure 4.

Selective labeling of incorporated Sec on live *E. coli* cell surface in the presence of endogenous Cys. (a) Scheme showing the procedures for Sec incorporation and labeling on *E. coli* cell surface. (b) *E. coli* cells expressing Sec-incorporated outer membrane protein was selectively labeled by the Alexa Fluor™ 488 dye at pH 5.0.

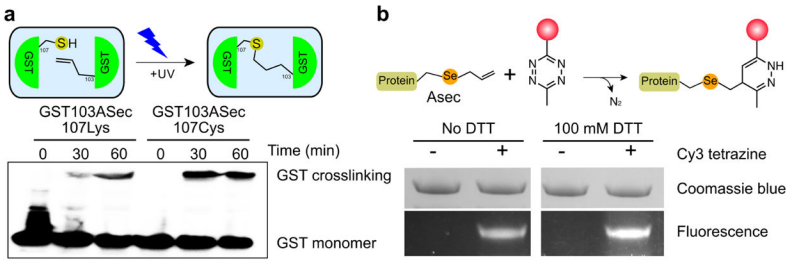


Figure 5.

Genetically incorporated ASec enables photo-induced GST dimerization in living cells and site specific protein labeling through IEDDA. (a) Western blot analysis of GST dimerization through thiol-ene reaction upon UV illumination of living cells. (b) Purified MBP-Z with ASec incorporated was fluorescently labelled with Cy3 tetrazine. Top: Coomassie stained SDS-PAGE gel; Bottom: fluorescence image of the same gel before Coomassie blue staining.

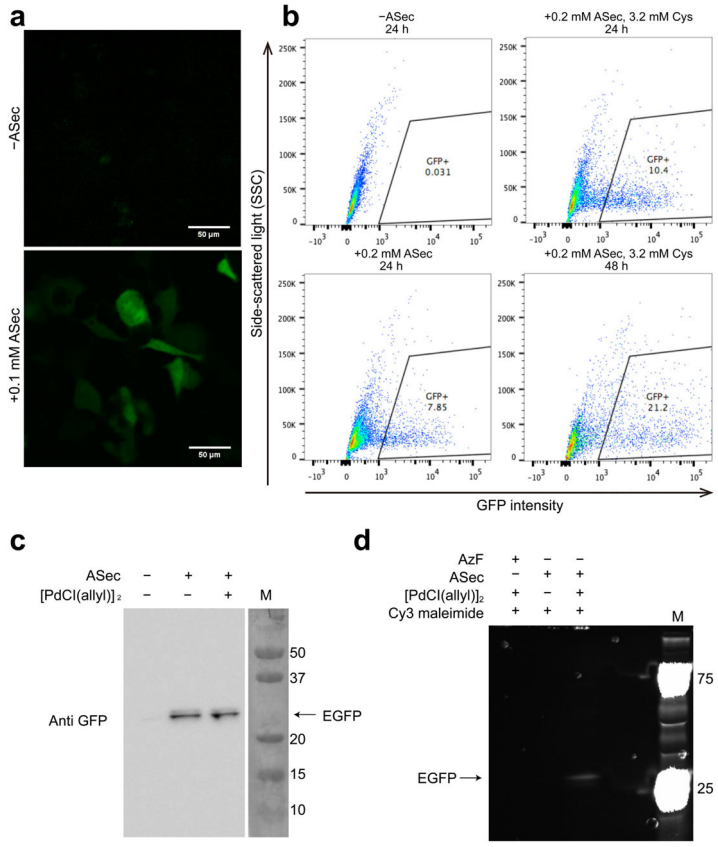
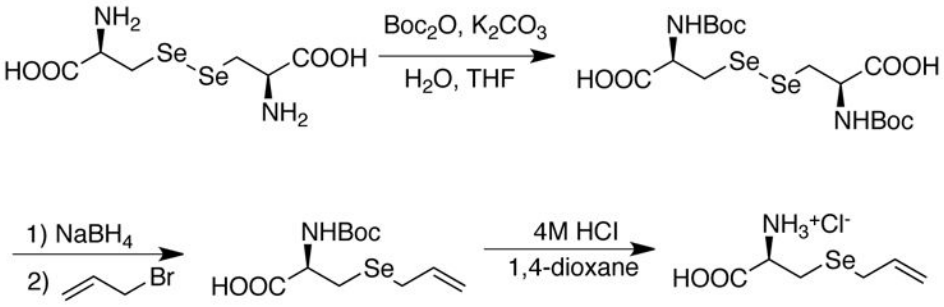


Figure 6.

Genetic incorporation of ASec and its deprotection in mammalian cells. (a) Confocal fluorescence image to detect the incorporation of ASec into EGFP-182TAG in HeLa cells. (b) FACS analyses of ASec incorporation into EGFP-182TAG in HeLa cells under conditions indicated above. The usage of ASec above 0.2 mM led to increased toxicity. Adding Cys rescued the toxicity, and thus increased incorporation efficiency. The data point of 0.2 mM ASec alone after 48 h was not collected due to ASec toxicity. (c) Western blot analysis of HeLa reporter cell lysate to detect ASec incorporation into EGFP. (d) Fluorescence gel image of various EGFP samples labeled with Cy3 maleimide. Deprotection of ASec into Sec in EGFP in HeLa cells led to Cy3 labeling of Sec.



Scheme 1.

Synthesis of ASec starting from semi-protected L-selenocysteine.