

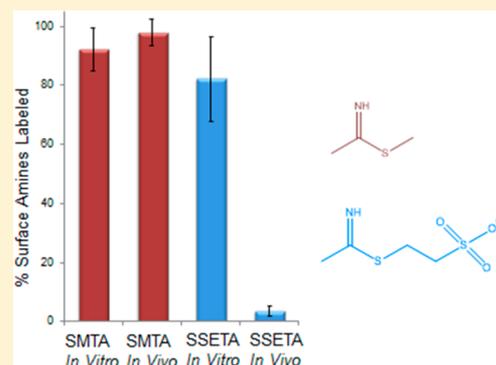
# In Vitro and In Vivo Chemical Labeling of Ribosomal Proteins: A Quantitative Comparison

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**S** Supporting Information

**ABSTRACT:** Thioimides have emerged as reagents for probing the protein structure, folding, and interactions under physiological conditions. The same properties that give thioimides biological relevance make these molecules ideal candidates for use in vivo. Through labeling of ribosomal proteins, we have quantified the in vivo and in vitro reactivity of two thioimides: S-methylthioacetimidate (SMTA) and a novel, charge-carrying analogue, S-sulfethylthioacetimidate (SSETA). In vitro experiments demonstrate that both amidinating reagents can probe the protein structure. Under comparable in vivo conditions, SMTA is found to be membrane-permeable while SSETA is not. The use of mass spectrometry with permeant and impermeant thioimides promises insights into the membrane topology and protein structure in the native environment.



Covalent modification combined with mass spectrometry (MS) has become a supplemental technique for deriving protein structural information by probing the surface topology.<sup>1–3</sup> Although lacking the resolution of X-ray crystallography, covalent modification can be used to study aqueous proteins and protein complexes under native conditions. Recently, covalent labeling has been used to explore the dynamics of protein structure, folding, and interactions.<sup>4–19</sup> Because observation of the native structure is a main goal, covalent reagents that minimally alter the system of interest are preferred, and amidinating reagents have grown in popularity. In the presence of proteins, amidinating reagents react with primary amines from solvent-exposed lysine residues and N-termini. Amidination has both biological and analytical advantages. While adding minimal bulk, the reaction preserves an amine's physiological positive charge and, consequently, its electrostatic interactions. Studies have documented that amidination's minimal invasiveness preserves the protein structure and enzymatic activity.<sup>20–33</sup> In terms of MS, the amidino group's slight increase in  $pK_a$  facilitates ionization.<sup>34</sup>

Despite their advantages, early amidinating reagents had significant drawbacks. The first class of amidinating reagents, collectively known as oxyimides, required the use of pH 10 or above.<sup>35,36</sup> Below pH 10, oxyimides are ineffective amidinating reagents because of their participation in side reactions.<sup>36–38</sup> To address this problem, a class of structural analogues known as thioimides was developed.<sup>39</sup> A thioimide contains a thiol in place of the oxyimide's alcohol, and this difference confers significant advantages. Because thiols are better leaving groups than alcohols, thioimides are able to amidinate at or below the physiological pH without participating in side reactions. Proteins labeled with thioimides retain more enzymatic activity than those labeled

with oxyimides.<sup>39</sup> Because of their enhanced physiological relevance, thioimides have recently supplanted oxyimides as amidinating reagents of choice for studies of the protein structure, folding, and interactions under native conditions.<sup>4,5,22,27–33,40,41</sup>

The properties that make thioimides more biologically relevant than oxyimides also make thioimides ideal candidates to replace oxyimides for use in vivo. In vivo experiments with oxyimides have indicated that cellular metabolism and growth persist after amidination.<sup>42,43</sup> Nevertheless, these experiments require exposure of cells to pH values above 10, and this impacts the physiological relevance of such studies. At lower pH values, complete amidination is difficult to achieve with oxyimides, and side reactions such as cross-linking perturb the native structure. The use of thioimides in vivo should circumvent the major disadvantages of oxyimides. It is predicted that thioimides will completely amidinate in vivo at pH 7.4.

That imides have disparate membrane permeabilities adds an analytical dimension to the use of these reagents in vivo. At alkaline pH, uncharged oxyimides enter into cells and amidinate intracellular amines.<sup>43</sup> After the addition of a fixed charge such as a sulfo group, oxyimides are blocked from entering cells and label only extracellular amines. This differential capability has allowed oxyimides to assist in locating amine-containing biomolecules with respect to the cellular membrane.<sup>37,43–52</sup> The method used to detect labeling in these membrane-based studies has been either fluorescence or autoradiography, neither of which is amenable to the

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quantification of labeling at specific amines. In vivo thioimide labeling with MS detection should allow for physiological modification of intra- and extracellular amines and detection at residue-level resolution.

In the present work, we explore whether thioimides at pH 7.4 possess the same ability to label amines inside and outside of cells, analogous to oxyimides. Upon movement from alkaline to physiological pH, ionization of the imide nitrogen complicates predictions about the thioimide membrane permeability. The  $pK_a$  value of an imide nitrogen is approximately neutral.<sup>43</sup> At the alkaline pH values used with oxyimides, the imide nitrogen is uncharged, and oxyimides readily enter cells. However, at the physiological pH, a higher proportion of imide nitrogen atoms are charged. We investigated whether this would prevent *S*-methylthioacetimidate (SMTA) from entering cells and labeling amines from ribosomal proteins. Recent MS experiments have yielded insight about ribosomal proteins and their post-translational modifications,<sup>53,54</sup> and in vitro experiments from several bacterial species have demonstrated that thioimide ribosomal labeling is consistent with X-ray crystallographic data.<sup>30–33</sup> Here we compare the in vivo and in vitro labeling of cytosolic ribosomes with SMTA. We describe the synthesis of *S*-sulfethylthioacetimidate, the first thioimide synthesized with a fixed charge, and investigate the membrane permeability of this new reagent relative to SMTA.

## ■ EXPERIMENTAL PROCEDURES

**Materials.** Deuterated dimethyl sulfoxide (DMSO), deuterium oxide, magnesium chloride hexahydrate, and 2-mercaptoethanol were purchased from Aldrich (Milwaukee, WI). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), a hydrogen chloride solution (4 M in dioxane), magnesium acetate tetrahydrate, phenylmethylsulfonyl fluoride (PMSF), potassium hydroxide, and sodium 2-mercaptoethanesulfonate were obtained from Sigma (St. Louis, MO). Ammonium chloride, potassium chloride, and sodium chloride were supplied by Mallinckrodt (Phillipsburg, NJ). Sucrose was purchased from IBI Scientific (Peosta, IA) and acetonitrile (ACN) from Honeywell (Muskegon, MI), and bacto-tryptone and bacto-yeast extract were purchased from Becton (Sparks, MD). Glacial acetic acid was obtained from EM Science (Gibbstown, NJ) and formic acid from JT Baker (Phillipsburg, NJ). Tris(2-carboxyethyl)phosphine (TCEP) was supplied by Thermo (Rockford, IL).

**Synthesis and Characterization of *S*-Sulfethylthioacetimidate (SSETA) and *S*-Methylthioacetimidate (SMTA).** SSETA was prepared from acetonitrile and 2-mercaptoethanesulfonate by the Pinner synthesis.<sup>55</sup> Sodium 2-mercaptoethanesulfonate (23 mmol) was finely ground and added to acetonitrile (2600 mmol), which had been dried with Baker 5A molecular sieves (supplemental Scheme 1 in the Supporting Information). The resulting suspension was placed on ice and stirred constantly. A total of 550 mmol of anhydrous HCl (4 M in dioxane) was slowly poured into the suspension. Further aliquots of anhydrous HCl (275 mmol) were added at 30 and 60 min. The reaction mixture was allowed to reach room temperature overnight, and the next day a small sample of the mixture was analyzed by NMR (supplemental Figures 1 and 2 in the Supporting Information). After NMR had confirmed its purity, the product was isolated by allowing the suspended solid to settle at the bottom of the flask. The supernatant was then removed with a syringe. The product was dried and stored in a

vacuum desiccator. SMTA was prepared as previously described by Beardsley and Reilly.<sup>27</sup>

<sup>1</sup>H NMR experiments were performed over a time course to investigate the hydrolysis of SMTA and SSETA. A small volume of 200 mM reagent was brought to neutral pH at room temperature and placed in the same buffering conditions as those used for labeling (25 mM HEPES/50 mM potassium chloride/10 mM magnesium chloride, pH 7.4). The solution contained 50:50 (v/v) water/deuterium oxide. The sample was loaded into a Varian Inova 400 MHz NMR spectrometer. Spectra were acquired at 1-min intervals. The absolute intensity mode was used to allow for comparison between spectra. In the SMTA experiment, the area between approximately 2.37 and 2.48 ppm was integrated in each spectrum. This area captured SMTA's methanethiol 3 proton singlet and no other peaks. In the SSETA experiment, the area between 2.27 and 2.37 ppm was integrated in each spectrum. This area captured SSETA's lone methyl 3 proton singlet and no other peaks.

### SMTA and SSETA In Vivo Labeling Conditions.

*Escherichia coli* K12 cells were grown in 500 mL of autoclaved LB broth overnight at 37 °C while being shaken at 200 rpm. Cells were pelleted and suspended in 50 mL of a reaction buffer (50 mM HEPES/100 mM potassium chloride/20 mM magnesium chloride, pH 7.4). A total of 6.4 mL of 400 mM SMTA was brought to neutral pH in 300 mM potassium hydroxide and immediately added to an equal volume of suspended cells. Because SMTA is isolated as an acidic salt, the above concentration of base is needed to bring the solution to neutral pH before the reagent is added to cells. The reaction vessel was shaken at 225 rpm at room temperature. Every 15 min as necessary, a few microliters of either 1 M potassium hydroxide or 1 M hydrogen chloride were added to bring the pH to 7.4, as measured by a calibrated electronic probe (IQ Scientific).

Half of the reaction mixture was collected at 15 min and the other half at 45 min. At each time, the reaction mixture was centrifuged at 14100g for 1 min to pellet cells out of the buffer containing SMTA. The cell pellet was then resuspended with 12.8 mL of a reaction buffer without SMTA. Cells were spun a second time at 14100g for 1 min, and the pellet was resuspended in Spedding's Buffer A in preparation for lysis. The process of collecting each fraction took 10 min. After the in vivo reaction, ribosomes were isolated as described by Spedding.<sup>56</sup> Following this procedure, we previously demonstrated that essentially every feature in a matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS spectrum of the resulting sample corresponded to a ribosomal protein.<sup>57</sup> The SSETA in vivo reaction conditions were identical with those of SMTA except that, at 15 and 30 min, a volume equal to that of the reaction mixture of pH-adjusted 400 mM SSETA was added.

**SMTA and SSETA In Vitro Labeling Conditions.** A total of 500  $\mu$ L of 400 mM SMTA was added to an equal volume of isolated ribosomes stored in the reaction buffer. PMSF (3 mM) was added to the buffer to discourage hydrolases, and 3 mM 2-mercaptoethanol was added to provide reducing conditions. The ribosomes were present in the buffer at a concentration of 1 mg/mL, as determined by a Bradford assay using bovine serum albumin as a standard. The reaction was performed at room temperature and shaken at 225 rpm. The pH was kept at 7.4 as described above.

Half of the reaction mixture was collected at 15 min and the other half at 45 min. At these times, the ribosomal proteins

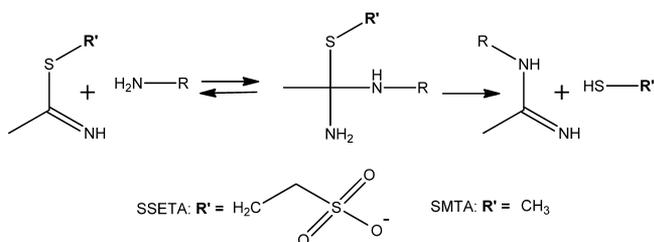
were concentrated using an Amicon Ultra 100K centrifugal filter (Eschborn, Germany). After concentration, rRNA was precipitated by adding glacial acetic acid and 1 M magnesium chloride so that the resulting solution contained a ratio of 3:6:1 (v/v/v) concentrated proteins/glacial acetic acid/1 M magnesium chloride. The process of quenching the reaction took 10 min. The SSETA *in vitro* reaction conditions were identical with those of SMTA except that additional reagent was added at 15 and 30 min as described above. After labeling and rRNA extraction, 2  $\mu\text{L}$  of 1 M TCEP was added to 32  $\mu\text{L}$  of ribosomal protein extract from the 45-min SMTA *in vitro* experiment. After 1 h of exposure to TCEP at room temperature, this reduced sample was analyzed separately from the unreduced sample.

**Liquid Chromatography–Electrospray Ionization MS (ESI-MS) and Whole-Protein MS.** For each preparation, 60  $\mu\text{g}$  of protein extract as determined by Bradford assay was loaded onto a C4 reversed-phase column (Thermo Hypersil-Keystone Pioneer, 1  $\times$  100 mm, Bellefonte, PA). A 55-min gradient that ramped from 10% to 60% acetonitrile was used to elute ribosomal proteins from the column. Water and acetonitrile mobile phases contained 0.1% formic acid. The effluent was split from a flow rate of 50  $\mu\text{L}/\text{min}$  down to 10  $\mu\text{L}/\text{min}$  and infused into an ESI source with an applied voltage of +3.0 kV. A quadrupole TOF mass spectrometer (Q-TOF, Waters, Manchester, U.K.) with a maximum resolution of 5000 Da and a protein mass accuracy of 1–2 Da was used to acquire whole-protein MS spectra. Spectra were deconvoluted using Bioanalyte ProTrawler/Regatta (Portland, ME).

## RESULTS AND DISCUSSION

**SMTA *In Vitro* and *In Vivo*.** The reaction between a thioimide and an amine proceeds by nucleophilic attack of the amine on the thioimide carbon to form a tetrahedral intermediate.<sup>39</sup> Scheme 1 displays the amidination reaction

**Scheme 1. Amidination Scheme Displaying the Moieties Specific to SMTA and SSETA**

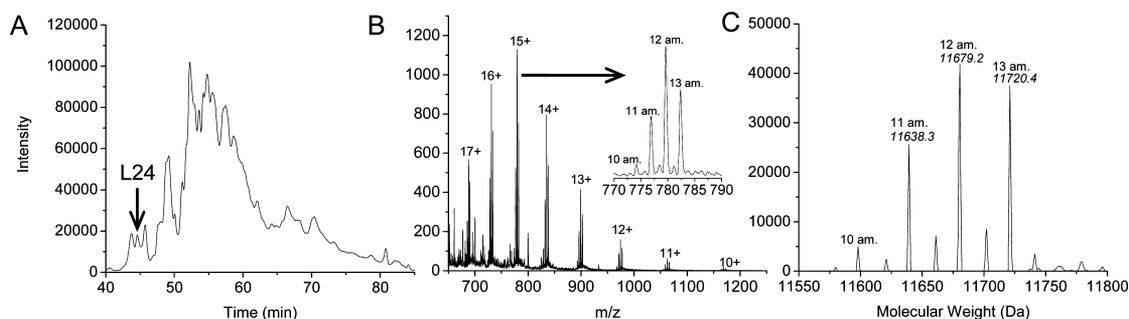


and highlights the moieties specific to SMTA and SSETA. After formation of the intermediate, the thiol is irreversibly eliminated, and the stable amidino product is formed. Both SMTA and SSETA increase the mass of lysine residues and N-termini by 41 Da. These two molecules are distinguished solely by the identity of their leaving group, methanethiol for SMTA and 2-mercaptoethanesulfonate for SSETA, as shown in Scheme 1.

*In vitro* and *in vivo* labeling experiments of ribosomal proteins with SMTA were initially compared. For *in vitro* experiments, ribosomes were isolated from *E. coli* cells and exposed to an excess concentration of SMTA at physiological pH. Acidic conditions were used to terminate the amidination reaction *in vitro* and to extract rRNA from the ribosomal proteins. In the *in vivo* experiments, *E. coli* cells were exposed to SMTA at physiological pH. The reaction was quenched by brief centrifugation sufficient to pellet cells, not ribosomes. The initial pelleting was followed by resuspension and a second pelleting. With each pelleting, any extracellular ribosomes and excess labeling reagent would be removed from the sample. Thus, the ribosomes that were analyzed in the *in vivo* experiments were located within *E. coli* cells during the reaction. After cells had been labeled *in vivo*, their ribosomes were isolated and the ribosomal proteins extracted. Ribosomes were analyzed after 15 and 45 min of exposure to the reagent in order to assess whether a longer exposure time resulted in a significant increase in labeling.

A typical total-ion chromatogram for SMTA-labeled proteins is displayed in panel A of Figure 1. Panels B and C display raw and deconvoluted ESI-MS spectra obtained from the L24 peak of the chromatogram. (Deconvoluted spectra of unlabeled and labeled ribosomal proteins are compared in Figure 1 of ref 31.) The inset to panel B emphasizes that, at each charge state in the raw spectrum, a distribution of peaks is detected corresponding to the range of amidination states. Protein identifications were made based on known *E. coli* ribosomal masses and their previously characterized post-translational modifications.<sup>31</sup> In addition, characteristic extents of amidination and reversed-phase retention times were also employed in the identification of ribosomal proteins. Amidinated L24 was detected with 10, 11, 12, and 13 amidinos added. With each amidination state weighted by the intensity of its corresponding peak, the average extent of modification of an amidinated protein was calculated. For L24, this value is 12.2.

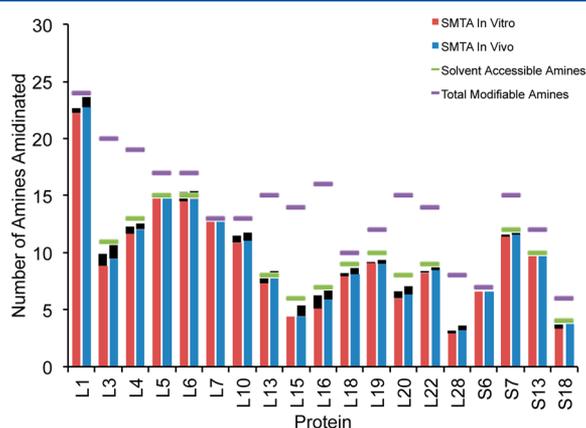
The extent of modification of ribosomal proteins under saturation labeling conditions enables the membrane permeabilities of SMTA and SSETA to be compared. The calculated



**Figure 1.** Experimental observation of ribosomal protein L24: (A) Reversed-phase total-ion chromatogram with the peak corresponding to L24 indicated. (B) Raw ESI-MS spectrum displaying the charge state distribution of L24. The range of amidination states of the 15+ charge state is displayed in the inset. (C) Deconvoluted ESI-MS spectrum.

extent of modification of a protein relies on a distribution of peaks, not on the intensity of any one peak. The value calculated is the same regardless of the amount of sample analyzed or the performance of the mass spectrometer. Using 1D separation, we detected masses corresponding to 48 of 54 ribosomal proteins in various amidination states. A total of 19 of these 48 proteins were detected in all four labeling experiments, and the average extent of modification of these 19 proteins was used to compare the abilities of SMTA and SSETA to amidinate in the cytosol. Previously, we reported that a 2D separation results in the detection of more bacterial ribosomal proteins.<sup>58,59</sup> However, the point of the present experiments was to compare the labeling of SMTA and SSETA, and 19 ribosomal proteins were considered to be an adequate sample for this study.

Figure 2 displays the results of the *in vivo* and *in vitro* SMTA experiments. Green bars represent the number of solvent-



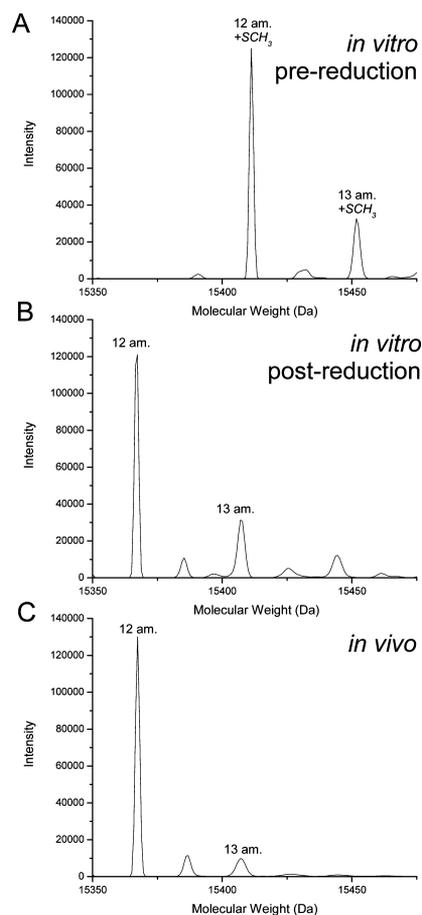
**Figure 2.** Comparison of SMTA *in vitro* and *in vivo* ribosomal-labeling experiments. The black bars indicate additional modifications achieved between 15 and 45 min of exposure to the reagent. Proteins for which solvent accessibility data are not included do not appear in the crystal structure.

accessible amines for each protein, as reported previously.<sup>31</sup> In that earlier study, MS/MS data on amidinated proteins were used to confirm protein mass measurements and identifications, and prior studies of post-translational modifications of these proteins were noted. The solvent accessibility was determined by manual inspection of the *E. coli* ribosomal crystal structure using the software PyMOL. It was also demonstrated in that earlier work that relatively lengthy reaction times that enable saturation labeling lead to excellent agreement with crystal structure estimates of the solvent accessibility. In Figure 2, the number of modifiable amines for each protein is indicated with purple bars. Black bars represent additional modifications achieved between 15 and 45 min of exposure to the reagent. The agreement between *in vivo* and *in vitro* extents of modification was remarkable considering that many other amine-containing molecules that should react with amidinating reagents are present in cells. It also implies a similarity in the structures of *in vivo* ribosomes and intact, isolated ribosomes. Both *in vivo* and *in vitro* extents of modification matched closely with the number of exposed amines determined from the ribosomal X-ray crystal structure.

With the exception of S6 and L7, fewer than the total number of amines in each protein were amidinated. In cases such as L3, L13, and L20, fewer than half of the total number of

modifiable amines were amidinated. The correspondence between *in vivo* and *in vitro* extents of modification suggests that *in vitro* conditions, in general, allow for accurate modeling of the ribosome's native structure. The data also demonstrate that little additional labeling was achieved after 15 min of exposure to the reagent. For the *in vitro* experiment, 5% of total labeling was achieved between 15 and 45 min of exposure to the reagent. The *in vivo* value was 6%. That so little subsequent labeling was achieved shows that 15 min of exposure was sufficient for SMTA to label both extra- and intracellular amines.

The *in vivo* experiments also presented evidence of the cell's reducing strength. Previously, we have demonstrated that exposure to thioimidates leads to modification of cysteine residues through the formation of disulfide bonds between the cysteine sulfur and the thioimide's thiol leaving group.<sup>29</sup> For SMTA, this modification is detected as a 46 Da shift corresponding to the mass of the leaving methanethiol group. Figure 3 shows the deconvoluted MS spectra of L11, a protein that contains one cysteine residue, from *in vitro* and *in vivo* 45-min labeling experiments. Panel A shows *in vitro* labeled L11. Despite the presence of a reducing reagent in the reaction buffer, *in vitro* experiments yielded methanethiol adducts (46 Da) in L11 and other cysteine-containing ribosomal proteins. To investigate the reduction of these adducts, *in vitro* labeled

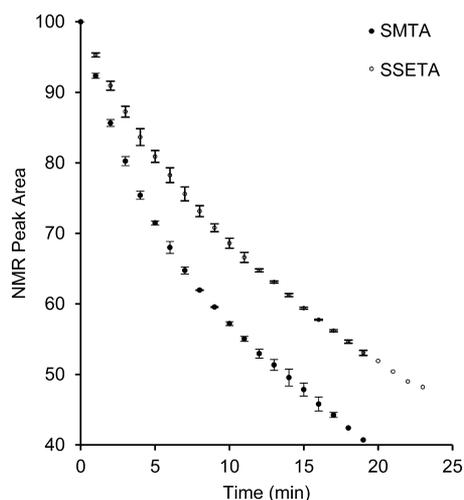


**Figure 3.** Deconvoluted ESI-MS spectra of ribosomal protein L11. L11 detected (A) *in vitro* with 12 and 13 amidinos added, along with a 46 Da mass shift corresponding to a methanethiol adduct, (B) *in vitro* after reduction of the thiol adduct with TCEP, and (C) *in vivo* without the thiol adduct.

ribosomal proteins were exposed to 50 mM TCEP, a strong reducing reagent. Panel B shows the results of *in vitro* labeling of L11 after exposure to TCEP. The 46 Da shift disappears from the spectrum, indicating that the methanethiol adduct has been reduced. *In vivo* labeled L11 is shown in panel C. Intriguingly, in *in vivo* experiments, L11 and all other cysteine-containing ribosomal proteins that we detected did not display a 46 Da mass shift. The absence of cysteine modification *in vivo* suggests that the conditions within *E. coli* cells are sufficiently reducing to prevent formation of the disulfide adduct.<sup>60</sup>

**SSETA *In Vitro* and *In Vivo*.** We expected that the fixed-charge SSETA would label extracellular amines at physiological pH without labeling intracellular ones. In the design of ribosomal experiments with SSETA, several controls were implemented in order to allow for a meaningful comparison between the SSETA and SMTA experiments. The same concentration of ribosomal proteins was used in both SMTA and SSETA *in vitro* experiments. *In vivo* labeling with SMTA and SSETA was performed on fractions from the same culture of *E. coli* cells. The ribosomal isolation and detection methods were also equivalent for experiments with both reagents.

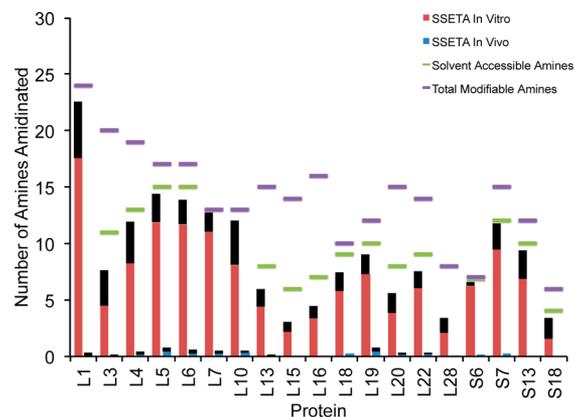
Initial experiments indicated that the fixed-charge SSETA was a less efficient amidinating reagent than SMTA, a result consistent with previous oxyimidate experiments.<sup>43</sup> The half-lives of SMTA and SSETA in a reaction buffer at pH 7.4 were measured by <sup>1</sup>H NMR to investigate whether instability in water accounted for SSETA's decreased amidinating efficiency. Reagent peaks were integrated at 1-min intervals, and the results are displayed in Figure 4. When the data were modeled



**Figure 4.** Time dependence of SMTA and SSETA NMR characteristic peaks. Reagents are in 50:50 (v/v) water/deuterium oxide at pH 7.4. Peak areas at 0 min in both experiments were arbitrarily set to 100.

using first-order kinetics, the half-lives of SMTA and SSETA were found to be  $15.6 \pm 0.3$  and  $22.4 \pm 0.7$  min, respectively. The longer half-life of SSETA suggests that rapid hydrolysis is not the reason for SSETA's decreased amidination efficiency relative to SMTA. We hypothesize that the reaction of an amine with SSETA is less productive because of the different charge states of SMTA's and SSETA's imidate nitrogen at pH 7.4 and the increased sterics of SSETA relative to SMTA.

Figure 5 displays the results of the SSETA labeling experiments *in vitro* and *in vivo*. Black bars display the additional modifications obtained between 15 and 45 min of



**Figure 5.** Comparison of SSETA *in vitro* and *in vivo* ribosomal protein-labeling experiments. The black bars indicate additional modifications achieved between the 15- and 45-min fractions. Although too low in several cases to be visible in the bar graph, SSETA *in vivo* extents of modification are displayed for each protein.

exposure to the reagent. Purple and green bars again display the number of modifiable amines for each protein and the number of solvent-accessible amines as determined from the X-ray crystal structure.<sup>31</sup> *In vitro* SSETA labeled a number of amines corresponding to or less than the number of solvent-accessible amines determined from the X-ray crystal structure. This result indicates that, like SMTA, SSETA is sensitive to the native structure of ribosomal proteins and does not cause denaturation. In these experiments, extra SSETA was added at 15-min intervals in order to approach complete amidination. The same chasing procedure was performed with SSETA *in vitro* and *in vivo* to enable a valid comparison between the extents of labeling in the two cases.

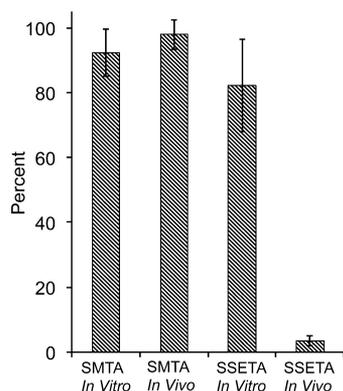
The ability of SSETA to amidinate ribosomal proteins *in vitro* at physiological pH contrasted starkly with its inability to amidinate the ribosomal proteins inside of intact cells. The ribosomal stalk protein L7 shows SMTA and SSETA labeling patterns that reflect the general trend observed. Each of L7's 13 modifiable amines is solvent-exposed. After 45 min of exposure to SMTA, the extents of modification of L7 are 12.96 *in vivo* and 12.80 *in vitro*. Upon exposure to SSETA for 45 min, L7's extents of modification are 13.00 *in vitro* and 0.55 *in vivo*. The significantly lower value with SSETA *in vivo* indicates that, under conditions in which SMTA completely amidinates cytosolic proteins, SSETA does not enter intact cells in sufficient abundance to yield full amidination.

SSETA was less permeable than SMTA but not completely impermeable to the cellular membrane. The minimal extents of amidination observed *in vivo* show that some SSETA was able to enter into cells. For SSETA, 61% of total *in vivo* amidination occurred between 15 and 45 min of exposure to reagent. This increasing modification with time suggests that, if cells are exposed long enough, some amidination of intracellular amines can be achieved even with a charged reagent. Therefore, if the goal of a labeling experiment is to distinguish what is inside of a cell from what is outside, then reaction conditions must be optimized to accomplish this.

In the SSETA *in vitro* experiments, some proteins achieved complete modification of solvent-accessible sites while others did not. Several proteins such as S6 and L7 that reside on the surface of the ribosome were completely modified by SSETA *in vitro*. Core proteins such as L15 and L16 were undermodified with SSETA *in vitro* compared both with the X-ray crystal

structure solvent accessibility and with the SMTA in vitro and in vivo extents of modification. These core proteins have a smaller ratio of solvent-accessible amines to total amines than the average ribosomal protein. Because the proteins under-modified with SSETA are significantly shielded, the few amines that are reactive may be partially blocked and slower to react with thioimidates. It is also possible that electrostatic repulsion between SSETA's fixed negative charge and negatively charged rRNA decreases SSETA's ability to amidinate certain ribosomal proteins. A higher amidinating efficiency and the absence of a negative charge may explain why SMTA is able to label certain residues, while SSETA is not.

**Comparison of the SMTA and SSETA Results.** These experiments have demonstrated that conditions can be found such that SMTA is capable of both complete intra- and extracellular labeling but SSETA only performs extracellular labeling. Figure 6 displays the average percent of solvent-



**Figure 6.** Average percent of solvent-accessible amines labeled after 45 min of exposure to each reagent in vivo and in vitro. Error bars depict 1 standard deviation in each direction.

accessible amines labeled after 45 min of exposure to each reagent in vivo and in vitro. Because the solvent accessibility is determined from the X-ray crystal structure, only proteins that appear in the MRE 600 *E. coli* ribosomal crystal structure were included in this calculation. The average percent of solvent-accessible amines labeled with SMTA was  $92.3 \pm 7.3\%$  for in vitro experiments and  $97.9 \pm 4.6\%$  for in vivo experiments. The same values for SSETA were  $82.2 \pm 14.3\%$  and  $3.5 \pm 1.6\%$ , respectively. These results demonstrate SMTA's ability to label solvent-accessible extra- and intracellular amines under the same conditions in which SSETA labels only extracellular amines.

While the ratio of reagent to ribosomal amines can easily be calculated, the ratio of reagent to total cytosolic amines is unknown because of the abundance of biomolecules that contain amines. This uncertainty makes it worth considering whether the drop in labeling was due to the diminished effectiveness of SSETA and not its impermeability. If the drop in SSETA labeling were predominantly due to increased amine concentration in vivo, we would expect a corresponding drop with SMTA between in vitro and in vivo experiments because the same concentration of cells in vivo and ribosomes in vitro were used for both reagents. Because we do not observe this drop with SMTA, an increased cytosolic amine concentration does not explain the lack of in vivo labeling with SSETA.

## CONCLUSION

We have quantitatively compared the ability of two thioimidates to label ribosomal proteins in vitro and in vivo. The extent of modification of ribosomal proteins with SMTA in vivo was shown to be remarkably consistent with in vitro labeling. We observed evidence of relatively strong reducing conditions within *E. coli* cells. The first charged thioimide SSETA was synthesized, and its ability to amidinate was characterized in vitro. Under conditions comparable to those used with SMTA, SSETA successfully labeled ribosomal proteins in vitro but incorporated almost no labels in vivo, demonstrating its diminished permeability compared with SMTA. By the combination of the differential permeability of thioimidates with peptide mass spectrometry, it will be possible to examine amine membrane sidedness at single-residue resolution. Such experiments promise detailed insight into the structure and dynamics of transmembrane proteins.

## ASSOCIATED CONTENT

### Supporting Information

Pinner synthesis of SSETA, NMR spectrum of SSETA in deuterated DMSO, and expansion and integration of the NMR spectrum from supplemental Figure 1. This material is available free of charge via the Internet at <http://pubs.acs.org>.

## AUTHOR INFORMATION

### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

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