Selenoglutathione: Efficient Oxidative Protein Folding by a Diselenide[†]

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ABSTRACT: Diselenide bonds are intrinsically more stable than disulfide bonds. To examine how this stability difference affects reactivity, we synthesized selenoglutathione (GSeSeG), an analogue of the oxidized form of the tripeptide glutathione that contains a diselenide bond in place of the natural disulfide. The reduction potential of this diselenide bond was determined to be -407 ± 9 mV, a value which is 151 mV lower than that of the disulfide bond in glutathione (GSSG). Thus, the diselenide bond of GSeSeG is 7 kcal/mol more stable than the disulfide bond of GSSG. Nonetheless, we found that GSeSeG can be used to oxidize cysteine residues in unfolded proteins, a process that is driven by the gain in protein conformational stability upon folding. Indeed, the folding of both ribonuclease A (RNase A) and bovine pancreatic trypsin inhibitor (BPTI) proceeded efficiently using GSeSeG as an oxidant, in the former case with a 2-fold rate increase relative to GSSG and in the latter case accelerating conversion of a stable folding intermediate to the native state. In addition, GSeSeG can also oxidize the common biological cofactor NADPH and is a good substrate for the NADPH-dependent enzyme glutathione reductase ($k_{cat} = 69 \pm 2\ s^{-1}$, $K_m = 54 \pm 7\ \mu M$), suggesting that diselenides can efficiently interact with the cellular redox machinery. Surprisingly, the greater thermodynamic stability of diselenide bonds relative to disulfide bonds is not matched by a corresponding decrease in reactivity.

Nature incorporates the essential trace element selenium into proteins as selenocysteine (Sec[1]), the 21st amino acid (*1*). Selenium and the biologically more common chalcogen sulfur are similar in many respects, including electronegativity, atom size, and accessible oxidation states. However, there are some clear differences between selenocysteine and its sulfur counterpart cysteine (Cys), including side-chain pK_a (5.2 for selenocysteine vs 8.3 for cysteine) (*2*), nucleophilicity (selenocysteine > cysteine) (*3*), and reduction potential (~140 mV lower for selenocystine compared to cystine) (*4*, *5*). Presumably, the chemical properties of selenium underlie the biological utility of this residue (*6*). Selenocysteine is often found in enzymatic active sites, where its known functions include acting as either a nucleophile, a metal ligand, or a redox element (*7*, *8*). While cysteine can assume such roles, in biological systems this amino acid is also known to perform acid/base chemistry and to form disulfide bonds, either as a catalytic redox element or as a means to impart structural stability.

To compare the properties of a disulfide bond with an analogous diselenide bond, the ubiquitous tripeptide glutathione (γ-Glu-Cys-Gly), in which γ-Glu designates an amide linkage between the side chain of glutamate and the amino group of cysteine, presents a particularly appealing system. By cycling between its thiol and disulfide forms, glutathione acts as a cellular redox buffer, and the unique γ-Glu linkage minimizes the degradation of this short peptide by endopeptidases (*9*). The ratio of reduced to oxidized glutathione (GSH and GSSG, respectively) is maintained by specific oxidoreductases. Glutathione has also found widespread application as a redox reagent for *in vitro* protein folding. Optimized conditions for this process often include a mixture of GSH and GSSG (*10*−*13*), at concentrations similar to those observed *in vivo* (*14*). During protein folding, GSSG directly oxidizes the protein and GSH enhances disulfide bond isomerization. The initial oxidation of a reduced protein is a relatively fast process; the isomerization of partially oxidized species to the native fold is generally rate determining (*15*).

Here, we report the synthesis and characterization of a "site-directed mutant" of glutathione, in which cysteine has been replaced by selenocysteine. We find that the diselenide form of selenoglutathione[2] (GSeSeG) can undergo efficient reduction, mediated by protein-based thiols, with either NADPH or a folding protein acting as an electron donor. These observations expand the functional scope of selenocysteine in biochemical processes.

MATERIALS AND METHODS

Materials. Buffers were prepared with ultrapure water. All chemicals were purchased from Sigma-Aldrich, Fluka, or

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[1] Abbreviations: Sec, selenocysteine; Cys, cysteine; DTT, dithiothreitol; GSSG, oxidized glutathione; GSH, reduced glutathione; GSeSeG, oxidized selenoglutathione; GSeH, reduced selenoglutathione; cCMP, cyclic cytidine-2′,3′-monophosphate; NADPH, nicotinamide adenosine dinucleotide phosphate; BPTI, bovine pancreatic trypsin inhibitor; RNase A, ribonuclease A; GR, glutathione reductase; GRX3, glutaredoxin 3.

[2] Three names have been proposed for the tripeptide γ-Glu-Sec-Gly: selenoglutathione (*16*), Se-Se-glutathione (*17*), and glutaselenone (*18*). We find "selenoglutathione" to be the most intuitively descriptive of these names and so adopt this nomenclature here.

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Acros. Glutathione reductase (GR) and ribonuclease A (RNase A) were obtained from Sigma-Aldrich. Bovine pancreatic trypsin inhibitor (BPTI) was a generous gift of Bayer AG.

Synthesis of Boc-Glu(α-OtBu)OPfp. The side-chain carboxylate of glutamate was activated by Pfp esterification following the procedure of Kisfaludy and Schön (*19*). Briefly, commercially available glutamate bearing Boc and *t*-butyl protecting groups at its α-amino and α-carboxylate moieties, respectively (Boc-Glu(α-OtBu)-OH, 0.76 g, 2.5 mmol), was dissolved in DMF (100 mL), and pentafluorophenol (0.5 g, 2.75 mmol) was added under nitrogen. After cooling to 4 °C, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (0.55 g, 2.75 mmol) was added under constant stirring and a continuous nitrogen flow. After 1 h, the reaction mixture was allowed to warm to room temperature and was stirred for an additional 12 h. EtOAc was added and the organic phase washed with water (4×), 1 M HCl, water, $NaHCO_3$, water, and brine and then dried with $MgSO_4$. Concentration under reduced pressure afforded a colorless oil, from which a white solid precipitated upon the addition of hexane. Pure product was obtained by recrystallization from EtOAc. [90%; HRMS calcd (M + H) 470.1524, found 470.1501; ^{1}H NMR (300 MHz, $CDCl_3$) δ = 1.36 (s, OC(CH_3)), 1.39 (s, OC(CH_3)), 1.84 (m, H-3B), 2.09 (m, H-3A), 2.27−2.45 (m, H-4), 4.15 (dd, J_1 = 8.0, J_2 = 13.0, H-2), 5.25, 6.29 (d, J = 8, NH).]

Synthesis of Fmoc-Sec(Mob)-OPfp. A selenocysteine derivative suitable for use in solid-phase peptide synthesis was prepared following the procedure of Quaderer et al. (*20*). Briefly, selenocystine was reduced, the selenol was protected with *p*-methoxybenzyl chloride (Mob), an Fmoc protecting group was added to the amine, and the carboxylate was activated by esterification with Pfp. The yield, molecular mass, and ^{1}H NMR spectrum of the purified product were in agreement with the published values.

Synthesis of Selenoglutathione (GSeSeG). GSeSeG was previously prepared in solution by Frank (*17*), Theodoropoulos et al. (*16*), and Tamura et al. (*18*). We synthesized GSeSeG using solid-phase peptide synthesis on an ABI 433A automated peptide synthesizer at a 0.25 mmol scale, following a protocol similar to that used by Cavero et al. for GSSG (*21*). The amino acids Fmoc-Sec(Mob)-OPfp and Boc-Glu(α-OtBu)-OPfp were sequentially coupled (by a HOBt/HBTU free coupling procedure) to preloaded Fmoc-Gly-WANG resin, using minimal amounts of piperidine for Fmoc deprotection. The Boc protecting group was used with glutamate to prevent racemization. Cleavage of the resin-bound peptide was performed in one step by addition of a cleavage cocktail containing TFA/TMSBr/thioanisole/*m*-cresol (750/132/120/50) and shaking at 4 °C for 2 h under an inert atmosphere. After workup and RP-HPLC purification, the fully deprotected peptide was obtained in 33% yield as yellow crystals of the diselenide. [HRMS calcd (M + H) 709.0409, found 709.0415; ^{1}H NMR (300 MHz, D_2O) δ = 2.2 (m, 2H, Glu-β-CH_2), 2.5 (m, 2H, Glu-γ-CH_2), 3.2 (m, 1H), 3.5 (m, 1H, Sec-β-CH_2), 3.8 (t, 1H, Glu-α-CH), 3.9 (s, 2H Gly-CH_2), 4.8 (D_2O and α-proton Sec).]

Determination of Reduction Potential. A published protocol for the equilibration of GSSG with DTT_{red} was used to obtain the reduction potential of GSeSeG (*22*). The equilibrium constant (K_{eq}, eq 1) was determined by averaging the values from ten individual experiments. The formal reduction potential of GSeSeG ($E^{\circ\prime}_{GSeSeG}$) was calculated using the Nernst equation (eq 2), where n is the number of transferred electrons (= 2), F is Faraday's constant (96 500 C/mol), R is the universal gas constant (8.314 J/K/mol), T is the temperature (298 K), and $E^{\circ\prime}_{DTT}$ is the formal reduction potential of DTT (−327 mV). As a control, the disulfide bond reduction potential of GSSG was determined in the same way.

$$K_{eq} = \frac{[DTT_{ox}][GSeH]^2}{[DTT_{red}][GSeSeG]} \quad (1)$$

$$E^{\circ\prime}_{GSeSeG} = E^{\circ\prime}_{DTT_{red}} + \frac{RT}{nF} \ln K_{eq} \quad (2)$$

Although high purity glutathione and DTT were purchased, it was still necessary to remove trace amounts of contaminants. Therefore, GSH, GSSG, DTT_{red}, and DTT_{ox} were each purified by RP-HPLC. Since TFA salts of glutathione and GSeSeG were obtained, stock solutions were carefully neutralized prior to use. Buffer and stock solutions were degassed by high-vacuum/argon cycles for 30 min. The concentrations of stock solutions were determined for GSH and DTT_{red} by Ellman's assay (*23*), for GSSG and GSeSeG by a standard colorimetric assay (*24*), and for DTT_{ox} by UV absorbance (ϵ_{283nm} = 273 M^{-1} cm^{-1} (*25*)). The reactions were initiated by addition of DTT_{red}, using a gastight syringe, to the degassed reaction vessel containing GSSG or GSeSeG, <1% DTT_{ox}, and pH 7.0 buffer (100 mM Tris-HCl plus 2 mM EDTA). Starting concentrations of DTT_{red} and GSSG or GSeSeG were equimolar (3 μM or 8 μM). Aliquots were withdrawn, quenched with one-fifth volume of 1 M HCl, and directly injected onto an analytical RP-HPLC column (Waters Polarity, dC18). The species were separated isocratically using 0.1% aqueous TFA. To check for adventitious oxidation, total free thiol concentration was determined by Ellman's assay for every quenched aliquot. The concentrations of the species at equilibrium were calculated from the observed peak areas and corresponding calibration curves. The concentration of GSeH at equilibrium was calculated from the initial and equilibrium concentrations of GSeSeG.

Oxidative Folding of Ribonuclease A (RNase A). The oxidative renaturation of RNase A was studied spectrophotometrically using the continuous assay of Lyles and Gilbert (*12*), which is based on the ability of properly oxidized and folded RNase A to catalyze the hydrolysis of cyclic cytidine-2′,3′-monophosphate (cCMP) (ϵ_{296nm} = 0.19 mM^{-1} cm^{-1} at pH 8.0) to CMP (ϵ_{296nm} = 0.38 mM^{-1} cm^{-1}). The protein (0.5 mM) was reduced by incubation overnight in Tris-HCl (pH 8) with 2 mM EDTA, 6 M Gdm-HCl, and 140 mM DTT. Fully reduced RNase A was purified by RP-HPLC, lyophilized, dissolved in 10 mM HCl to a final concentration of 500 μM, and stored at −20 °C. Folding assays were performed in a Perkin-Elmer Lambda-20 UV−vis spectrophotometer thermostatted at 25 °C. Reactions were initiated by adding reduced RNaseA (5 μM) to a solution containing 20 μM of oxidant (GSSG or GSeSeG), 4.5 mM cCMP, and 0.1 M Tris-HCl buffer (pH 8.0). The concentrations of active RNase A present over the course of the experiment were calculated as described (*26*).

Oxidative Folding of Bovine Pancreatic Trypsin Inhibitor (BPTI). The oxidative renaturation of BPTI was monitored based on the method of Weissman and Kim (*27*). BPTI was purified by RP-HPLC before reduction to remove a minor contaminant containing an oxidized methionine. The purified native BPTI (0.5 mM) was reduced for 4 h in the presence of 2 mM EDTA, 6 M Gdm-HCl, and 150 mM DTT. Reduced BPTI was purified by RP-HPLC, lyophilized, dissolved to a final concentration of 500 μM in 10 mM HCl, and stored at −20 °C. To induce oxidative folding, reduced BPTI (30 μM) was incubated, under constant stirring, with 150 μM of folding reagent (GSSG or GSeSeG) in folding buffer (containing 100 mM Tris-HCl and 1 mM EDTA, pH 8.7). At various intervals, 50 μL aliquots were removed, quenched with 5 μL of 1 M HCl in prechilled HPLC vials, and directly injected onto the same RP-HPLC column used for the reduction potential determinations described above. Separation of the folding reaction components was achieved with a gradient of 5:95 to 50:50 acetonitrile/0.05% TFA:water/0.1% TFA over 30 min (1 mL/min flow rate). The HPLC peak assignment for native BPTI was confirmed by coinjection of the oxidative folding product with an authentic standard of native BPTI, and the concentration of native BPTI was quantified by the relative extinction coefficients of BPTI ($\epsilon_{220\ nm} = 6.5 \times 10^4\ M^{-1}\ cm^{-1}$) and an internal standard (caffeine, $\epsilon_{272nm} = 9930\ M^{-1}\ cm^{-1}$ (*28*)).

For anaerobic folding of BPTI, the same procedure was used but the reactions were performed in degassed reaction vials connected to a Schlenk line under a continuous positive pressure of argon. To minimize the number of septa punctures, fewer aliquots were taken relative to the aerobic BPTI folding assay. To check for background oxidation (air leakage), reduced BPTI, dissolved in buffer containing neither GSSG nor GSeSeG, was incubated in parallel and afforded no native BPTI over a period of 4 h.

Kinetics of Reduction by Glutathione Reductase (GR). The catalytic activities of GR with either GSSG or GSeSeG as substrate were assayed according to the method of Carlberg and Mannervik (*29*). Initial velocities were determined by monitoring the absorbance at 340 nm and 25 °C. The assay was initiated by the addition of GR (0.2 nM final concentration) to a solution containing 100 mM sodium phosphate (pH 7.0), 2 mM EDTA, 100 μM NADPH, and substrate (concentrations ranged from 10 μM to 200 μM).

RESULTS

Synthesis of Selenoglutathione. GSeSeG was synthesized by solid-phase peptide synthesis on preloaded Fmoc-Gly-Wang resin using Fmoc-Sec(Mob)-OPfp (*20*) and Boc-Glu(α-OtBu)-OPfp (*19*) (Figure 1). After cleavage from the resin and RP-HPLC purification, GSeSeG was obtained as a yellow crystalline material in 33% yield, based on resin-loading. Characterization by NMR and MS gave values in agreement with those reported (*18*).

Determination of Reduction Potential. To determine the difference in stability between the disulfide bond of GSSG and the diselenide bond of GSeSeG, the reduction potentials of both compounds were determined by measuring their equilibrium constants for reduction by dithiothreitol (DTT_{red}). Both GSSG and GSeSeG were individually equilibrated (anaerobically) with DTT, and, after equilibrium had been

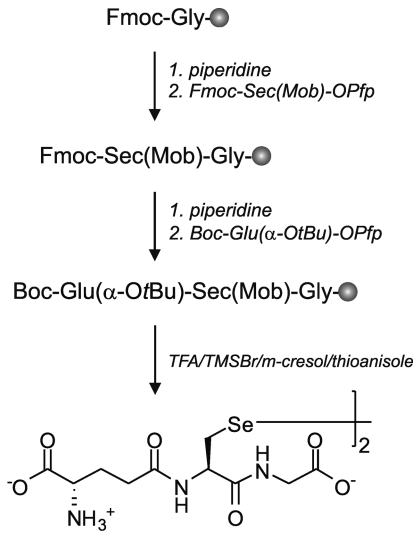


FIGURE 1: Solid-phase peptide synthesis of selenoglutathione (GSeSeG) using preloaded Fmoc-Gly-Wang resin. Fmoc-Sec(Mob)-OPfp (*20*) and Boc-Glu(α-OtBu)-OPfp (*19*) were synthesized according to previously reported procedures.

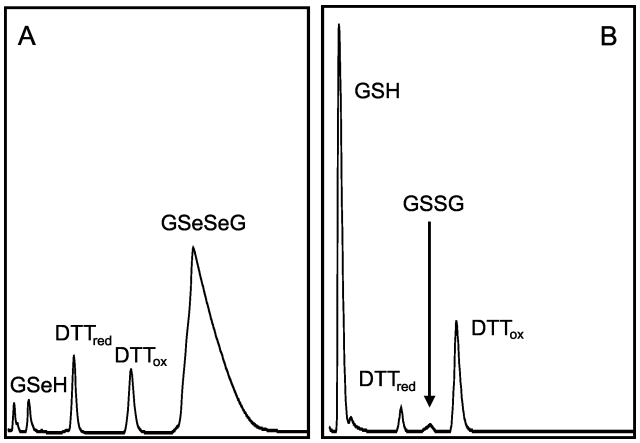


FIGURE 2: Representative RP-HPLC chromatograms of equilibrated (120 min) mixtures of (A) GSeSeG and DTT and (B) GSSG and DTT.

reached, aliquots were quenched with acid and analyzed by RP-HPLC (Figure 2). The reduction potentials for the disulfide and diselenide were calculated from the equilibrium constants ($K_{eq} = 251 \pm 7$ M for GSSG and $K_{eq} = 1.91 \pm 0.70$ mM for GSeSeG) and the known reduction potential of DTT (−327 mV). For GSSG, the value obtained for $E^{\circ\prime}_{GSSG}$ was -256 ± 5 mV, which is in good agreement with previously reported values (*22*, *30*). For GSeSeG, the value obtained for $E^{\circ\prime}_{GSeSeG}$ was -407 ± 9 mV. Further, the reaction between GSeSeG and DTT_{red} went to equilibrium much faster than that between GSSG and DTT_{red} (see Figure S1 of the Supporting Information).

Oxidative Folding of RNase A. To address the question of whether GSeSeG can oxidize protein thiols during folding, we compared the folding behavior of reduced RNase A in the presence of either GSSG or GSeSeG. The folding of RNase A, which has eight cysteines that form four disulfide bonds in the native state, was performed using standard conditions and was monitored by a continuous UV spectrophotometric assay based on the ability of oxidized, native RNase A to hydrolyze cCMP. The concentration of active RNase A at any time was calculated from the first derivative of the absorbance versus time plot (*26*). As shown in Figure

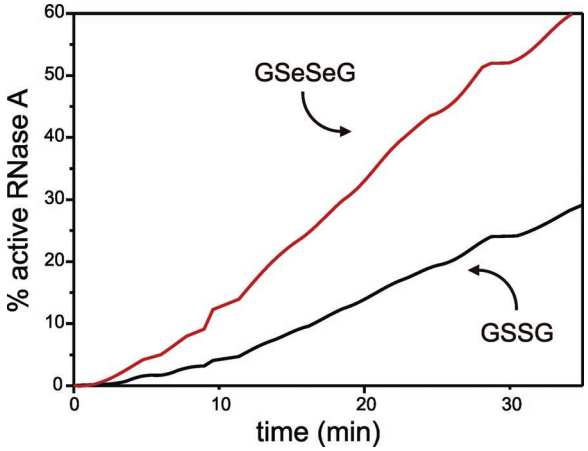


FIGURE 3: Oxidative folding of RNase A. Time courses (averages of six independent measurements) for the increase in native RNase A produced by the addition of GSeSeG (20 μM) or GSSG (20 μM) to reduced RNase A (5 μM). Folding progress was monitored by the production of CMP from cCMP (4.5 mM), which is catalyzed by native RNase A. The percent of active RNase A was calculated from the slopes of the corresponding absorbance vs time curves (see Figure S2 of the Supporting Information), as described (*26*).

3, GSeSeG speeds the aerobic folding of reduced RNase A by a factor of 2 compared to GSSG. Further, both oxidants produced equivalent total yields of native RNase A (see Figure S3 of the Supporting Information).

Oxidative Folding of BPTI. To examine the generality of GSeSeG's folding efficacy, we used HPLC to monitor the oxidative folding of reduced BPTI, which has six cysteines that form three disulfide bonds in the native state. We compared the folding behavior of GSeSeG and GSSG under aerobic (Figure 4) and anaerobic conditions (Figure 5), using standard conditions developed for BPTI folding with GSSG (*27*). Under aerobic conditions, the complex kinetic behavior of BPTI folding with GSSG can be subdivided into three phases: a short (<5 min) lag, followed by a fast phase (yielding ~50% native protein), and then a slow linear phase (Figure 4C). Aerobic folding of BPTI by GSeSeG showed no lag phase, a shorter fast phase (yielding ~13% native protein), and a linear phase with a larger slope. Due to this increased slope, the folding of BPTI by GSeSeG is finished after 4 h while the folding by GSSG is only about two-thirds complete.

Anaerobically, only the fast and linear phases were observed with both oxidants (Figure 5C). The rates and yields of native protein produced by the fast and slow linear phases were similar for aerobic and anaerobic folding by GSSG. In contrast, the fast phase of anaerobic folding by GSeSeG produced a higher yield of properly oxidized BPTI compared to aerobic folding by this oxidant. Further, the slope of the linear phase for GSeSeG is about two times slower under anaerobic conditions relative to aerobic conditions. Nonetheless, GSeSeG again shows a higher yield of native BPTI after 4 h compared to GSSG (90% for GSeSeG and 67% for GSSG).

Kinetics of Reduction by GR. As an alternative to unfolded proteins, NADPH represents another electron donor for the biochemical reduction of a disulfide bond. For example, GSSG is reduced by glutathione reductase (GR), an NADPH-dependent flavoprotein that regulates the oxidation state of glutathione *in vivo*. We found that this oxidoreductase is also

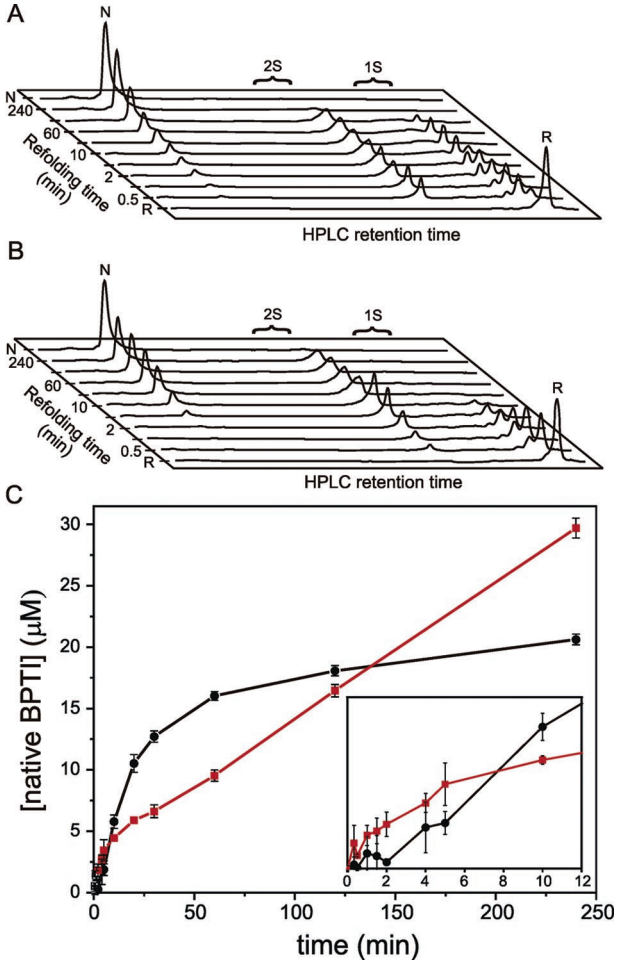


FIGURE 4: Aerobic folding of BPTI. (A) and (B) show representative HPLC chromatograms obtained at various times (0.5, 1, 2, 5, 10, 30, 60, 120, and 240 min) after the addition of reduced BPTI (30 μM) to air-exposed folding buffer containing either 150 μM GSeSeG (A) or 150 μM GSSG (B). Aliquots withdrawn at each time point were quenched with 1 M HCl and directly injected onto the HPLC. The peaks corresponding to reduced BPTI ("R"), native BPTI ("N", identity checked by coinjection with native BPTI), one-disulfide-containing intermediates ("1S"), and two-disulfide-containing intermediates ("2S") are indicated. Presumably the 2S peak is a mixture of both the N′ and N* intermediates (*27*). (C) The concentration of native BPTI over time is plotted for the folding reactions with GSSG (black lines, ●) or GSeSeG (red lines, ■). Each point represents an average of four independent experiments. The inset shows the increases in native BPTI over the first 12 min of the folding reactions.

able to catalyze diselenide reduction in GSeSeG via two active-site cysteines (Figure 6). The kinetic parameters (at 25 °C, pH 7.0) for the reduction of GSSG and GSeSeG by NADPH (100 μM, $E^{\circ\prime}_{NADP+} = -315$ mV) (*31*) and GR were similar, with $k_{cat} = 450 \pm 10$ s^{-1}, $K_m = 39 \pm 3$ μM for GSSG (in agreement with previously obtained values (*32*)) and $k_{cat} = 69 \pm 2$ s^{-1}, $K_m = 54 \pm 7$ μM for GSeSeG. The biggest difference is a 7-fold drop in k_{cat} for GSeSeG relative to GSSG.

DISCUSSION

Although selenium is essential to life, the roles played by this trace element in biological processes are not well understood (*33*). The majority of characterized selenoproteins are enzymes, which generally show enhanced catalytic

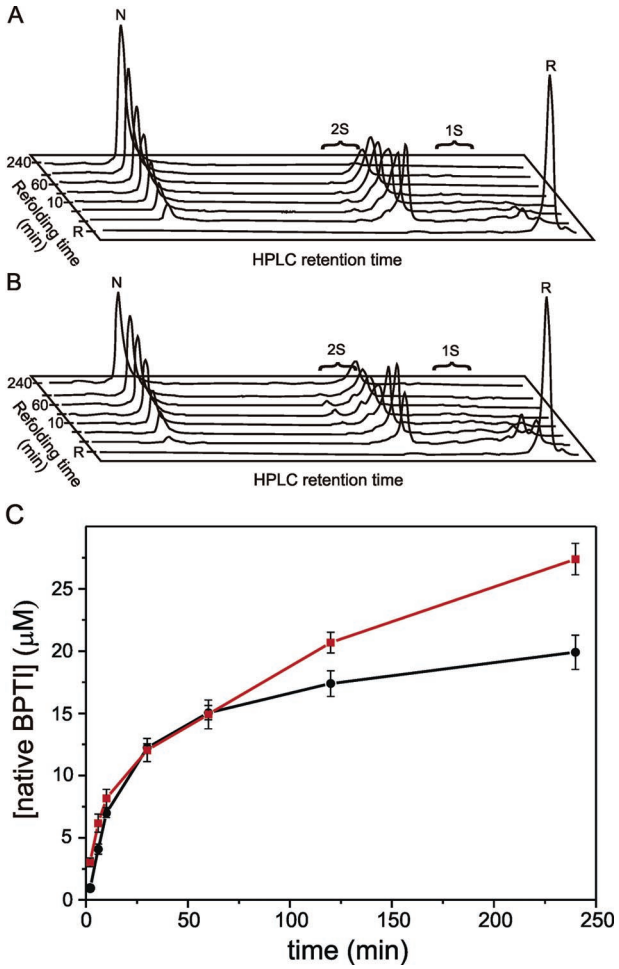


FIGURE 5: Anaerobic folding of BPTI. (A) and (B) show representative HPLC chromatograms obtained at various times (2, 6, 10, 30, 60, 120, and 240 min) after the addition of reduced BPTI (30 μM) to folding buffer containing either 150 μM GSeSeG (A) or 150 μM GSSG (B) and maintained under a positive pressure of argon. Aliquots withdrawn at each time point were quenched with 1 M HCl and directly injected onto the HPLC. The peaks corresponding to reduced BPTI ("R"), native BPTI ("N"), one-disulfide-containing intermediates ("1S"), and two-disulfide-containing intermediates ("2S") are indicated. Presumably the 2S peak is a mixture of both the N′ and N* intermediates (*27*). (C) The concentration of native BPTI over time is plotted for the folding reactions with GSSG (black line, ●) or GSeSeG (red line, ■). Each point represents an average of four independent experiments.

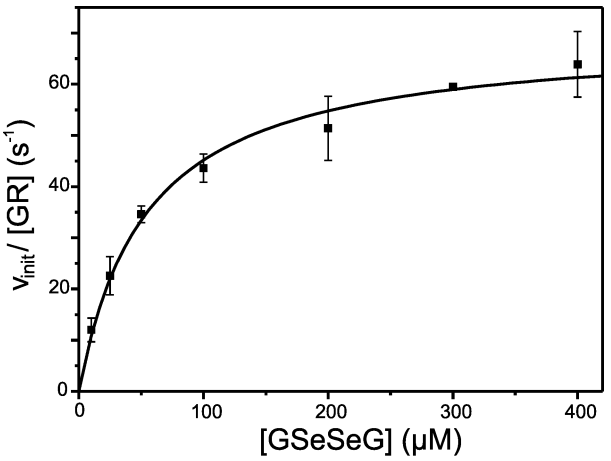


FIGURE 6: Kinetics of GSeSeG reduction catalyzed by glutathione reductase (GR). The GR-catalyzed reduction of GSeSeG by NADPH (100 μM) was carried out at pH 7.0 and 25 °C. The enzyme concentration was 0.2 nM. The solid line represents a fit to the Michaelis−Menten equation and gives a k_{cat} value of 69 ± 2 s^{-1} and a K_m value of 54 ± 7 μM. A similar plot for the GR-catalyzed reduction of GSSG is shown in Figure S4 of the Supporting Information.

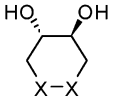
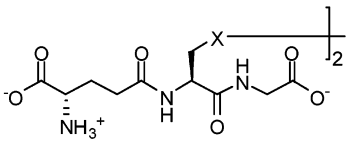
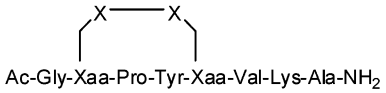
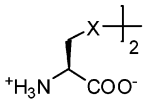
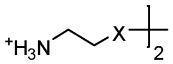
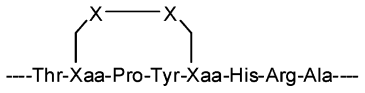
efficiency compared to sulfur-containing analogues (*7*, *8*, *34*). In selenoenzymes, an active-site selenium often either acts as a nucleophile or performs redox chemistry. For example, it has been proposed that deiodinases utilize a nucleophilic selenolate to convert thyroxine (T4) to triiodothyronine (T3) (*35*), and in the well-studied oxidoreductase glutathione peroxidase, the redox properties of selenocysteine are harnessed in a mechanism that involves conversion of the selenolate to both selenenic acid and selenosulfide-bonded intermediates (*36*). Using protein engineering techniques, non-natural selenoproteins have been generated in the laboratory. For example, a posttranslational serine-to-selenocysteine mutation endowed the protease subtilisin with a novel peroxidase activity (*37*). A number of other interesting selenocysteine-containing peptide and protein variants (*20*, *38*−*46*) have recently been obtained through various combinations of expressed protein ligation, solid-phase peptide synthesis, and (selenocysteine-mediated) native chemical ligation. Although diselenide bonds have not been identified in natural proteins (with the possible exception of the protein Sel P (*47*)), many of these engineered variants contain diselenides. Proteins containing diselenide bonds can be considerably more stable compared with disulfide-bonded proteins, which may increase their usefulness in medicinal or industrial applications (*46*). To better understand how diselenide bond incorporation might affect protein structure and function, the properties of diselenide-containing small molecules provide useful points of comparison.

Thermodynamic Diselenide Bond Stability. The average difference in reduction potential (or free energy) between analogous diselenides and disulfides is about 140 mV (or 6−7 kcal/mol), with $E^{\circ\prime}$ values clustering between −350 mV and −410 mV for the former and between −230 mV and −260 mV for the latter (Table 1). In practice, this difference has led to the observation that molecules containing diselenide cross-links cannot be appreciably reduced by thiol-bearing analogues, even with large excesses of reductant (*3*, *48*, *49*). Here, we show that GSeSeG and GSSG exhibit a similar difference in bond stability, as the $E^{\circ\prime}$ value of GSeSeG (−407 mV) is 151 mV lower than that of GSSG (−256 mV). Interestingly, the diselenide bond in an engineered variant of the protein GRX3 containing two Sec residues has an $E^{\circ\prime}$ value that is only 115 mV lower than that of its disulfide counterpart, presumably due to the influence of the protein fold (*43*).

The reduction potential of GSeSeG is slightly lower than that of other unconstrained linear diselenides, such as selenocystine ($E^{\circ\prime}_{\text{selenocystine}} = -383$ mV). However, this difference in stability (24 mV) is comparable to that between GSSG and cystine (18 mV). Indeed, the properties of GSeSeG fit well with what is known about the relative stabilities of diselenide bonds.

GSeSeG and Protein Folding. For structural disulfide bonds in proteins, the reduction potentials generally range between −350 mV and −470 mV (*50*). These remarkably low values are due to the energetic linkage between disulfide bond stability and protein conformational stability (*53*, *54*).

Table 1: Reduction Potentials of Disulfide- and Diselenide-Containing Compounds

Compounds	Structure	$E^{\circ\prime}$ (mV) X = S	$E^{\circ\prime}$ (mV) X = Se
DTT / DST		-327[a]	—[b]
GSSG / GSeSeG		-256[c]	-407[c]
GRX1 octapeptide fragment[d]	 Ac-Gly-Xaa-Pro-Tyr-Xaa-Val-Lys-Ala-NH_2	-235[c]	-387[f]
Cys / Sec		-238[g]	-383[h]
Cystamine / Selenocystamine		-236[i]	-352[j]
GRX3 (85 amino acid protein)[d]	 ----Thr-Xaa-Pro-Tyr-Xaa-His-Arg-Ala----	-194[k]	-309[l]

[a] Calculated from the published K_{eq} value using $E^{\circ\prime} = -288$ mV for the reference molecule (lipoic acid) (*30*). [b] The dithiol DTT was unable to significantly reduce this diselenide (DST), even at a large excess of reductant (*48*). [c] This work. [d] Xaa is either Cys or Sec. [e] Calculated from the published K_{eq} value (*44*) using $E^{\circ\prime} = -256$ mV for the reference molecule (GSSG). [f] Calculated from the published K_{eq} value (*44*) using $E^{\circ\prime} = -327$ mV for the reference molecule (DTT (*30*)). [g] Calculated from the published K_{eq} value (*5*) using $E^{\circ\prime} = -288$ mV for the reference molecule (lipoic acid (*30*)). [h] Calculated from the published K_{eq} value (*4*) using $E^{\circ\prime} = -327$ mV for the reference molecule (DTT (*30*)). [i] Calculated from the published K_{eq} value (*51*) using $E^{\circ\prime} = -256$ mV for the reference molecule (GSSG). [j] Calculated from the published K_{eq} value (*3*) using $E^{\circ\prime} = -327$ mV for the reference molecule (DTT (*30*)). [k] Calculated from the published K_{eq} value using $E^{\circ\prime} = -270$ mV for the reference molecule (thioredoxin) (*52*). [l] Calculated from the published K_{eq} value (*43*) using $E^{\circ\prime} = -270$ mV for the reference molecule (thioredoxin (*52*)).

In contrast, disulfide bonds within unfolded proteins should be (on average) approximately isoenergetic compared to those between small molecules (*55*). With a conventional oxidant such as GSSG, the gain in structural stability upon disulfide-coupled folding, typically 5−20 kcal/mol, drives protein oxidation to completion.

By replacing GSSG with GSeSeG, the oxidation of cysteine residues in proteins becomes more energetically challenging. Nonetheless, this diselenide oxidant gives a high yield (≥90%) during the anaerobic folding of BPTI. Apparently, the gain in conformational stability afforded by the formation of native disulfide bonds in this case (10.6 kcal/mol at pH 7.0 and 25 °C (*56*)) can offset the 5−7 kcal/mol[3] difference in intrinsic stability between disulfide and diselenide bonds. Further, the yields of folded proteins obtained using GSeSeG as an oxidant may generally increase with increasing pH as the stability difference between disulfide and diselenide bonds decreases (see Supporting Information).

Strikingly, a large excess of GSeSeG over proteinaceous cysteine pairs is not required to compensate for the intrinsic stability difference between disulfide and diselenide bonds. While the anaerobic folding yield is high for BPTI (and presumably would also be similar for RNase A, which has a conformational stability of 8.7 kcal/mol at pH 8.0 and 20 °C (*58*)), performing oxidative folding reactions with GSeSeG in the presence of air could help push folding reactions further for less stable proteins, due to the rapid

[3] Folding yield is sensitive to solution conditions. Here, the effect of replacing the disulfide bond of glutathione with a diselenide was assessed using standard pH values, temperature, and oxidant concentrations that were developed for protein folding using GSSG. $E^{\circ\prime}$ values specifically represent the stability of a disulfide or diselenide bond at pH 7.0. Above this pH value the stability difference between GSSG and GSeSeG should decrease (see Supporting Information). For example, at pH 8.7, the condition at which BPTI folding was performed, the intrinsic stability difference between the disulfide of GSSG and the diselenide of GSeSeG should diminish to ca. 5 kcal/mol (*57*).

reoxidation, by O_2, of selenols generated *in situ*. Thus, the ability of GSeSeG to oxidize reduced, unfolded proteins should be fairly general, and this reagent might even serve as a catalyst for protein oxidation.

The effect of diselenide bonds on oxidative protein folding has been previously examined in apamin, an 18 amino acid peptide containing two disulfide bonds (*39*). That study involved the pairwise replacement of cysteine residues by selenocysteines. For variants with non-native selenocysteine pairs, air oxidation of the reduced peptides resulted in the formation of a non-native arrangement, containing one disulfide bond and one diselenide bond, preferentially over the native arrangement, which would consist of two selenosulfide bonds. In this system, the modest stabilization provided by the structure of the native fold (ΔG_{unfold} = 4.5 kcal/mol at pH 7.0 and 20 °C (*59*)), relative to the non-native folds, is apparently not sufficient to overcome the energetic benefit of diselenide bond formation. These apamin analogues provided thermodynamically stable models of kinetically unstable intermediates in the folding pathway of the wild-type peptide, and this strategy seemed to offer a potentially general way to study mechanisms of oxidative protein folding. Our results suggest that while this approach may work well for short peptides with low conformational stabilities, it might not be generally applicable to larger proteins. The pH-dependence of the reduction potential difference[3] suggests that such thermodynamic trapping will be maximized at lower pH values.

Despite the thermodynamic disadvantage of using a diselenide oxidant, the rate of oxidative folding need not be impaired by the substitution of GSSG with GSeSeG. During the oxidative folding of RNase A by GSeSeG, the lag phase is shorter and the maximal rate is higher compared to GSSG. This efficiency is surprising in light of the low folding rates obtained using DTT_{ox} (*60*), which is both the thermodynamically weakest and kinetically slowest protein oxidant among disulfide reagents (including GSSG, cystine, and 2-hydroxyethyldisulfide (*10*, *61*)). For the refolding of reduced RNase A, 100 mM DTT_{ox} displayed a long lag phase (~15 min) and, following the lag, was still slightly slower than with an optimized glutathione redox buffer (containing 0.77 mM GSSG) (*13*, *60*). The greater efficiency of GSeSeG, relative to GSSG, benefits from the intrinsically faster reduction of diselenides by thiols compared to the reduction of disulfides by thiols (*43*, *49*). Indeed, GSeSeG exhibits a shorter lag time at the beginning of the RNase A folding reaction relative to GSSG, which is consistent with faster nonspecific protein disulfide bond formation. The oxidation of RNase A produces selenols and thiols from GSeSeG and GSSG, respectively. As selenols are better nucleophiles than thiols (*3*), improved catalysis of rate-determining disulfide bond isomerizations (despite the presumably rapid depletion of selenol by O_2) might provide a further advantage to GSeSeG.

The mechanistic differences between GSeSeG and GSSG are more readily apparent in the BPTI folding experiments. The BPTI folding pathway has been described in detail and proceeds via intermediates containing one and two disulfide bonds (*27*, *62*). Under strongly oxidizing conditions, roughly half of the reduced BPTI molecules quickly reach the native state (~1 h at pH 8.7 and 25 °C); the other half become trapped as a stable, native-like intermediate (N*), which contains native disulfide bonds between residues 5 and 55 and between residues 14 and 38 but lacks the native 30−51 disulfide (*27*). Conversion of N* to the true, fully oxidized native state proceeds slowly and involves a rate-determining disulfide bond isomerization step.

Under anaerobic conditions with GSSG as the oxidant, our data paint a similar picture. Although the HPLC peak resolution was not optimal, we clearly detect a spectrum of late-eluting singly disulfide-bonded species (labeled as 1S in Figure 5) which rapidly convert to two-disulfide intermediates (2S, presumably a combination of the two-disulfide intermediates N′ and N* described by Weissman and Kim (*63*))[4] and are in turn converted to native BPTI. Qualitatively, the anaerobic folding profile obtained with GSeSeG is similar, although the spectrum of one-disulfide intermediates differs somewhat. Interestingly, while GSSG and GSeSeG seem to produce similar partitioning between the N′ and N* pathways, conversion of N* to native BPTI appears to be accelerated in the presence of GSeSeG, perhaps due to the ability of GSeH to catalyze disulfide bond isomerization (*3*).

The folding behavior of BPTI with GSSG under aerobic conditions is similar to that seen under anaerobic conditions, although the observed lag and persistence of 1S species (Figure 4) suggest kinetic trapping of unproductive one-disulfide intermediates. With GSeSeG, the difference between aerobic and anaerobic conditions is particularly striking. Specifically, the fraction of reduced BPTI that rapidly folds is considerably lower (~13%), which probably reflects altered partitioning between the two main folding pathways leading to a greater accumulation of the N* intermediate. This altered partitioning might stem from relatively slow isomerization of one-disulfide intermediates as a consequence of rapid selenol depletion in the presence of air. Further, the slope of the linear phase for GSeSeG is significantly larger under aerobic conditions than anaerobic conditions, whereas the slope of the linear phase for GSSG is unaffected by the presence of oxygen. Apparently, out of the four folding conditions examined here, the presence of GSeSeG and air both favors the formation of the N* intermediate and most readily facilitates its conversion to native BPTI. Although our data suggest that the greater BPTI folding efficiency of GSeSeG relative to GSSG derives from its greater kinetic effectiveness in rescuing the unreactive N* intermediate, a more comprehensive analysis will be required to understand the influence of chalcogen change on the complex reaction equilibria and disulfide isomerization steps.

Substrate for GR. The catalytic efficiency of GR, an enzyme that has evolved to near perfection for the specific reduction of GSSG by NADPH, with the substrate analogue GSeSeG provides another means to assess the biochemical reactivity of a diselenide bond. The reduction potentials of $NADP^+$ (−315 mV) and GSeSeG (−407 mV) indicate that,

[4] These tentative HPLC peak assignments are based on comparison with published BPTI folding profiles (*27*, *65*). Generally, reduced BPTI elutes late, native BPTI elutes early, and folding intermediates elute in between. Singly disulfide-bonded intermediates elute later than doubly disulfide-bonded intermediates. Further, folding intermediates containing two disulfide bonds become more highly populated and break down more slowly than other intermediates. The assignments of the 1S and 2S intermediates in Figures 4 and 5 are consistent with these observations. Unfortunately, the N′ and N* species were not resolved in our HPLC chromatograms, which precludes a quantitative assessment of the partitioning between the two major folding routes.

in principle, this diselenide can be significantly reduced by nicotinamide. For example, with equimolar starting concentrations of 100 μM for substrate and cofactor, two-thirds of GSeSeG should be reduced by NADPH at equilibrium. Indeed, we find that GSeSeG is efficiently reduced by NADPH and GR.

This enzyme utilizes a ping-pong mechanism and possesses active-site cysteines that interact with glutathione and cycle between the thiol and disulfide states. Within each half-reaction a chemical step partially limits turnover (*64*); therefore, the K_m values are functions of the rate constants for both halves. The similar K_m values for GSSG and GSeSeG suggest similar ratios of catalytic efficiencies between both half-reactions. Further, the overall structural changes induced by replacement of the substrate disulfide bond with a diselenide are not likely to significantly perturb ground-state binding by the enzyme, especially since the most tightly bound part of glutathione, the zwitterionic γ-glutamyl end, is identical for GSSG and GSeSeG (*66*). However, the turnover number, k_{cat}, for GSeSeG is lower by a factor of 7, despite the intrinsically lower kinetic barrier for the reduction of diselenides relative to disulfides (*43*, *49*). Deprotonation of a histidine residue in the active site by the thiolate of reduced glutathione is thought to be the rate-limiting step of the half-reaction involving GSSG (*64*), which could perhaps explain the lower k_{cat}, as a selenolate is a weaker base. Nevertheless, reduction of the diselenide bond by NADPH and enzymic thiols, the standard machinery for biochemical reductions, readily occurs.

Perspectives. The synthesis of the selenium analogue of glutathione provides a means to study the intrinsic stability and reactivity of the diselenide bond. Protein-based thiols are capable of reducing the diselenide bond in GSeSeG, in spite of both the higher innate stability of diselenide bonds relative to disulfide bonds and the apparent absence of diselenides in biological systems. The reduction of diselenide bonds can be driven by the lowering of free energy in native disulfide bonds that accompanies protein folding. Given the more efficient folding seen for both BPTI and RNase A, GSeSeG should be a generally useful protein oxidant (in the presence or absence of air) and may find practical application for preparative protein folding *in vitro*. As selenols oxidize rapidly in air, GSeSeG may even work catalytically, which would further increase its practical utility. GSeSeG (and its low reduction potential) might also prove useful for determining the stabilities of structural disulfide bonds in native proteins. Finally, GSeSeG is a good substrate for GR, suggesting that diselenides can efficiently interact with the cellular redox machinery. Although it remains unknown whether diselenide bonds play any roles in biology, the *in vitro* properties of the non-natural peptide selenoglutathione demonstrate the great biochemical potential of such cross-links.

SUPPORTING INFORMATION AVAILABLE

Additional kinetic data for the experiments described here and calculations of the pH dependence of reduction potentials. This material is available free of charge via the Internet at http://pubs.acs.org.

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