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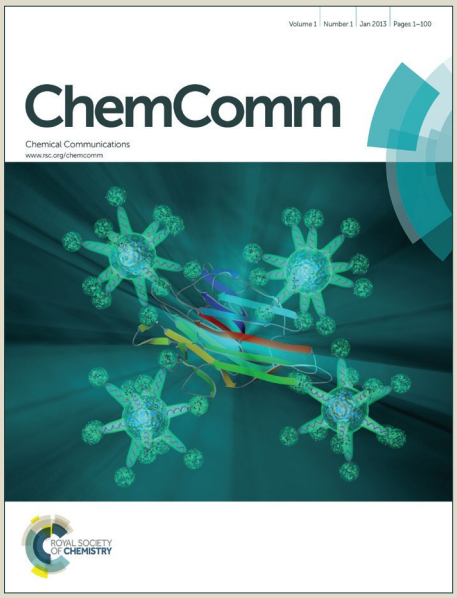

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Small Molecule Diselenide Additives for *In Vitro* Oxidative Protein Folding

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The *in vitro* oxidative folding of disulfide-rich proteins can be challenging. Here we show a new class of small molecule diselenides, which can be easily prepared from inexpensive starting materials, used to enhance oxidative protein folding. These compounds were tested on a model protein, bovine pancreatic trypsin inhibitor. Two of the tested diselenides showed considerable improvement over glutathione and were on par with the previously described selenoglutathione.

The *in vitro* refolding of disulfide-rich proteins can be a challenging task,[1, 2] although this process is generally spontaneous because all the information required for correct folding of a protein into its native state is found in the amino acid sequence.[3] However, as the number of disulfide bonds increases, proteins rich with cysteine (Cys) residues may exhibit slow folding and low yields of native protein due to multiple possible intermediates, including trapped intermediates. Trapped intermediates are long-lived species highly stabilized by disulfide bonds, in which reduced cysteine residues are buried in the protein interior and rendered inaccessible for oxidation. To facilitate oxidative refolding, small compound additives are frequently used, typically, a redox buffer composed of reduced and oxidized glutathione (GSH and GSSG, respectively, Figure 1a) or other thiol-containing additives.[4-6]

Previous studies showed that the oxidative refolding of disulfide-rich proteins in the presence of selenoglutathione (Figure 1a), a selenocysteine (Sec)-containing analog of glutathione, performs better than glutathione in this respect for many proteins, both in terms of the rate of refolding and the yield of native state proteins.[7-11] Furthermore, selenocystamine, a commercially available diselenide, was shown to enhance the *in vitro* oxidative folding of two proteins

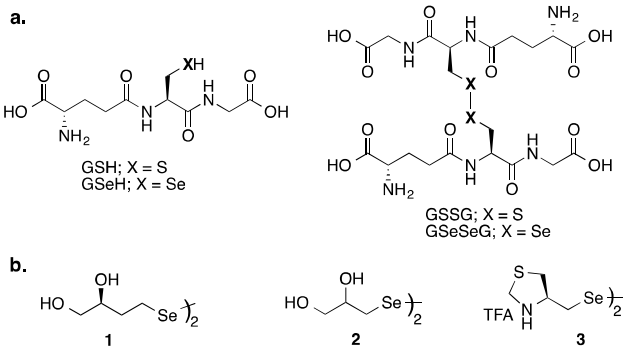

Figure 1. **a**. Structures of reduced and oxidized glutathione (X = S; GSH and GSSG) and selenoglutathione, (X= Se; GSeH and GSeSeG), respectively; **b**. Structures of small molecule diselenides **1**-**3** tested as additives for *in vitro* oxidative protein folding.

RNase A and bovine pancreatic trypsin inhibitor (BPTI), although it provided lower yield of native protein than GSeSeG.[8] Later Beld et al. tested other commercially available small molecule diselenides and compared them to their disulfide counterpart as additives for oxidative folding *in vivo*.[12] All diselenides showed better oxidative folding enhancement than their disulfide analogs. In particular, selenocystamine showed the best *in vivo* activity,[12] perhaps due to its smaller size compared to aromatic diselenides tested and its enhanced solubility, hence was chosen for further studies. More recently, selenocystamine improved the accumulation of disulfide containing proteins in chloroplasts of eukaryotic green algae.[13]

Selenium is a particularly effective electrophile and nucleophile in nucleophilic exchange reactions,[14, 15] due to its greater polarizability in comparison to sulfur and the lower pK_a for selenol side-chain of Sec compared to thiol of Cys (5.2 versus 8.5, respectively).[16] Owing to the rapid oxidation of selenols by atmospheric oxygen, catalytic amounts of diselenides can be used for protein refolding under aerobic conditions.[8, 12] It was also suggested that folding enhancement observed with diselenides is due to enhanced thiol-disulfide exchange reactions, which can rescue trapped intermediates.[11]

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Electronic Supplementary Information (ESI) available: [details of synthetic procedures for diselenides **1**-**3** and full characterizations are available in the supplementary information]. See DOI: 10.1039/x0xx00000x

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Furthermore, Sec has been used as an *intra*molecular catalyst for oxidative protein refolding through Cys-to-Sec substitutions into the protein sequence.[17-24] Strategic Sec substitution into disulfide-rich polypeptides has proven to be advantageous in facilitating this process and providing higher yields of native structures.[21-24] Recently, we reported that Sec-substitution into BPTI could be exploited as a tool to facilitate the folding process and to steer folding in predictive and more productive routes.[21, 23, 24]

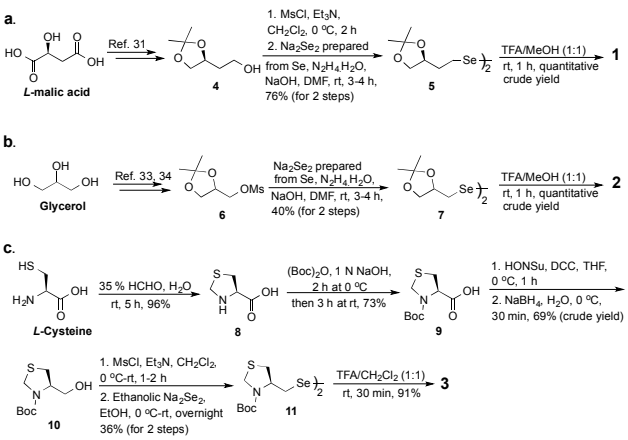
Small seleno-compounds containing diselenide or selenosulfide bonds have been reported. Seleno-analogs of lipoic acid have been previously synthesized;[25] however, their use in protein folding was never demonstrated. In addition, Tomoda and coworkers reported the synthesis of diselenothreitol (DST^{ox}),[26] an analog of the well-studied additive for protein folding, oxidized dithiothreitol (DTT^{ox}). However, DST was found to exhibit much lower redox potential than DTT and was therefore suggested to be a poor additive for protein folding.[26] Recently, Raines and coworkers showed that an analog of reduced dithiothreitol DTT^{red}, dithiobutylamine (DTBA), in which an amino group is present, was a superior reductant for certain proteins over DTT^{red}, due the lower pK_a values of its thiols.[27] Later, Raines and coworkers reported the synthesis of a seleno-DTBA analog and its use in protein reduction but did not extend the study to oxidative protein refolding.[28]

These studies show that small molecule diselenides may become useful additives for *in vivo* and/or *in vitro* protein refolding in academic laboratories and pharmaceutical industries, by enhancing oxidative protein refolding and increasing the recovery of native protein structures, for example, from inclusion bodies.

The most studied seleno-additive is selenoglutathione (GSeH), which, like glutathione (Figure 1a), is a tripeptide with a negative net charge at neutral pH (−2 for GSeH vs. −1 for GSH). Its oxidized dimeric form, GSeSeG, contains an *inter*molecular diselenide bond instead of a disulfide. The preparation of selenoglutathione requires multi-step synthesis from protected Sec, which also requires multi-step synthesis,[29, 30] followed by peptide synthesis, which also uses a non-standard protected glutamate.[7] In contrast, smaller molecule diselenides (e.g. **1**-**3** in Figure 1b) can be readily prepared from commercially available and cheap starting materials (Scheme 1). These kind of smaller diselenides with different functionalities and with different net charge may turn to be advantageous in protein refolding, one good example is selenocystamine *vide supra*.[12]

Here we designed a straightforward synthesis of the diselenide molecules **1-3** (Scheme 1), and study the efficacy in promoting oxidative folding of model protein, BPTI.

Our aims in synthesizing diselenides **1**-**3** were threefold. First, we wanted to synthesize simpler, less costly alternatives to selenoglutathione. Second, we wanted to reduce the size of these additives relative to selenoglutathione (Figure 1), in order to afford better penetration into the protein interior of



Scheme 1. Chemical synthesis of diselenides **1**, **2** and **3**.

trapped intermediates, where free cysteines are usually inaccessible to oxidant. Third, we wanted to explore the effect of changing the net charge of the diselenides **1**-**3**. The hydroxy and amino polar groups in **1**-**3** ensure enhanced solubility in buffer, and their net charge is altered from negative in selenoglutathione to neutral in **1** and **2**, and positively charged in **3**. Changing the net charge can be useful for certain proteins, as recently described for the reduction of proteins by superior reductant DTBA and seleno-DTBA.[27, 28]

Our results indicate that **2** and **3** enhance the refolding of BPTI in the same order as with selenoglutathione and are almost 10-fold better than glutathione.

The diselenides **1**-**3** were prepared from commercially available starting materials; *L*-malic acid, glycerol, and *L*-cysteine, respectively. *L*-malic acid (Scheme 1a) was converted to acetonide protected alcohol **4** via a known procedure.[31] Then **4** was mesylated, followed by treatment with Na_2Se_2 (generated from Se, $N_2H_4{\cdot}H_2O$, and NaOH in DMF)[32] giving diselenide **5** in 76% yield. Acetonide **5** deprotection was achieved by TFA, providing diselenide **1** in quantitative crude yield. The product was purified via C_{18} preparative reversed-phase (RP) HPLC and confirmed by ^{1}H-, ^{13}C- and ^{77}Se-NMR and ESI-MS (see SI).

In the same manner, glycerol (Scheme 1b) was converted to its acetonide protected mesylate **6** by a known procedure.[33, 34] Mesylate **6** was treated with Na_2Se_2[32] to give diselenide **7** in 40% yield. Acetonide **7** deprotection was performed in the same manner as with **5**, giving diselenide **2** in quantitative crude yield. The product was purified by preparative RP-HPLC and confirmed by ^{1}H-, ^{13}C- and ^{77}Se-NMR and ESI-MS (see SI).

Finally, thiazolidine (Thz) **8** (Scheme 1c) was obtained in excellent yield (96%) from a solution of *L*-cysteine and 35% HCHO in water.[35, 36] Treatment of **8** with $(Boc)_2O$ and 1N NaOH afforded Boc-Thz-carboxylic acid (**9**), which is also commercially available, in 73% yield. Compound **9** was converted to its –ONSu activated ester using *N*-hydroxysuccinimide (HONSu) and dicyclohexylcarbodiimide (DCC) in THF at 0 °C, and subsequent reduction[37] with aqueous $NaBH_4$ at 0 °C gave alcohol **10** in 69% crude yield. The crude alcohol was treated with MsCl and Et_3N in CH_2Cl_2 to give the

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corresponding mesylate **10a**, which was treated directly with *in situ* generated ethanolic Na_2Se_2[38] to afford diselenide **11** in 36% total yield. Diselenide **11** was subjected to Boc-deprotection with 50% TFA in CH_2Cl_2 at room temperature to give diselenide **3** as a TFA salt in 91% crude yield, which was purified by preparative RP-HPLC and confirmed by ^{1}H-,^{13}C- and ^{77}Se-NMR and ESI-MS (see SI).

To elucidate the efficacy of diselenides **1-3** in promoting oxidative protein folding, we chose BPTI as our model system.[39-41] The oxidative renaturation of BPTI (Figure 2) was monitored based on the method of Weissman and Kim.[41]

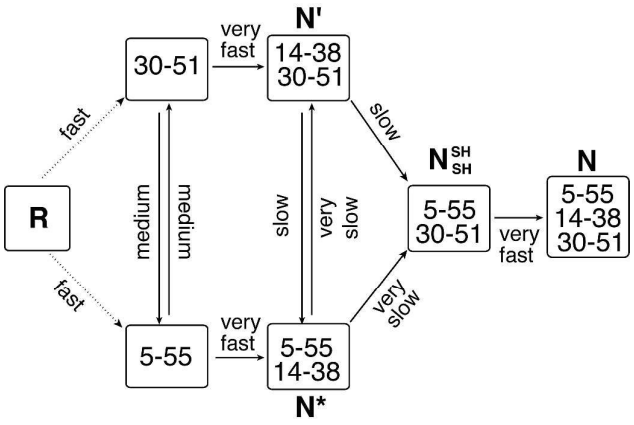

Figure 2. Folding mechanism of BPTI.[41] The initial oxidation of the reduced (**R**) form is fast and affords many intermediates containing one disulfide bond, which rearrange (dotted arrows) to give [30-51] and [5-55] intermediates. Qualitative estimates of the relative rates of the other steps are indicated. Oxidation of the single disulfide intermediates give [14-38, 30-51], **N'**, and [5-55, 14-38] **N***, respectively. The conversion of these trapped intermediates is rate-limiting and goes through unfolding and isomerization to give [5-55, 30-51] N^{SH}_{SH}, which is readily oxidized to the native (**N**) state.

The folding reactions were carried out in degassed buffer under aerobic conditions. Oxidized GSSG or diselenides **1-3** (final concentration 150 µM) was added to reduced BPTI (30 µM) in Tris buffer (100 mM Tris-HCl, 200 mM KCl, 1 mM EDTA, pH 8.7). At determined time points, 80 µL aliquots were removed, quenched with HCl (30 µL of 2 M), and injected into a Waters ACQUITY UPLC using Waters HSS T3 column (1.8 µm, 2.1 x 100 mm), heated at 40 °C, and eluted with a 10:90 to 26.5:73.5 gradient (buffer A = 0.1% TFA in acetonitrile : buffer B = 0.1% TFA in water) over 5 min, increasing to 40:60 (A : B) over 19 min. The chromatograms were monitored at 214 nm. For kinetic analysis, the sum of all peaks of the protein states was assigned as 100%. The peak assignment for native BPTI (**N**) was confirmed by co-injection with an authentic standard, and the peaks of the intermediates **N'** and **N*** that accumulate during folding were assigned according to their mass and previous literature.[11, 21, 23, 41] Under these conditions, oxidative refolding of BPTI in the presence of GSSG was still not complete after 20 h (Figure 3a).[21, 23]

In the initial stages of BPTI renaturation, diselenide **1** exhibited a folding behaviour slower than that with GSSG up to 20 min, where N* was the dominate intermediate (Figure 3b).

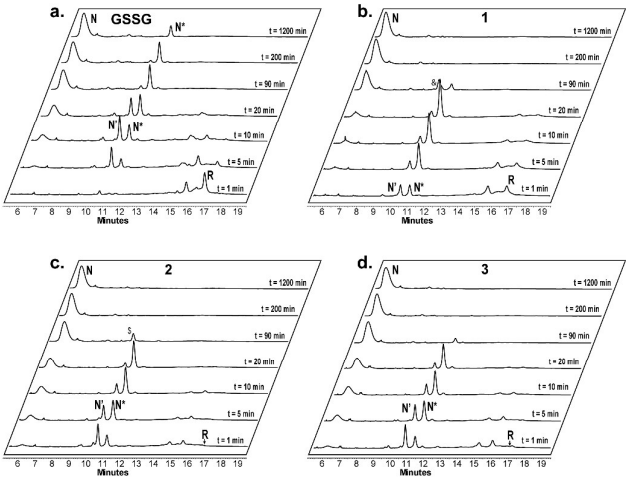

Figure 3. Oxidative folding of BPTI using **a**. GSSG; **b**. diselenide **1**; **c**. **2**; and **d**. **3**. All experiments were carried in parallel; the oxidant (final concentration 150 µM) was added to reduced BPTI (final concentration 30 µM) in Tris-HCl buffer at pH 8.7 . At time points, samples were taken and quenched.[11] & is impurity co-eluted with **N'**, $ is **N*** eluted earlier than anticipated.

However, in contrast to GSSG, the folding with **1** was complete within 200 min, while folding with GSSG was not complete even after 20 h (Figure 3b). BPTI folding with diselenides **2** and **3** was substantially faster than with GSSG (Figure 3c-d). The initial oxidation of reduced protein by **2** and **3** were very rapid and comparable to that observed with GSeSeG,[11] where after only 1 min, the two trapped intermediates **N'** and **N*** dominate.[11] Diselenides **2** and **3** continue to enhance the folding throughout the folding process, resembling the kinetics of GSeSeG (Figure 4). Although the structures of **1** and **2** look similar, an additional methylene group in diselenide **1**, in comparison to **2** seem to cause some effects on folding rates, for example through steric hindrance, although diselenide **1**-**3** provided quantitative final yield of folded protein.

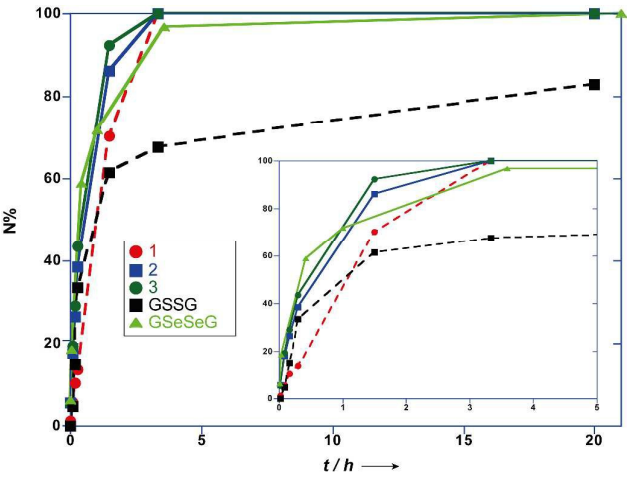

Figure 4. Folding of BPTI with GSSG, diselenides **1**-**3**, and GSeSeG; the data points for GSeSeG were taken from Ref 11.

Since oxidative folding is performed under aerobic conditions, and because selenols are quite sensitive to air oxidation, the

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concentration of the diselenide oxidant is not altered throughout the folding process.[8, 12] We believe that these small molecule diselenides (and perhaps others) may become useful additives for *in vitro* and/or *in vivo* protein refolding in academia and biopharmaceutical industries.

Conclusions

A new class of small molecule diselenides was prepared from commercially available and cheap starting materials. Our results indicate that these synthetic diselenides are superior to glutathione, which is typically used as additive for *in vitro* oxidative folding. Two of the diselenides demonstrated folding capabilities similar to that of selenoglutathione, which was previously shown to enhance the oxidative folding of many proteins, different in size, number of disulfide bonds and folding mechanisms. This class of small molecule diselenides may demonstrate even better folding capabilities with other proteins, for example those exhibiting a different folding mechanism than BPTI.

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