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JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Utilizing Selenocysteine for Expressed Protein Ligation and Bioconjugations

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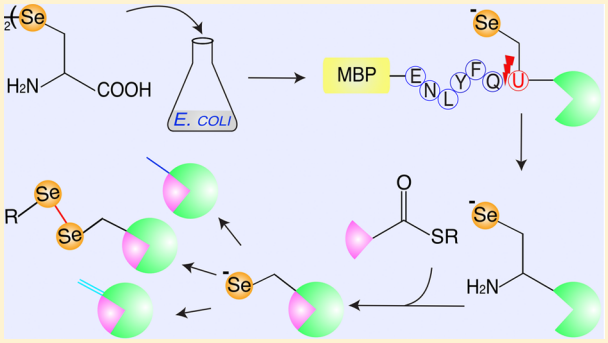
Supporting Information

ABSTRACT: Employing selenocysteine-containing protein fragments to form the amide bond between respective protein fragments significantly extends the current capabilities of the widely used protein engineering method, expressed protein ligation. Selenocysteine-mediated ligation is noteworthy for its high yield and efficiency. However, it has so far been restricted to solid-phase synthesized seleno-peptides and thus constrained by where the selenocysteine can be positioned. Here we employ heterologously expressed seleno-fragments to overcome the placement and size restrictions in selenocysteine-mediated chemical ligation. Following ligation, the selenocysteine can be deselenized into an alanine or serine, resulting in nonselenoproteins. This greatly extends the flexibility in selecting the conjugation site in expressed protein ligations with no influence on native cysteines. Furthermore, the selenocysteine can be used to selectively introduce site-specific protein modifications. Therefore, selenocysteine-mediated expressed protein ligation simplifies incorporation of post-translational modifications into the protein scaffold.



INTRODUCTION

Native chemical ligation (NCL) is a biosynthetic method for protein preparation that relies upon an amide-forming reaction to generate proteins from their respective fragments.[1−3] Many proteins require NCL preparation due to their toxicity to cells, their inability to fold or assemble correctly in the host, or because their preparation involves post-translational modifications that cannot be carried out by the host or in cell-free systems. The majority of NCL reactions are accomplished via expressed protein ligation (EPL) in which at least one of the fragments to be ligated was prepared by heterologous expression. The ligation is driven by a cysteine-mediated attack on a protein fragment terminating with a thioester.[4,5] EPL is widely used but suffers from several limitations. First the ligation efficiency can be low, particularly for large fragments, because the reaction needs to be carried out at a slightly basic pH to allow Cys deprotonation, which unfortunately also accelerates the thioester hydrolysis. Second, the cysteine cannot be desulfurized selectively if the protein contains other cysteines that are necessary for its function. Cysteine may have structural, catalytic, or signaling functions and is often necessary for function. Third, since the cysteine is necessary for ligation, it restricts the choice of ligation site or alternatively leaves a cysteine instead of the original residue, introducing a mutation. However, the ligation efficiency can be significantly boosted by using selenocysteine (Sec, U), which is a better nucleophile than cysteine.[6,7] Furthermore, Sec's low pK_a (∼5.2) allows the reaction to occur at lower pH values, thereby minimizing undesired side reactions including competing thioester hydrolysis. Sec-mediated ligation also offers much greater flexibility in the choice of ligation site because, following ligation, the Sec can be selectively converted into an alanine or serine under mild conditions.[8−11] Due to the abundance of these amino acids, it is straightforward to generate the protein of interest with the desired sequence with no trace of the ligation. The distinct advantage of Sec deselenization to Ala is that it is compatible with the presence of cysteines in the target protein. In contrast, Cys desulfurization to Ala will eliminate all cysteines indiscriminately, including those that are indispensable for function.[8,9,12] Moreover, due to its specialized chemistry, Sec can be specifically manipulated while leaving any of the 20 canonical amino acids unaffected.[13−17] Therefore, Sec's chemical reactivity can be further exploited to specifically label the protein via selenylsulfide (Se−S), diselenide (Se−Se), and selenoether (C−Se−C) bonds, or via conversion to dehydroalanine (Figure 1). Dehydroalanine is a highly reactive electrophile that readily reacts with nucleophiles, linking them permanently to the protein of interest.

The power of Sec-mediated over Cys-mediated ligation was noted by seminal work on native chemical ligation.[18,19] Yet, despite its many advantages, it was demonstrated for only a handful of proteins because of restrictions on where the Sec can be incorporated. This is because current methods rely exclusively on seleno-fragments prepared by solid-phase peptide synthesis, a powerful technique yet one that is

Received: October 27, 2016
Published: February 10, 2017

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430−3437

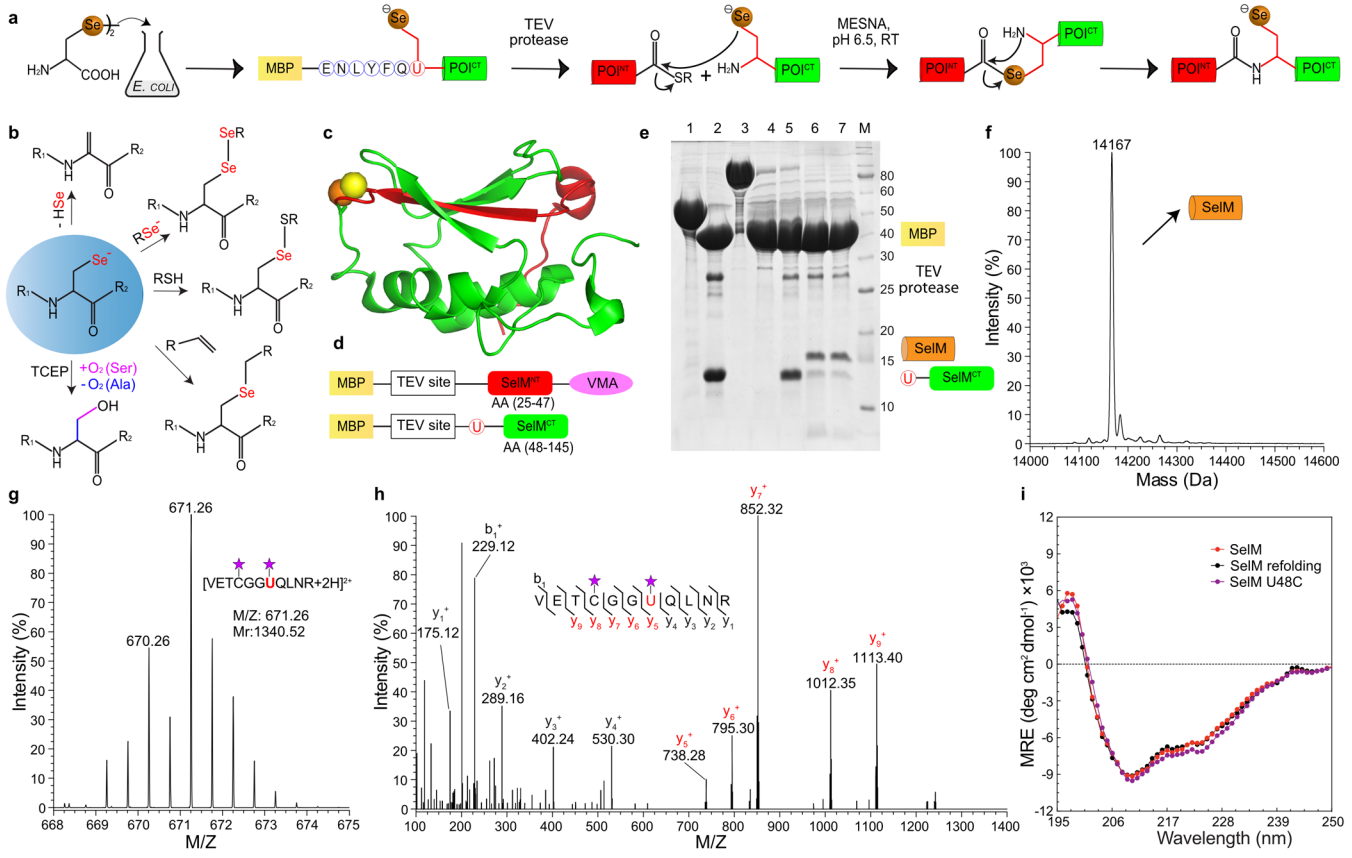


Figure 1. (a) Principle of Sec-mediated expressed protein ligation (Sec-EPL). The seleno-fragment, fused with MBP, is expressed in *E. coli* in selenocystine enriched medium. The complementary thioester protein fragment is prepared via intein technology. TEV protease cleaves MBP from the seleno-fragment, and the protein of interest (POI) is then spontaneously generated through amide bond formation. (b) Applications of Sec-mediated ligation. Panels c–h: Sec-EPL preparation of SelM. (c) Design of SelM fragments. Red: thioester fragment; Green: seleno-fragment; Yellow: sulfur atom; Orange: selenium atom. (d) The design of thioester ($SelM^{NT}$) and seleno ($SelM^{CT}$) fragments. SelM's ER targeting sequence (residues 1 to 24) was omitted, as it is cleaved *in vivo*. (e) SelM ligation at 25 °C and pH 7.0 monitored by SDS-PAGE under reducing conditions: Lane 1: MBP-$SelM^{CT}$; lane 2: same as lane 1 but after TEV protease cleavage; lane 3: MBP-$SelM^{NT}$-VMA; lane 4: MBP-$SelM^{NT}$ thioester following cleavage of MBP-$SelM^{NT}$-VMA with thiols and subsequent purification; lanes 5–7: the formation of SelM monitored on days 0, 1, and 2; lane M: molecular mass standards. $SelM^{NT}$ cannot be detected because of its 2 kDa mass. (f) Deconvoluted ESI-MS of intact SelM. (g) ESI-MS spectrum of the SelM peptide containing the Sec residue alkylated with iodoacetamide (purple star). (h) Tandem MS sequencing of the peptide from panel G confirms Sec's presence in SelM. Fragment ions that contain Sec are colored red. (i) Superimposed CD spectra of SelM, before and after refolding, and SelM U48C prepared by heterologous expression.

constrained by peptide size and properties.[20−22] Accordingly, placement of Sec at loci distant from the C-terminus is technically demanding, and the majority of Sec-driven EPL reactions were carried out with short peptides. Indeed, due to these limitations, only a few selenoproteins with a Sec-peptide exceeding 25 residues have been synthesized at this point, and merely three native selenoproteins have been produced in this manner.[23−26] To remove these constraints, and to allow high yield as well as readily accessible Sec-mediated ligation, we developed a broadly applicable method employing heterologous expression for the preparation of Sec-containing protein fragments (Figure 1), which we have abbreviated as Sec-EPL. In Sec-EPL, seleno-fragments are prepared by heterologous expression and stabilized by a fusion partner. The seleno-fragment is then released immediately prior to ligation to minimize potential aggregation. This assists Sec-driven EPL ligations, particularly those with large or insoluble fragments. The procedure is simple, is cost-effective, and offers high-yield preparation of both selenoproteins and conventional (i.e., without selenium) targets. The primary advantage is that a broad range of the seleno-containing fragments can be generated and subsequently ligated. This facilitates the preparation of demanding targets such as large proteins. It is also suitable for introducing multiple post-translational modifications by manipulating both Sec and Cys in proteins for site-specific dual-labeling.

RESULTS

Principles of Selenocysteine—Expressed Protein Ligation. The challenge in heterologous expression of protein fragments with Sec is that while Sec is a genetically encoded amino acid, its incorporation into proteins is distinct from that of the 20 canonical amino acids.[27,28] Uniquely, Sec insertion requires a dedicated suite of proteins and a structural element in the protein mRNA to reprogram the opal codon UGA to encode for Sec. While it is possible to utilize the native cellular machinery to prepare selenoproteins, the incorporation efficiency remains low due to the opal codon being decoded as a termination signal.[29] More recently, engineering of the Sec-specific tRNA removed the restrictions on the transcribed sequence, affording a flexible platform for heterologous

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430−3437

expression of selenoproteins with yield constantly improving.[30−33] Other methods include manipulations of unconventional amino acids inserted using recombinant systems for amber codon suppression,[14,34] the substitution of all sulfur atoms into selenium,[35] linking the Sec enzymatically before a Lys or Arg,[36] and chemical synthesis.[25] Nevertheless, placement of Sec at a desired position in a protein remains more demanding than routine protein work and so far suffers from low yield. Since our goal is to readily prepare Sec-fragments with high yield, we have opted to prepare seleno-fragments using a method in which Sec is incorporated by misloading the cysteinyl-tRNA with Sec (Figure 1a) using defined *E. coli* growth medium supplemented with selenocystine.[37] Our procedure was adapted from protocols developed for enrichment of Sec and selenomethionine in proteins for crystallographic phasing[38,39] but simplified to minimize growth media exchange and optimize yield. Similar to those reports, our modified procedure leads to about 90% substitution of all Cys residues to Sec (examples are shown in the next section). For selective placement of Sec in only one location in the protein, the Sec-fragment cannot contain Cys residues because the Sec incorporation method results in a global replacement of all Cys with Sec. However, Cys residues can be placed without restrictions in the complementary fragment. The seleno-fragment is expressed fused with maltose binding protein (MBP). This inhibits its aggregation, increases both yield and solubility, and offers an affinity tag for purification. Using fusion partners allows the expression of diverse seleno-fragments at high yield and removes the constraint on their size or properties. However, as shown in Figure 1a, in order for the ligation to take place, the Sec must be the first residue at the N-terminus of the seleno-fragment, so that the selenolate can attack the thioester bond of the complementary protein fragment to generate the amide bond. To that end, we demonstrate that the seleno-fragment can be cleaved directly before the Sec, using tobacco etch virus (TEV) protease (see later). Once freed, the exposed Sec attacks the thioester fragment and the ligation proceeds through a spontaneous Se → N acyl shift to form the protein of interest.[5] Thus, it is key to Sec-EPL success that the seleno-fragment is released just prior to ligation to avoid aggregation and that the TEV protease releases the seleno-fragment with a free N-terminus Sec. Depending on the needs for subsequent purification steps, the TEV protease can be present during or removed ahead of the ligation. An advantage of retaining the TEV protease is that the release of the Sec-fragment is immediately followed by a ligation, which minimizes the chances of aggregation of unstable protein fragments. In what follows, we demonstrate the broad applicability of our Sec-EPL method, as summarized in Figure 1b.

Sec-EPL of Selenoproteins. First, Sec-EPL allows the preparation of selenoproteins, a family of enzymes that is predominantly involved in signal transduction and detoxification pathways.[27,28,40] Despite their physiological importance, many of their functions remain unknown, as their specialized biosynthesis renders them difficult to obtain for characterization (see above).[27] The largest class of selenoproteins possesses a thioredoxin (Trx) fold. It is an early evolutionary tertiary protein structure that is commonly found in enzymes that catalyze a broad spectrum of reactions, such as oxidation, reduction, or thiol disulfide exchange.[41,42] In humans, members of this class (SelH, SelM, SelT, SelV, SelW, and Sep15) are implicated in signal transduction and regulation of gene expression.[43] However, so far, no Sec-containing species from this class has been characterized *in vitro*. We made selenoprotein M (SelM) as a representative member of the group. SelM was linked to obesity, but its precise function or protein partners remain unknown.[27] Human SelM contains Sec at the 48th residue out of 145, in a Cys-X-X-Sec redox motif located on a loop adjacent to the first β sheet (where X stands for any amino acid; Figure 1c).[44] Figure 1d and S1 detail the construction of the two fragments for ligation: MBP-SelM^{CT} and MBP-SelM^{NT}-thioester. Sec was incorporated using a modified cysteinyl-tRNA misloading protocol (for details see Supporting Information). Following purification, the seleno-fragment SelM^{CT} was cleaved from its MBP partner by TEV protease. The TEV protease was selected due to its tolerance toward small amino acids at its recognition site ENLYFQ/P1′ (where P1′ stands for any canonical amino acid except P).[45] We demonstrated that TEV protease cleaves the peptide bond between Q and U, leaving a fragment with Sec at the N-terminus that is free to react with the thioester fragment (Figure 1e, S2−S4). The thioester fragment was generated by standard intein technology using *Saccharomyces cerevisiae* Vacuolar Membrane ATPase (VMA1) intein (Figure S5).[46] The ligation reaction under native conditions was over 90% completed after 1 day (Figure 1e, S6−S7). SelM was further purified to homogeneity and its identity confirmed by intact protein ESI-MS and tandem mass sequencing (Figure 1f-h and S8). The presence of Sec was confirmed from the isotopic distribution of the seleno-peptide VETCGGUQLNR (generated by a trypsin digestion) and its subsequent sequencing by tandem mass spectrometry. The resulting SelM is primarily the Sec-containing form as judged by intact ESI-MS (Figure 1f). This is because even though a slight amount of Cys-containing fragment is present during the ligation, at pH < 7 the Sec-fragment is at least 10 times more reactive than the Cys-fragment.[5] We found in multiple systems that MBP was helpful for increasing the yield and solubility. Other fusion proteins worked well, but MBP was the most robust. Furthermore, pMAL vectors and our modified pMAL cloning vector (Figure S1) were the most robust for achieving high Sec incorporation, including multiple Sec (Figure S9). The ligation yield is compatible with that needed for biochemical and structural characterization: 10 mg of SelM from ligation utilizing MBP-SelM^{NT}-VMA expressed in 6 L of LB medium and MBP-SelM^{CT} from 2 L of defined growth medium. The circular dichroism (CD) spectra of SelM before and after refolding, and that of SelM U48C prepared by conventional heterologous expression (Figure S10) exhibited a high degree of similarity when superimposed (Figure 1i). This indicates that, after ligation, SelM folded spontaneously to adopt its native fold.

In addition, we have prepared selenoprotein W (SelW), a cell cycle regulator that interacts with signaling proteins.[47,48] Like SelM, its enzymatic function is unknown. In contrast to SelM, in SelW the active site is stabilized by an extensive network of aromatic residues.[49] Consequently, its preparation requires refolding (Figure S11−S16). Human and other mammalian SelW have been reported to be glutathiolated on their nonactive cysteines.[25,50] Thus, in preparation for activity assays and studies of SelW's interactions with protein partners, we selected *Gallus gallus* SelW, which contains only one Cys at the active site.

Sec-EPL of Nonselenoproteins. Importantly, Sec-EPL can generate a wide range of proteins without introducing mutations because the Sec can be readily and selectively

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430−3437

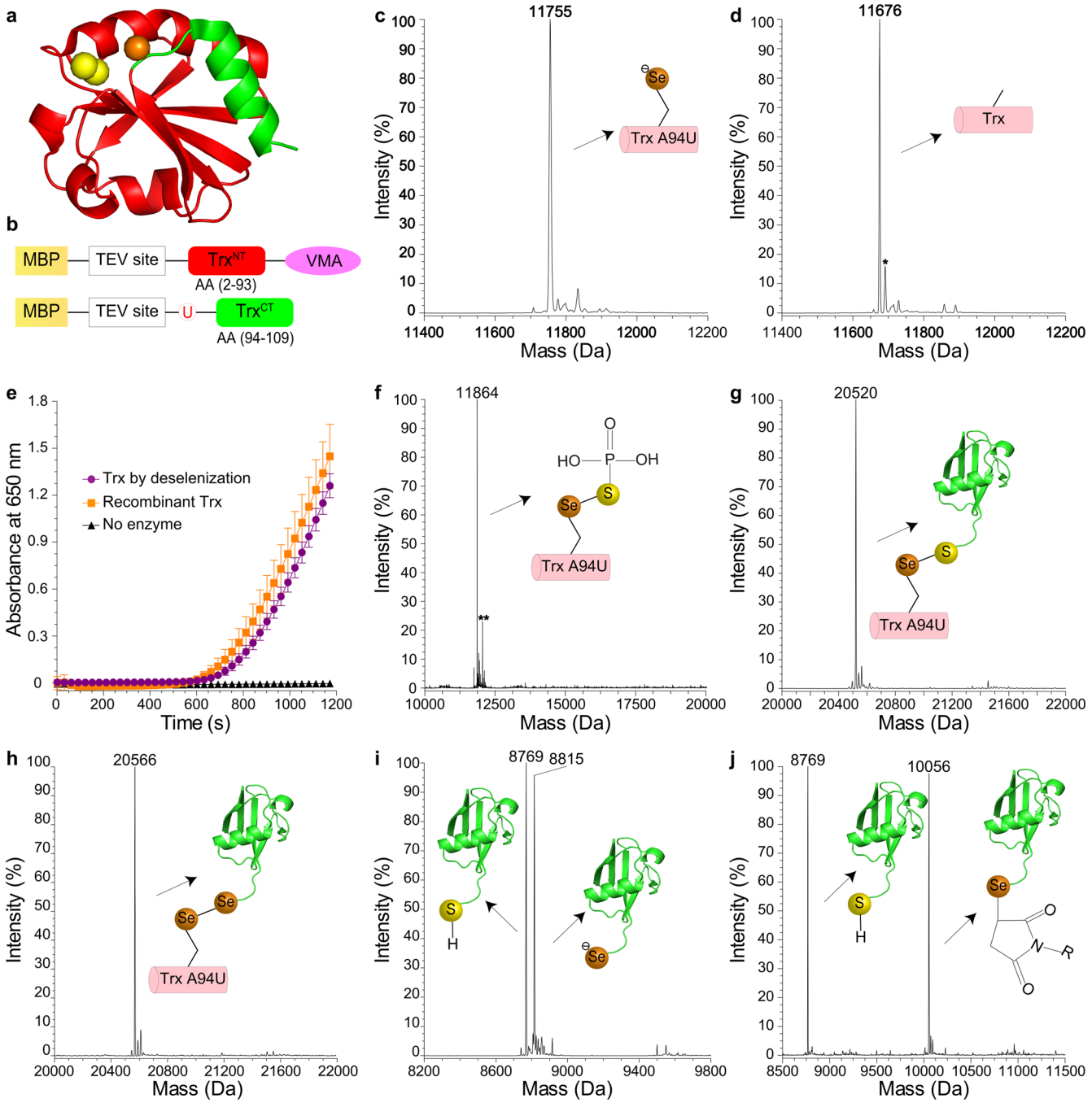


Figure 2. Sec-EPL can be used to generate nonselenoproteins and to introduce a unique site for labeling. (Panels a–d) Chemical ligation of *E. coli* Trx with Sec at position 94 and subsequent deselenization to form wild-type Trx. (a) The structure of Trx A94U based on PDB entry 2TRX. Red: thioester fragment; Green: seleno-fragment; Yellow: sulfur atoms; Orange: selenium atom. (b) Design of thioester (Trx^{NT}) and seleno (Trx^{CT}) fragments. (c) Deconvoluted ESI-MS of Trx A94U. (d) Deconvoluted ESI-MS of wild-type Trx generated by deselenization of Sec to Ala (the * denotes Trx A94S). (e) The enzymatic activity of Trx generated by deselenization and that of Trx prepared by heterologous expression are similar as measured by insulin turbidity assays. Data represent mean ± s.d. (n = 3). (f) Labeling of Trx A94U with a thiophosphate (the peak marked with ** is a contamination from the commercial thiophosphate). (g) Conjugation of Trx A94U to ubiquitin G76C via the formation of a selenylsulfide bond. (h) Conjugation of Trx A94U to ubiquitin G76U via the formation of a diselenide bond. (Panels i–j) Due to Sec's low pK_a, it can be selectively alkylated. (i) A mixture of ubiquitin G76C and ubiquitin G76U prior to alkylation. (j) The same sample from panel i alkylated with $MM(PEG)_{24}$. Only ubiquitin G76U was alkylated.

deselenized into either serine or alanine.[10,11] Therefore, it is possible to convert Sec into an abundant amino acid, leaving no trace of the original ligation method. The ligation site then can be selected to increase the probability of the fragments reacting, such as loops and other surface accessible regions. To demonstrate the conversion and its specificity, we prepared a Sec-containing form of *E. coli* Trx using residue 94 as the ligation site (Figure 2a–c, Figures S17–S22; figures show Trx samples following purification). Trx A94U was converted to native Trx by treating it with 200-fold excess tris(2-carboxyethyl)phosphine (TCEP) and dithiothreitol (DTT) under anaerobic conditions to promote deselenization of Sec

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430–3437

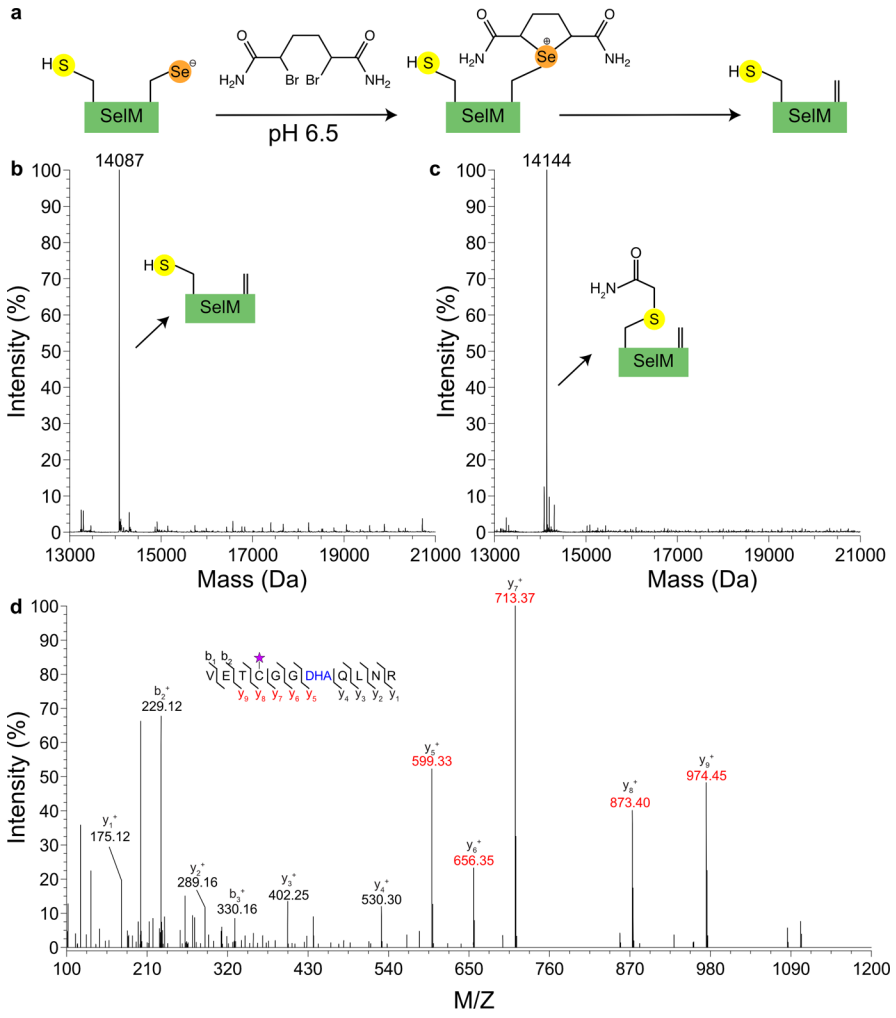


Figure 3. Chemoselective dehydroalanine formation from Sec. (a) Sec deselenization into dehydroalanine can be promoted through two successive alkylation steps. The reaction can be carried out selectively, even when cysteines are present, due to the difference between the Sec and Cys pK_a's. (b) Dehydroalanine formation in SelM at pH 6.5 showing complete conversion. (c) The sample from panel b treated with excess iodoacetamide at pH 7.0. The sole Cys was available for alkylation at that pH even though it was in proximity to the dehydroalanine site. (d) The tandem mass of trypsin-digested sample from panel c further confirms the presence of dehydroalanine in position 48. Red color indicates ions containing dehydroalanine. The purple star denotes alkylation by iodoacetamide.

into alanine (Figure 2d, Figure S23), while under aerobic conditions Sec turned into Ser (Figure S24). Trx has two catalytic Cys residues, which were not affected by this mild treatment. The disulfide reductase activity of Trx formed by deselenization was comparable to that of Trx prepared by heterologous expression (Figure 2e, Figures S25–S26).

Site-Specific Conjugation of Post-translational Modifications. Subsequently, Trx A94U was used to selectively introduce post-translational modifications and for conjugating proteins. Trx A94U provides an excellent test case because its catalytic cysteines are highly reactive and can in theory be modified as well. A phosphate group was introduced selectively on U94 by reacting reduced Trx A94U with thiophosphate (Figures 2f and S27). We next tested the ability to link proteins via the Sec in a reversible fashion. Trx A94U was conjugated to ubiquitin G76C via a selenylsulfide (Se–S) bond, and to ubiquitin G76U via a diselenide (Se–Se) bond (Figures 2g–h and S28). Such conjugations are reversible and can be conveniently manipulated in a selective manner. The diselenide bond has a lower redox potential than that of the disulfide bond and can be retained under mild reducing conditions for downstream applications such as activity assays or for keeping the diselenide linkage while reducing undesired disulfide bonds.[51] Finally, we also demonstrate that it is possible to selectively target Sec over Cys in a mixture of ubiquitin G76C and ubiquitin G76U (Figure 2i–j). Ubiquitin G76U formed a stable selenoether (C–Se–C) bond with the alkylating reagent methyl-PEG-maleimide, while ubiquitin G76C was unmodified. Overall, these examples demonstrate the well-documented ability to selectively modify Sec, even when cysteines are present,[15] while further corroborating that no side reactions, such as Sec elimination, take place (as detected by mass spectrometry).

Another promising Sec-EPL application is to promote selenium elimination from Sec to form dehydroalanine, which serves as an electrophilic site for Michael addition reactions, typically with thiol nucleophiles. Dehydroalanine provides a unique site to introduce conjugates that closely resemble the proteins original biological modifications.[12,52] In addition, a biocompatible reaction that forms C–C bonds has recently vastly diversified the kind of protein modifications that can be attached to dehydroalanine.[53] Dehydroalanine is typically

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430–3437

incorporated into protein by desulfurization of cysteine,[12] via overoxidation of nonconventional amino acids, or phosphate removal from a phosphoserine.[14,54,55] Yet, introducing dehydroalanine chemoselectively remains challenging when proteins have multiple cysteines. We tested Sec deselenization by bis-alkylation-elimination using α,α'-dibromo-adipyl(bis)-amide (DBAA), which was previously reported not only to be highly efficient for cysteine desulfurization but also to have the least side reactions.[12] It was also recently used to promote dehydroalanine formation chemoselectively from Sec in peptides without modifying other amino acids. However, the reactions were carried out at very low pH and under denaturing conditions that are incompatible with labeling of most proteins.[56] We show that DBAA can be used to selectively generate Sec in Cysteine-containing proteins under mild reaction conditions (Figure 3). This selectivity was demonstrated using SelM because its Sec and Cys are in close proximity, which makes it a challenging target. When the reaction was carried out at pH 6.5, over 95% of Sec were deselenized to dehydroalanine, as is apparent from the mass loss of 81 Da compared to that of the unreacted protein (Figure 3a–b), corresponding to a selenium atom and two protons. When the sample containing dehydroalanine was incubated with the alkylating reagent iodoacetamide, the protein acquired a mass of 57 Da, which corresponds to Cys45 alkylation with one iodoacetamide, demonstrating that the Cys remained exposed and available for labeling (Figure 3c–d). Reactions at higher pH were not selective to Sec and instead targeted both Cys and Sec, as expected based on their pK_a (Figure S29). Hence, Sec-EPL allows introducing dehydroalanine selectively in proteins that contain cysteines as long as the reaction pH is poised lower than the cysteines' pK_a (Figure S30). This demonstrates that Sec-mediated ligation and conversion to dehydroalanine can be applied broadly for the incorporation of diverse post-translational modifications. Furthermore, if the labeling reaction with dehydroalanine is carried out at pH < 7, then additional conjugates can be introduced by modifying cysteines at a higher pH in a separate step.

DISCUSSION

Sec-EPL is as straightforward to implement as Cys-mediated EPL but offers higher ligation efficiency by allowing the reaction's pH to be lower than 7, which minimizes the competing thioester hydrolysis. In addition, this method offers the highest flexibility for the choice of conjugation site among all EPL approaches because deselenization is selective over desulfurization, allowing Cys in the protein to be retained.[8,9] It also requires milder reagents than desulfurization, which does not damage the protein. For bioconjugations and post-translational modifications, the Sec provides a site-specific handle for chemical conjugation that can be selectively targeted over other residues. These advantages are well documented for ligation with seleno-peptides but are here enabled for research with heterologous Sec-containing proteins extending the range of possible targets. The approach is robust without a need to optimize expression or purification conditions for different targets. It is also compatible with both denaturing and native conditions, which can be helpful in assembly of proteins from respective pieces. Such multistep ligations are popular for Cys-driven peptide ligations of complex proteins,[57] but not for proteins due to low yield. Yet, it is a powerful approach for introducing multiple post-translational modifications and bioconjugates in different positions into the protein scaffold.

Here, our Sec-EPL method was introduced for proteins that contain no native cysteines past the Sec, as the currently utilized incorporation method would replace native cysteines in the C-terminal segment of the protein with Sec. Because of cysteines' low abundance, a large number of proteins will not be affected by this issue. However, to extend the methodology to proteins that are affected, purification of the desired seleno-fragment based either on differences in Sec and Cys's pK_a's or variances in the rates of covalent bonds formation could be utilized. Nevertheless, we believe that multistep reactions—building proteins through multiple ligation steps—would provide a more general solution to this limitation. To that end, the high ligation efficiencies presented here provide a promising first step.

Sec-EPL enables the introduction of Sec residues and thus selenylsulfide and diselenide bonds in proteins to generate new functions or folding paths.[58] The experiments presented here demonstrate the application of Sec-EPL for production of both naturally occurring and engineered selenoproteins. In particular, we show the successful production of several members of the large class of selenoproteins with a thioredoxin fold,[59] which can currently only be prepared by techniques that require expert knowledge, such as total chemical synthesis[25] and engineered Sec-specific tRNAs.[30−33] Due to the high yields of the procedure, all preparations resulted in sufficient amounts for the proteins' biochemical and biophysical characterization. Such information will be valuable for identifying selenoproteins' enzymatic functions, the contribution of Sec, their roles in sensing and signaling of oxidative stress, and potentially new applications.

In addition we have demonstrated the broad applicability to nonselenoproteins. We have taken advantage of Sec's unique chemistry and known reactivity, which offers paths for site-specific incorporation of bioconjugations, and the coupling of two proteins. As we have shown here, Sec-EPL is compatible with the presence of cysteines and it is possible to adjust the reaction conditions to preferentially label Sec. As we have shown here, Sec can be used to reversibly introduce bioconjugates via selenylsulfide and diselenide bonds and irreversibly via selenoether bonds. We have also demonstrated a selective generation of dehydroalanine from Sec in proteins containing Cys with no damage to other residues under biocompatible conditions. The ability to generate it in proteins with multiple cysteines without the need to rely on unnatural amino acid formation should be advantageous for downstream applications, including introducing post-translational modifications that closely resemble those that occur *in vivo*, e.g. similar charge, length, and conformational degrees of freedom. Therefore, Sec-EPL can be used to incorporate multiple post-translational modifications by manipulation of Sec and Cys residues in proteins.

In conclusion, our work provides new tools for preparing and modifying proteins. The Sec-EPL procedure is versatile, is robust, is straightforward to implement, and requires no synthesis or special expertise with selenium chemistry. Overall, Sec-EPL extends the ease and generality of biosynthetic protein preparations, extends the class of proteins that can be prepared, and offers new avenues to exploit Sec's high chemical reactivity.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b10991.

DOI: 10.1021/jacs.6b10991
J. Am. Chem. Soc. 2017, 139, 3430−3437

Details of the experimental methods used in this manuscript, addgene plasmid numbers, the expression, purification and characterization of protein fragments, as well as the selective generation of dehydroalanine and protein tagging (PDF)

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Colin Thorpe and Joe Fox for helpful discussions and Zhihao Zhuang for generous contribution of DBAA for initial tests. Q.C. acknowledges support by chemical biology interface training grant award T32GM008550 from the National Institute of General Medical Sciences. The Delaware COBRE program supported this project 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. This work was primarily supported by the National Science Foundation under Grant No. MCB-1054447 "CAREER: Reactivity of Selenoproteins" and Grant No. MCB-1616178.

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