Selenomethionine as an expressible handle for bioconjugations

Dillon T. Flood[a], Jordi C. J. Hintzen[a], Kyle W. Knouse[a], David E. Hill[b], Chenxi Lu[a], Philip A. Cistrone[a], Jason S. Chen[c], Takanori Otomo[d], and Philip. E. Dawson[a,1]

[a]Department of Chemistry, Scripps Research, La Jolla, CA 92037; [b]The Warren and Katharine Schlinger Laboratory for Chemistry and Chemical Engineering, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA 91125; [c]Automated Synthesis Facility, The Scripps Research Institute, La Jolla, CA 92037; and [d]Department of Integrative Structural and Computational Biology, The Scripps Research Institute, La Jolla, CA 92037

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Site-selective chemical bioconjugation reactions are enabling tools for the chemical biologist. Guided by a careful study of the selenomethionine (SeM) benzylation, we have refined the reaction to meet the requirements of practical protein bioconjugation. SeM is readily introduced through auxotrophic expression and exhibits unique nucleophilic properties that allow it to be selectively modified even in the presence of cysteine. The resulting benzylselenonium adduct is stable at physiological pH, is selectively labile to glutathione, and embodies a broadly tunable cleavage profile. Specifically, a 4-bromomethylphenylacetyl (BrMePAA) linker has been applied for efficient conjugation of complex organic molecules to SeM-containing proteins. This expansion of the bioconjugation toolkit has broad potential in the development of chemically enhanced proteins.

bioconjugation | selenomethionine | protein chemistry

Protein bioconjugation has been used to create constructs vital for elucidating pathways underlying human disease as well as several important classes of established therapeutics, including constrained peptides, antibody–drug conjugates (ADCs), and polyethylene glycol-modified proteins (1–7). The most useful protein modification strategies are chemoselective, are site specific, and have fast reaction kinetics (since biomolecules are usually modified at submillimolar concentrations) (8). While proteins can be assembled synthetically or semisynthetically, the ability to modify biologically expressed proteins allows for efficient bioproduction of modified domains. Most bioconjugation strategies target the nucleophiles of naturally occurring amino acids, such as the primary amines present at the protein N terminus or on lysine residues (9, 10) and the cysteine thiol (1, 2). Less common strategies target tyrosine (11, 12), arginine (13), and methionine (14), but these procedures are usually sluggish or dependent on complex reagents (10, 15). More recently, a redox-based linker has been developed for methionine-based sulfilimide bioconjugation (16–20), as well as a photo-redox–enabled method (21), demonstrating the potential utility of this residue.

Enhanced site selectivity and chemo-selectivity can be obtained through biological expression with unnatural amino acids (UAAs) (22–25). Amber suppression and genetic code expansion allow for the incorporation of unique and orthogonal groups, such as ketones and azides, for use in oxime ligations and Cu(I)-catalyzed azide-alkyne cycloaddition, respectively. Although amber expression is frequently utilized with success in the laboratory, the synthesis of unnatural amino acids and scale up can be limiting. Alternatively, certain UAAs, like selenomethionine, can be introduced through auxotrophic expression. Auxotrophic expression of selenomethionine (SeM) is an efficient and robust technique that has been utilized for the production of heavy labeled proteins by crystallographers for decades (26–28). Another popular technique utilizes Met auxotrophic systems to introduce azidohomoalanine or homopropargylglycine into expressed proteins. These selectively reactive click handles have been employed successfully in bacterial and mammalian cells for recombinant protein or direct cell surface labeling (22, 29–31).

The selenomethionine selenoether displays interesting chemical properties, as selenoethers are uniquely nucleophilic and sensitive to mild redox transformations (32). Methionine (which is replaced by selenomethionine in auxotrophic systems) is the second rarest amino acid in vertebrates which allows surface-exposed residues to be engineered for selective chemical modification while native buried residues can be left unaltered as not to perturb protein structure (16). The unique chemical properties and the facile introduction into expressed proteins make selenomethionine a prime target for the development of additional protein chemistries. Also, SeM can be alkylated with benzyl bromides under conditions which do not modify Met; typically Met alkylation reactions require extended reaction times and high concentrations of alkylating reagents (high millimolar to saturating) (14, 33–35).

Selenomethionine has been shown to be selectively modified with benzyl bromides under acidic aqueous conditions, but the reaction has yet to be embraced as a protein bioconjugation strategy (34–36). Here, a detailed study of the selenomethionine benzylation reaction has been performed, leading to the identification of labeling reagents that form the basis of a highly chemoselective and tunable bioconjugation strategy (Fig. 1). The robust ligation kinetics of the newly identified 4-bromomethylphenylacetyl (BrMePAA) linker suggest the SeM benzylation reaction will be widely applicable as a protein-labeling strategy.

Significance

The manipulation of proteins in a site-selective manner underpins much of modern protein chemistry. A careful evaluation of selenomethionine benzylation led to the identification of a highly efficient bromobenzyl linker for protein bioconjugation. This selenonium-forming ligation reaction can efficiently link proteins to other complex molecules such as synthetic peptides, polymers, or reactive handles. The selenonium product is sufficiently stable at physiological pH, yet is efficiently cleaved by glutathione at intracellular concentrations. Selenomethionine benzylation is a broadly applicable tool for engineering of complex macromolecules.

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[1]To whom correspondence may be addressed. Email: dawson@scripps.edu.

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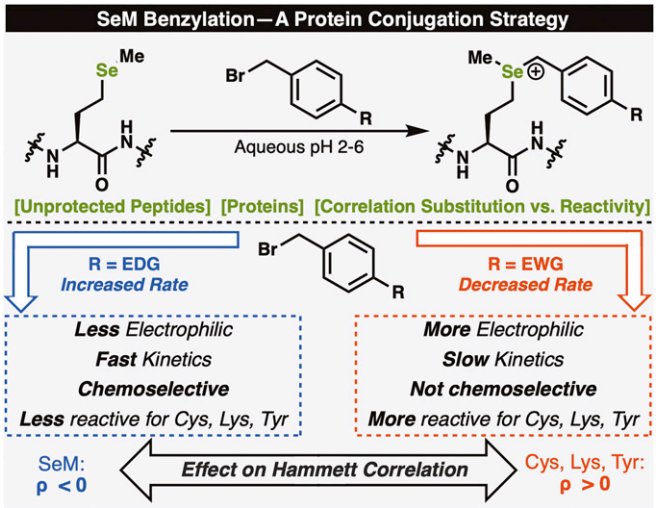

Fig. 1. The selenomethionine benzylation.

Results and Discussion

pH Independence of the Selenomethionine Benzylation. The pH dependence of SeM benzylation was explored. Peptide **1** (2.5 mM) (Fig. 2*A*) in a solution of 30% MeCN, 5% dimethylsulfoxide (DMSO), and 10 mM ascorbic acid was exposed to 2 equiv. BnBr in an aqueous solution, buffered at pH 2 to 7.4 (Fig. 2*B*). Consistent reaction profiles were observed for conversion of **1** to the corresponding benzylated species **2** which suggests that the SeM benzylation is pH independent, over the range of pH 2 to 7.4 (Fig. 2*B*). This observation is notable as all other proteogenic nucleophiles are protonatable and thus rendered less reactive as pH decreases.

Selenomethionine Orthogonality. Upon treating peptide 1 with maleimidopropioic acid, under standard Cys labeling conditions (1), no SeM conjugation is observed at pH 6 while only minor reactivity is observed at pH 7.5 (*SI Appendix*). This is consistent with our previous observation of the lack of reactivity of iodoacetamide at pH 2 (34) and may allow for SeM benzylation to be used in concert with or augment other bioconjugation techniques.

Selenomethionine Benzylation Kinetics. To evaluate the order of the reaction, initial rates were measured over a range of reactant concentrations for benzyl bromides with electron-donating (*p*-CH_2-COO^-) and electron-withdrawing (*p*-CN) substituents. Benzylation of **1** with bromomethylphenylacetic acid and *p*-cyanobenzylbromide shows a first-order rate dependence in both reactants (*SI Appendix*). The observed rate order implies that the reaction mechanism does not change across the electronic perturbations tested.

Hammett Study of Selenomethionine Benzylation. Benzyl bromides with electron-donating and -withdrawing substituents were evaluated in the selenomethionine benzylation reaction. Benzyl bromides with various parasubstituents were used in the benzylation of **1**, and log ($k_{p\text{-X}}/k_{p\text{-H}}$) was plotted against Hammett constants from the literature (37). The study shows a clear inverse Hammett correlation, with a ρ of −1.4 (Fig. 3*B*) (37, 38). Initial rate measurements revealed that the reaction is first order with respect to [peptide] and [BnBr]. The kinetic data were fitted to a second-order integrated rate equation to obtain rate constants of 0.07 $M^{-1}{\cdot}s^{-1}$ for the benzamide linker, 0.02 $M^{-1}{\cdot}s^{-1}$ for the quaternary ammonium linker, and 0.7 $M^{-1}{\cdot}s^{-1}$ for the BrMePAA linker (Fig. 3*C*). This phenomenon was unexpected, as most bimolecular substitution reactions, which are first order in both reactants, would be expected to show a positive Hammett correlation.

Benzylselenonium Conjugate Stability. The stability of the benzylselenonium adducts under physiological conditions was profiled. Stability in phosphate-buffered saline (PBS), pH 7.4, mimicking human blood plasma was explored with two distinct benzylselenonium derivatives of **1**. These constructs exhibited stability with half-lives of 43 and 24 d for the benzyl bromide and 4-Me benzylbromide adduct, respectively (Fig. 4*A*). To probe cytosolic stability, three benzylselenonium adducts of **1** were incubated in 7.5 mM glutathione (GSH) in PBS, pH 7.4 (39, 40). The conjugate with an electron-withdrawing group ($4NO_2$) degraded rapidly; less than 5% of the 4-nitrobenzyl bromide adduct remained after 2 h, while the electron-donating ($-CH_2COO^-$) analog remained 50% intact at the same time point (Fig. 4*B*). This stability profile could allow benzylated SeM species to be used in various biomolecular probe and therapeutic applications.

Selenomethionine Benzylation in Bioconjugation. The benzylation of selenomethionine displays multiple unique attributes that should allow it to be employed as a useful bioconjugation strategy. In contrast to most proteogenic nucleophiles, which become less reactive at low pH (as protonation occurs), the nucleophilicity of SeM is unaffected by pH. Similarly, the electronic dependence of the benzyl bromide linker on rate is the inverse of other proteogenic nucleophiles. Although SeM reacts fastest with electron-donating benzyl bromides, the proteogenic nucleophiles should react faster with benzyl bromides bearing electron-withdrawing substituents, as those result in a more electrophilic benzylic carbon. Therefore, the electronic demands and pH dependence of the SeM nucleophile are the opposite to those of most other nucleophiles present in biological macromolecules, which results in high chemoselectivity toward SeM. Importantly, SeM is orthogonal to commonly employed cysteine-labeling reagents such as maleimide and iodoacetamide.

It is noteworthy that the stability of the sulfonium linker can be modulated. The selenomethionine–sulfonium adducts display half-lives in biological environments that would allow them to be utilized for the creation of serum stable protein conjugates such as ADCs or constructs designed for controlled release (3, 5).

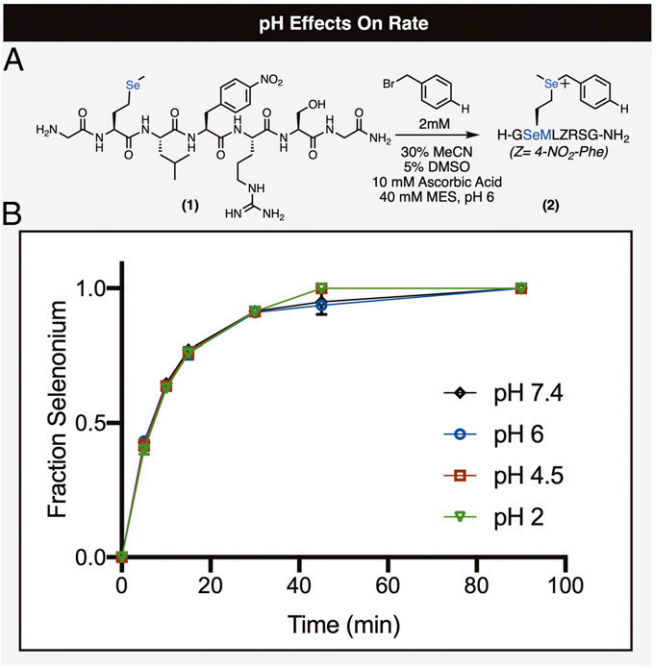

Fig. 2. (*A*) The selenomethionine benzylation reaction conditions. (*B*) pH dependence of SeM benzylation (2.5 mM peptide **1**, 5 mM BnBr, 30% MeCN, 5% DMSO, 1 mM Boc-Tyr-OH as internal standard). Some error bars are smaller than point markers.

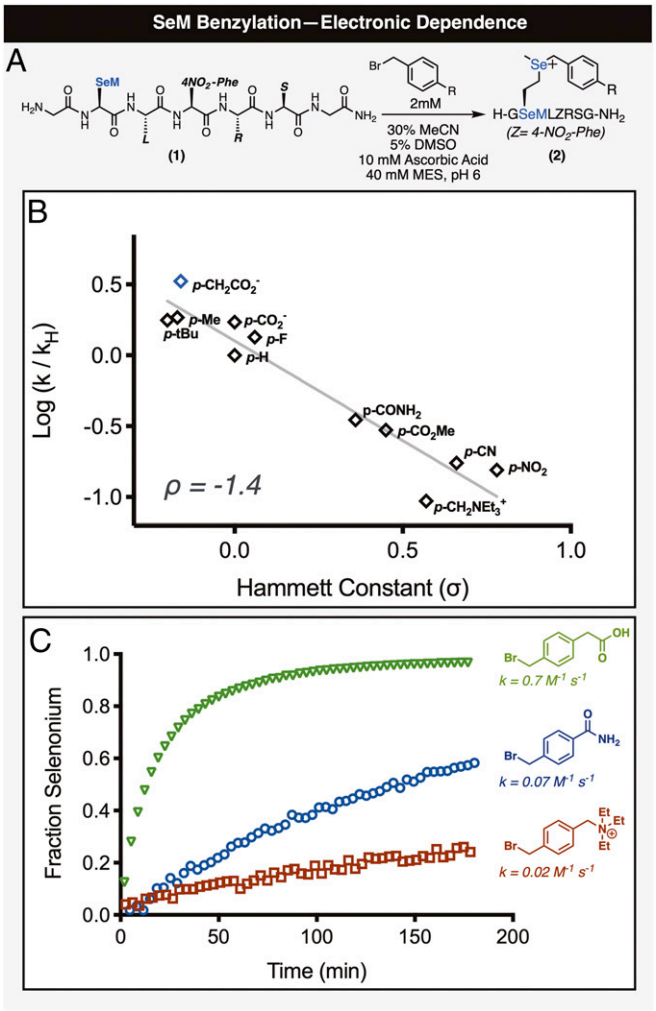

Fig. 3. (*A*) The selenomethionine benzylation reaction conditions. (*B*) Hammett correlation of the selenomethionine benzylation [the Hammett constant for p-$CH_2NEt_3^+$ was estimated from p-$CH_2N(Me)_3^+$]. (*C*) Conversion of **1** to the corresponding selenonium species of representative linkers. Shown are BrMePAA (Green), benzamide (blue), and quaternary ammonium linker (red) [1 mM **1**, 2 mM R-BnBr, 1 mM Boc-Tyr-OH (internal standard), 10 mM ascorbic acid, 40 mM MES pH 6, 30% MeCN, 5% DMSO in water]. Rate constants were derived by fitting rate conversion experiments to a second-order integrated rate law.

Also, since these benzyl selenonium linkers exhibit the opposite degradation kinetics in serum versus in cytosol, an interesting opportunity is presented. One can envision creating a tunable linker which exhibits a long, stable half-life while in circulation but upon cell internalization is readily cleaved by intracellular GSH to release a payload. The benzyl selenonium linkage is highly tunable, allowing one to use electronic perturbation to modulate conjugation and cleavage rates. Taken together these qualities should allow the benzylation of SeM to be readily applied to protein bioconjugation.

SeM Benzylation as a Strategy for Protein Bioconjugation. Insights gained from the Hammett study (Fig. 3*B*) led to the selection of BrMePAA as a general bioconjugation linker. BrMePAA is readily available, shelf stable, and easily incorporated into tags of interest by either solution or solid-phase methods. To test the utility of this linker, the albumin-binding, three-helix bundle SpA domain, **3**, was selected due to its utility as a half-life–extending moiety in vivo (Fig. 5*A*) (4, 41). The naturally occurring solvent-exposed Met9 was replaced with a SeM and Cys22 was added to evaluate chemoselectivity in the presence of a free thiol. In the first protein-labeling experiment, protein **3** was exposed to tag **4** at pH 6 for 6 h. This reaction resulted in 75% of the desired (SeM-conjugated) product and 25% of a side product in which both the SeM and the Cys were labeled (Fig. 5*A*). In a subsequent reaction, the pH was adjusted to 5 while leaving all other parameters constant. After 6 h, the monoselenonium product (82%) was observed along with 15% starting material, and only a trace of the doubly labeled material was observed (Fig. 5*A*). Using these selective conditions, two additional tags, a 3xFlag tag **5** and a chromophore-containing tag **6**, were used to label protein **3** (Fig. 5*B*). These tags label only the SeM residue, resulting in complete conversion to the tagged conjugate with no detectable double-labeled side product or starting material (Fig. 5*B*).

The protein GATE-16, an autophagic ubiquitin-like protein, was auxotrophically expressed with selenomethionine as a Tev fusion **7** (Fig. 6*A*). This construct contains four selenomethionines: two solvent exposed, one buried but in a flexible N-terminal region, and one fully buried in the core of the protein. GATE-16 also contains a solvent-exposed cystine. Treating **7** (200 μM) with tag **8** (800 μM, 4 equiv. relative to protein) at pH 5 for 12 h yielded a dual-labeled species with minor single and triple addition products (Fig. 6*C*). SeM over Cys selectivity was confirmed through subsequent treatment with *N*-ethylmaleimide (800 μM), which led to complete labeling of the solvent-exposed cysteine (*SI Appendix*). Regioselective labeling at SeM positions 3, 6, and 63 was confirmed by both trypsin and chymotrypsin digest and high-performance liquid chromatography–mass spectrometry (HPLC-MS) (*SI Appendix*). Importantly the buried SeM93 and exposed Cys17 showed no evidence of labeling with 8 (*SI Appendix*). Chemoselectivity for SeM over the other proteogenic nucleophiles, even thiols, suggests a broad utility in bioconjugation.

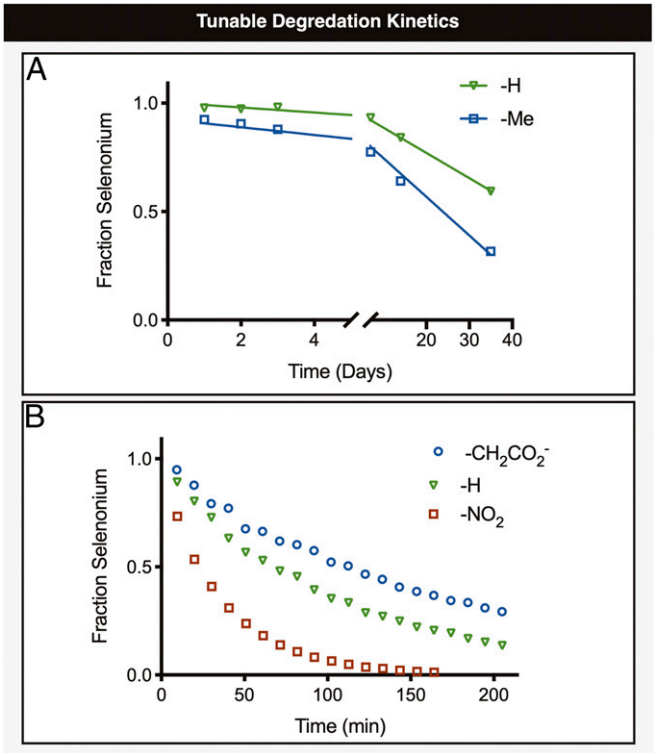

Fig. 4. (*A*) Benzyl-selenonium stability in plasma mimic (1 mM peptide, in PBS pH 7.4, monitored by HPLC). (*B*) Benzyl selenonium stability in cytosol mimic (1 mM peptide, in PBS pH 7.4, with 7.5 mM GSH, monitored by HPLC).

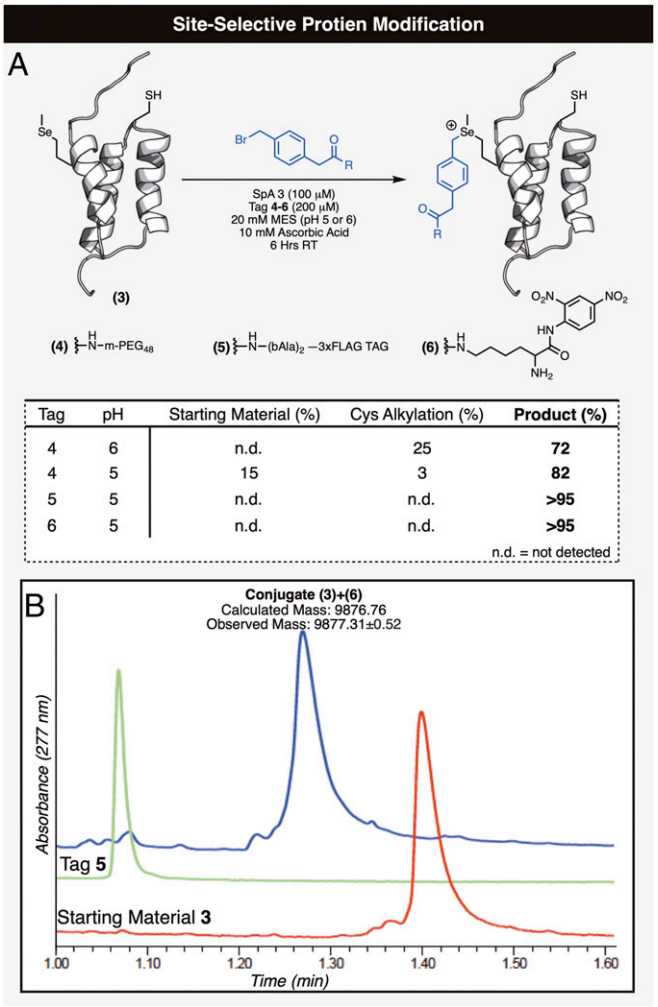

Fig. 5. (*A*) Modification of SpA (PDB: 1BDD) protein **3** with tags varying pH (0.15 mM **3**; 0.2 mM tag **4, 5,** or **6**; 20 mM MES; 10 mM ascorbic acid). (*B*) HPLC chromatogram of reaction of **3** and **5** (0.15 mM **3**, 0.2 mM tag **6**, 20 mM MES buffer pH 5). Deconvolution was performed manually from centroid collapsed peaks between *m/z* 600 and 1,250.

SeM maltose-binding protein (His-MBP-Tev) was also evaluated for selenomethionine bioconjugation. The MBP construct **9** contained seven SeM residues, one of which is fully solvent exposed (located in the unstructured C-terminal region, SeM389), two of which are partially solvent exposed (SeM 164 and 337), and four which are completely buried (SeM 220, 240, 346, and 356) (Fig. 7*A*). Treating MBP **9** (50 μM) with tag **8**, at pH 5 for 12 h, yielded a singly labeled major product with additional dual- and triple-labeled conjugate species observed (Fig. 7*C*). The conjugation sites were confirmed by trypsin digest to be SEM 164, 337, and 389 (*SI Appendix*). These conditions resulted in a primary conjugation product that was singly labeled at the most solvent-exposed residue, SeM389. These experiments suggest significant regioselectivity can be achieved in favor of exposed SeM residues and that minimally engineered proteins can be benzylated to form useful constructs. Alternatively, conservative Met to Leu mutations could be utilized to eliminate undesired reactivity as shown for Met-specific protein conjugates (19, 42).

Simultaneous, Orthogonal Labeling of SeM and Cys. Drawing inspiration from Palmer's early report (35), we strove to exploit the unique reactivity profiles of SeM and Cys in protein **7** to conjugate two distinct tags (Fig. 8*B*). Treating GATE-16 **7** (200 μM) with both BrMePAA tag **8** (800 μM) and maleimide tag **10** (800 μM), at pH 5 for 12 h, resulted in a conjugate that was fully labeled at Cys7 and had a similar SeM-labeling distribution as observed when labeling with tag **8** alone. When used in concert with established Cys modification reagents, the SeM benzylation allows for complex protein systems to be quickly and selectively modified in one pot, using two distinct tags.

Conclusion. In summary, we present a detailed study of the SeM benzylation reaction and demonstrate its utility for protein bioconjugation. The chemical orthogonality of the reaction toward all proteogenic nucleophiles as well as common thiol-labeling reagents is demonstrated. We have also outlined the stability of the selenonium conjugate under physiological conditions and suggested its lability to cytosolic glutathione could act as a release mechanism for the conjugate inside cells. Compatibility of the approach with auxotrophically expressed proteins suggests broad application. The SeM benzylation can also be used in concert with traditional thiol-maleimide chemistry to yield dual modified proteins. Importantly, the electronic optimization of the benzyl bromide electrophile identified BrMePAA as a readily introduced reactive linker that facilitates room temperature selective SeM labeling at submillimolar concentration. We anticipate that SeM benzylation using BrMePAA will be a valuable addition to the bioconjugation toolbox.

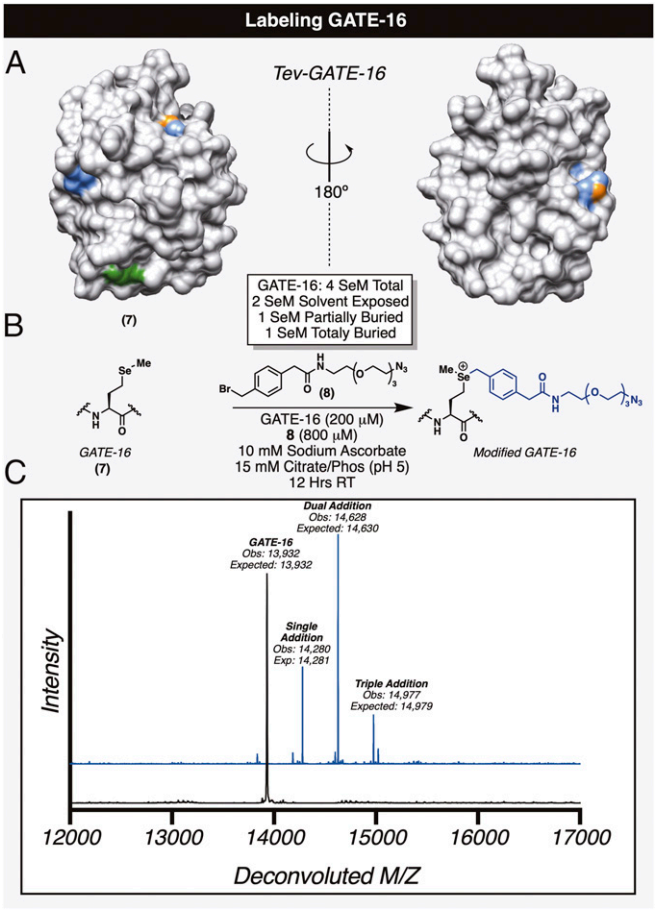

Fig. 6. (*A*) Tev-GATE-16 (PDB: 4CO7) **7** surface, SeM residues shown in blue, Se highlighted in orange, and the Cys residue shown in green. (*B*) Labeling conditions. (*C*) Mass spectrum of starting material and labeled product.

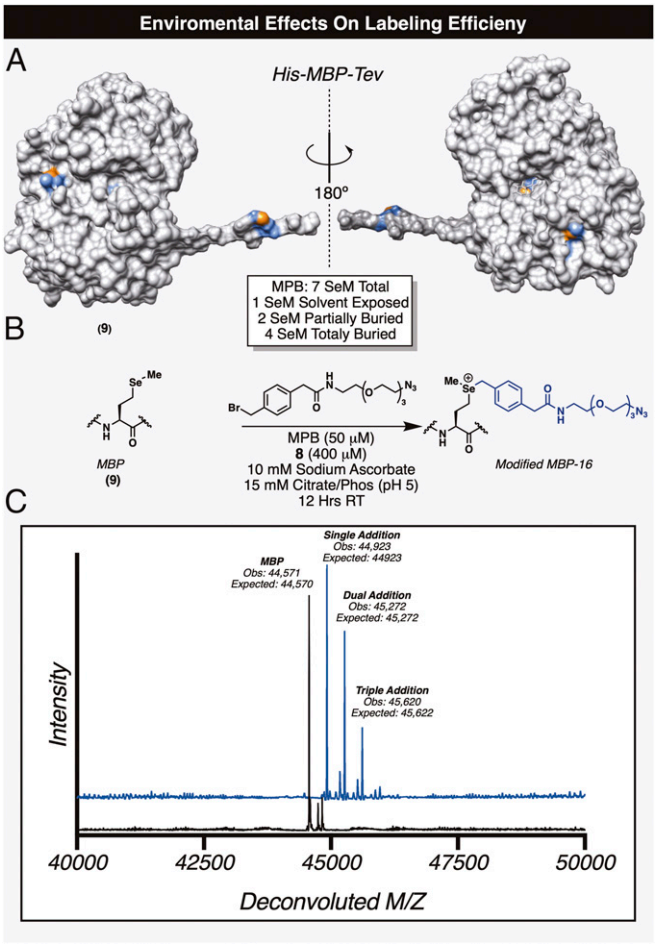

Fig. 7. (*A*) His-MBP-Tev (PDB: 1ANF) **9** surface, SeM residues shown in blue, Se highlighted in orange. (*B*) Labeling conditions. (*C*) Mass spectrum of starting material and labeled product.

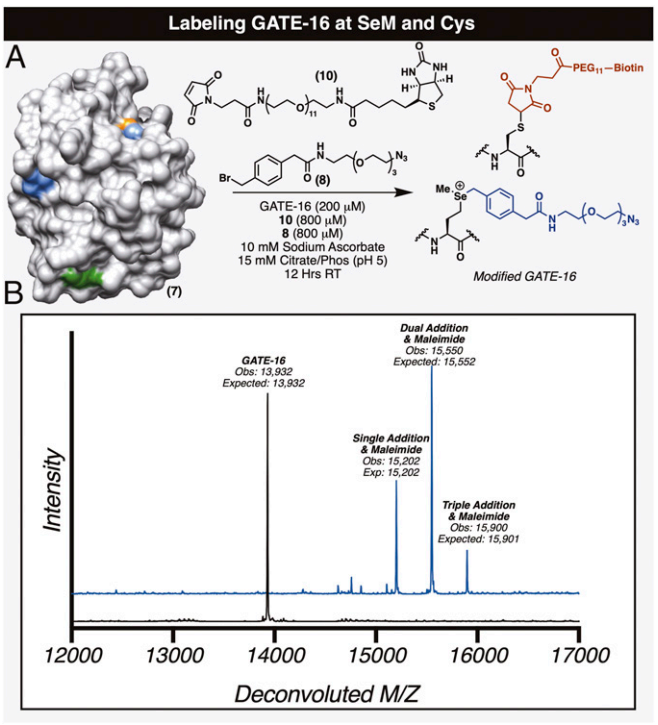

Fig. 8. (*A*) Tev-GATE-16 (PDB: 4CO7) **7** surface, SeM residues shown in blue, Se atom highlighted in orange, and the Cys residue shown in green. (*B*) Mass spectrum of starting material and labeled product.

Methods

Peptide and Protein Synthesis. Peptides were chain assembled by *N*-(9-fluorenylmethoxycarbony)–solid phase peptide synthesis, deprotected, and cleaved from the resin with trifluoroacetic acid/scavengers and purified by HPLC (43). The polypeptide corresponding to the SpA domain was assembled by native chemical ligation of two synthetic peptides and folded in PBS (*SI Appendix*) (43).

Monitoring of Reaction Progress. The consumption of peptide **1** was monitored by integration of ultraviolet (UV) absorbance ultrahigh-performance liquid chromatography (UPLC) 1-μL aliquots, by autosampler, at 3- to 12-min intervals. For all spectra, UV absorbance at 277 nm was auto-integrated against an internal standard (Boc-Tyr-OH). All mass spectra were obtained by integration of the total ion current (TIC) over the entire peak area.

Initial Rate Study. Substituted benzyl bromide (BrMePAA or *p*-CN BnBr) was reacted with peptide **1** in 30% acetonitrile, 5% DMSO, 40 mM mercaptoethylsulfonic acid (MES), 10 mM ascorbic acid at pH 6. Ascorbic acid was added to selectively reduce any SeM selenoxide side products while not reducing sulfoxides or disulfides (44). Reaction progress was monitored by UPLC-MS and the initial rate was determined through linear regression.

Hammett Analysis. Peptide **1** (1 mM) was reacted with 2 equiv. substituted benzyl bromide (X-BnBr) in a solution of 30% acetonitrile, 5% DMSO, 40 mM MES buffer, 10 mM ascorbic acid, pH 6. Reaction progress was monitored by UPLC-MS and rate constant was determined by fitting data to the second-order integrated rate law. The log of the ratio of the initial rate of each *p*-X BnBr to the initial rate of *p*-H BnBr was plotted against known *p*-Hammett constants.

Conjugate Stability.
Serum mimic conditions. Purified benzyl-selenonium adducts of peptide **1** were dissolved in PBS, pH 7.4 with Boc-Tyr-OH as an internal standard and sampled for 36 d.
Cytosol mimic conditions. Purified benzyl-selenonium adducts of peptide **1** were dissolved in PBS, pH 7.4 with 7.5 mM GSH and Boc-Tyr-OH as an internal standard. The mixture was monitored every 12 min for 3 h, by UPLC-MS.

Expressed Protein Labeling. Proteins **7** and **9** were diluted into buffer containing 15 mM phosphate-citrate, 10 mM ascorbic acid at specified pH. The conjugation was initiated by adding **8** (from a 20× or 50× stock in dimethylamine or DMSO). The final organic solvent concentration was less than or equal to 5% vol/vol. The reactions were allowed to incubate at room temperature for 12 h at which point they were analyzed by UPLC time-of-flight mass spectrometry. Chromatographic separation was not achieved, so the entire TIC peak was combined and deconvoluted by built-in Max Ent 1 software.

Data Availability. All study data are included in this article and/or *SI Appendix*.

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