DOI: 10.1002/cbic.200700745

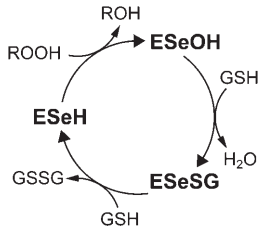
Selenoglutaredoxin as a Glutathione Peroxidase Mimic

Giulio Casi,[a] Gerard Roelfes,[b] and Donald Hilvert*[a]

Glutaredoxin (Grx1) from Escherichia coli *is a monomeric, 85-amino-acid-long, disulfide-containing redox protein. A Grx1 variant in which the redox-active disulfide was replaced with a selenocysteine (C11U/C14S) was prepared by native chemical ligation from three fragments as a potential mimic of the natural selenoenzyme glutathione peroxidase (Gpx). Selenoglutaredoxin, like the analogous C14S Grx1 variant, shows weak peroxidase activity. The selenol provides a 30-fold advantage over the thiol, but its activity is four orders of magnitude lower than that of bovine Gpx. In contrast, selenoglutaredoxin is an excellent catalyst for thiol–disulfide exchange reactions; it promotes the reduction of β-hydroxyethyldisulfide by glutathione with a specific activity of 130 units mg^{-1}. This value is 1.8 times greater than that of C14S Grx1 under identical conditions, and $>10^4$ greater than the peroxidase activity of either enzyme. Given the facile reduction of the glutathionyl-selenoglutaredoxin adduct by glutathione, oxidation of the selenol by the alkyl hydroperoxide substrate likely limits catalytic turnover and will have to be optimized to create more effective Gpx mimics. These results highlight the challenge of generating Gpx activity in a small, generic protein scaffold, despite the presence of a well-defined glutathione binding site and the intrinsic advantage of selenium over sulfur derivatives.*

Glutathione peroxidases (Gpx) efficiently catalyze the reduction of hydrogen peroxide and organic hydroperoxides by glutathione.[1–3] Their activity is believed to protect cells against the oxidative damage that is involved in the etiology of a variety of diseases, including emphysema, heart disease, and cancer.[3] The well-characterized cytosolic Gpx is a large homotetrameric protein.[4,5] Each subunit consists of 198 residues and adopts a thioredoxin-like α/β fold.[6] Its activity depends on a selenocysteine residue, which is incorporated into the protein cotranslationally at position 35.[7] The selenol side chain is relatively exposed on the surface of the protein, proximal to a binding site for glutathione, where it makes hydrogen-bonding interactions with a conserved tryptophan and a glutamine.[4,5] A catalytic mechanism that involves the interconversion of the selenol (ESeH), selenenic acid (ESeOH) and selenenyl sulfide (ESeSR) forms of the prosthetic group has been proposed (Scheme 1).[7] However, alternative catalytic cycles have not been ruled out, and the origins of the enzyme's high efficiency are unclear.[8]

It is possible to mimic the properties of Gpx by incorporating selenium into a variety of other protein scaffolds. For example, treating the serine protease subtilisin sequentially with phenylmethanesulfonyl fluoride and hydrogen selenide selectively converts Ser221 at the enzyme active site into a selenocysteine.[9] The resulting protein, selenosubtilisin, catalyzes the reduction of alkyl hydroperoxides by thiols.[10] Kinetic analyses,[11,12] site-directed mutagenesis,[13,14] ^{1}H and ^{77}Se NMR spectroscopy,[15,16] and crystallography[17] have afforded detailed insight into the influence of the active-site microenvironment on the reactivity of the selenium prosthetic group. The enzyme has also been used for the chiral resolution of alkyl hydroperoxides. It shows opposite selectivity but similar efficiency to that of horseradish peroxidase, despite a completely different mechanism.[18]



Scheme 1. Postulated catalytic cycle of the Gpx-catalyzed reduction of hydrogen peroxide by glutathione (GSH).

Although selenosubtilisin shows many similarities to Gpx, its preferred donor substrates are aryl thiols rather than glutathione[12] and it is substantially less efficient than the natural peroxidase,[12] which operates close to the diffusion limit.[19] The absence of a defined glutathione binding site and the placement of the selenol in a binding pocket rather than on the exposed surface of the protein might account for these differences. To develop more effective Gpx mimics, other protein scaffolds have been investigated, including trypsin,[20] glyceraldehyde-3-phosphate dehydrogenase (GAPDH),[21] glutathione S-transferase (GST),[22] and antibodies.[23] Nonetheless, with the exception of GST, which adopts a thioredoxin-like fold and contains a glutathione-binding site like Gpx, relatively modest activities have been reported.

Here we examine one of the smallest members of the thioredoxin family, *E. coli* glutaredoxin 1 (Grx1),[24] as a template for creating artificial peroxidases. This structurally well-characterized protein is monomeric and only 85 amino acids long. It possesses a redox-active disulfide at a position that is analogous to the selenocysteine in Gpx as well as a well-defined binding site for glutathione.[25,26] We envisaged that replacing the solvent-accessible Cys11 with selenocysteine, and the

[a] *G. Casi, Prof. D. Hilvert*
Laboratorium für Organische Chemie, ETH Zürich
Hönggerberg HCI F339, 8093 Zürich (Switzerland)
Fax: (+41) 44-632-1486
E-mail: hilvert@org.chem.ethz.ch

[b] *Dr. G. Roelfes*
Current address: Stratingh Institute for Chemistry, University of Groningen
Nijenborgh 4, 9747 AG Groningen (The Netherlands)

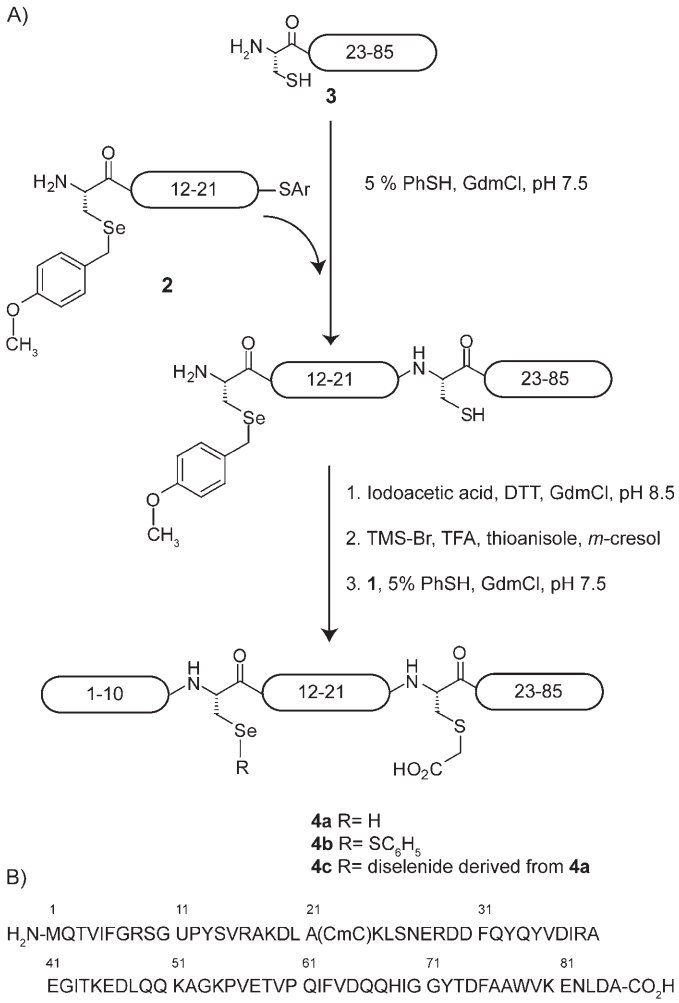
buried Cys14 with serine would convert this simple redox agent into a monomeric peroxidase with a preference for glutathione as the donor substrate. The small size of glutaredoxin makes it an ideal candidate for total chemical synthesis and, as outlined below, we successfully prepared selenoglutaredoxin by following a three-fragment chemical ligation scheme. Its redox activity was also examined and compared with that of the analogous cysteine-containing protein.

Results

Selenoglutaredoxin

Recombinant C14S Grx1 has been described previously,[27] and the structure of its mixed disulfide adduct with glutathione was determined by NMR spectroscopy.[28] We prepared the analogous C11U/C14S variant, selenoglutaredoxin, as a potential Gpx mimic. Although selenoproteins can be produced biosynthetically[29–31] or by post-translational modification,[9,20] we adopted a (semi)synthetic strategy.[32–40] Selenocysteine-mediated chemical ligation of synthetic peptide fragments[32,33,36] is an efficient method for producing artificial selenoproteins that obviates the need for especially reactive residues or special molecular biological methods.

A three-fragment ligation strategy was selected for the construction of selenoglutaredoxin that employed one conventional cysteine-based and one selenocysteine-based ligation to minimize the number of chemical transformations on the peptide containing the potentially labile selenocysteine residue. In an initial approach the protein was disconnected between residues 10–11 and 40–41. However, the use of intein chemistry[41] to produce the Grx1(41–85) fragment, and the solid-phase synthesis of the selenocysteine-containing middle fragment, Grx1(11–40), proved to be difficult and generally resulted in low yields. Therefore, in an improved design, the protein was disconnected between residues 10–11 and 21–22 (Scheme 2). Although this strategy requires mutating glutamate 22 to cysteine, carboxymethylation of this residue with iodoacetic acid after ligation affords a noncoded amino acid side chain that is sterically and electronically similar to the original glutamate.[37,42]



Scheme 2. Synthesis of selenoglutaredoxin. A) Semisynthetic route. B) Selenoglutaredoxin sequence with single-letter amino acid abbreviations; U = selenocysteine; CmC = carboxymethyl cysteine.

The N-terminal fragment Grx1(1–10) **1**, which was activated as a thioester, was prepared by solid-phase peptide synthesis using Fmoc chemistry. The thioester was obtained by treating a hydroxymethyl resin with alkylaluminum thiolate.[43] The middle fragment, C11U(Mob)/C14S Grx1(11–21) **2**, was synthesized on a 2-chlorotrityl resin.[44] Selenocysteine was introduced in the last step as an orthogonally protected Boc-Sec(Mob)-OPfp ester to minimize problems with racemization and β-elimination.[34] After mild acidic cleavage, the C-terminal carboxylic group of the protected peptide was directly converted to the corresponding thioester, and TFA cleavage of the protecting groups afforded the desired deprotected fragment **2**. The C-terminal fragment, E22C Grx1(22–85) **3**, was produced recombinantly and fused to an N-terminal decahistidine tag via a linker that contained a TEV protease cleavage site. Treatment of the purified fusion protein with S219P TEV protease afforded fragment **3** in good yield.

Selenoglutaredoxin was assembled from the three fragments in two ligation steps, starting from the C terminus (Scheme 2). Fragments **2** and **3** were coupled by a standard native chemical ligation protocol.[45] The reaction was performed with ca. 1 mM of each peptide in 100 mM phosphate buffer (pH 7.5) that contained 6 M GdmCl and 5% thiophenol for 16 h. The cysteine residue at position 22 was subsequently carboxymethylated with iodoacetic acid to give C11U(Mob)/C14S/E22CmC Grx1(11–85). Deprotection of selenocysteine, followed by selenocysteine-mediated native chemical ligation[32,33,36] with the Grx1(1–10) thioester **1**, afforded full-length C11U/C14S/E22CmC Grx1(1–85) as a mixed selenosulfide with thiophenol **4b** and a small amount of diselenide dimer **4c**.

Prior to folding, the peptide was converted to a mixed selenosulfide with glutathione. The protein was reduced with DTT and treated with excess oxidized glutathione to give the glutathione adduct in an overall 14% yield after HPLC purification. MALDI-TOF mass spectrometry confirmed the identity of the product. The lyophilized protein was folded as previously described.[46] Because the mixed selenosulfide disproportionates to the diselenide and oxidized glutathione upon prolonged dialysis, the lyophilized protein was generally dissolved directly in the folding buffer immediately prior to use. The resulting samples and the previously characterized glutathione adduct of recombinant C14S Grx1[46] exhibited essentially identical CD spectra (Figure 1). Folding yields typically ranged between 40 and 80%.

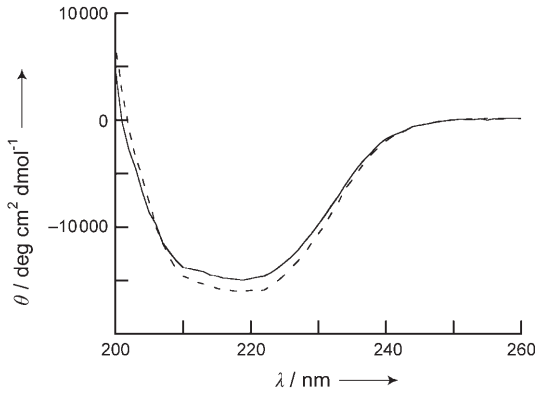


Figure 1. CD spectra of the glutathione adducts of selenoglutaredoxin (——) and C14S Grx1 (-----). The spectrum of refolded C14S Grx1 is superimposable on that of a sample that had never been unfolded. Measurements were performed with 10–20 μM of the desired protein in 50 mM phosphate buffer (pH 7.0) that contained 100 mM NaCl, 1 mM EDTA at 25 °C. The spectra were corrected for the background signal from the buffer.

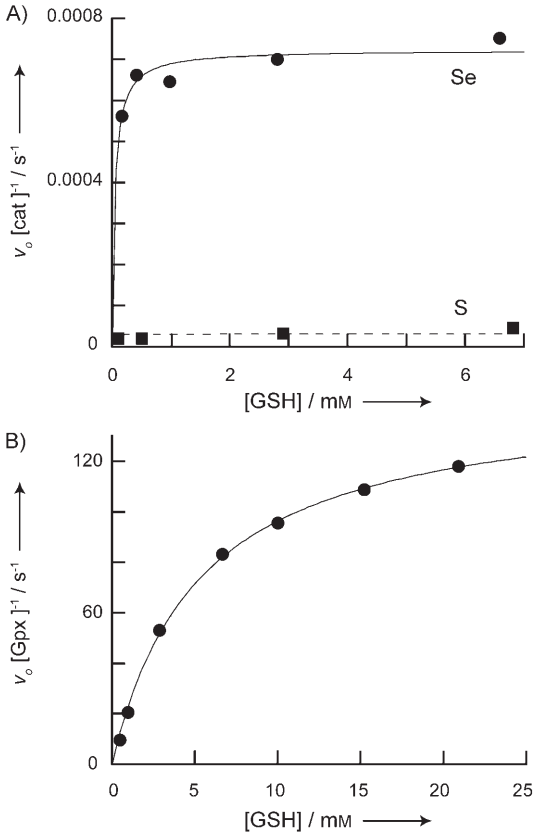


Figure 2. A) Peroxidase activity of selenoglutaredoxin (——) and C14S Grx1 (-----). B) Peroxidase activity of natural Gpx (——). The oxidation of glutathione with 100 μM *tert*-butylhydrogen peroxide was carried out at 25 °C in 50 mM phosphate buffer (pH 7.0), containing 100 mM NaCl, 1 mM EDTA, 210 μM NADPH, and 5 U mL^{-1} glutathione reductase by using either 30 μM selenoglutaredoxin, 100 μM C14S Grx1, or 1.5–6 nM Gpx as the catalyst. Initial rates were fitted to the Michaelis–Menten equation $v_o/[E] = k_{cat}[S]/(K_m+[S])$.

Peroxidase activity

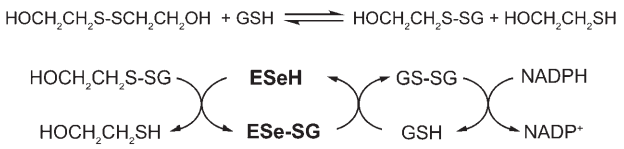
Selenoglutaredoxin and C14S Grx1 were assayed as potential catalysts for the reduction of *tert*-butylhydroperoxide by glutathione. Activity was monitored by a standard coupled assay in which the GSSG that was produced in the course of the reaction was catalytically reduced by NADPH with the enzyme glutathione reductase.[47] After subtraction of the spontaneous background reaction, only modest peroxidase activity could be attributed to the glutaredoxin variants. The selenocysteine-containing protein has an apparent k_{cat} of 7×10^{-4} s^{-1} which corresponds to a ca. 140-fold acceleration over background, whereas the cysteine-containing protein enhances the rate of reaction only four-fold (Figure 2A). In both cases the reaction rate is essentially independent of the glutathione concentration ($K_m \ll 0.01$ mM). For comparison, authentic bovine Gpx has a k_{cat} of 150 ± 3 s^{-1} and $K_m = 5.3\pm0.3$ mM (Figure 2B). At 1 mM glutathione, Gpx is $>10^4$ times more efficient than the artificial selenoenzyme, which is in turn 30 times more efficient that C14S Grx1.

To investigate the importance of the glutathione binding site for peroxidase activity, DL-dihydrolipoic acid was examined as an alternative reductant. Oxidation of the thiol can be monitored directly by the increase in absorbance at 330 nm, which is associated with the formation of the constrained intramolecular disulfide of lipoic acid. Consistent with expectations that intramolecular attack of the second substrate thiol would lead to rapid decomposition of the initially formed selenosulfide/disulfide adduct with the proteins, somewhat faster reaction rates were observed with dihydrolipoic acid compared to glutathione. The apparent k_{cat} for selenoglutaredoxin and C14S Grx1 were 4.8×10^{-3} s^{-1} and 1.5×10^{-4} s^{-1}; this corresponds to about 3000 and 90-fold accelerations over the spontaneous background reaction.

Grx activity

Because C14S Grx1, like wild-type Grx, efficiently catalyzes the reduction of low-molecular-weight glutathionyl mixed disulfides,[27] selenoglutaredoxin was also examined as a potential disulfide reductase. The standard assay monitors the thiol–disulfide interchange between glutathione and β-hydroxyethyldisulfide (HED).[48,49] Like the peroxidase assay that was described in the previous section, this process is coupled to NADPH via

glutathione reductase, leading to a NADPH-dependent disulfide reduction where GSH nominally plays a catalytic role (Scheme 3). In contrast to its modest peroxidase activity, selenoglutaredoxin is a surprisingly good catalyst for disulfide reduction, and it exhibits a specific activity of 130 units mg^{-1} in the HED assay. This value is 1.8 times higher than that of C14S Grx1, when measured under identical conditions (71 units mg^{-1}), and more than four orders of magnitude higher than the peroxidase activity of either protein (Figure 3). The high efficiency of the selenoglutaredoxin-catalyzed thiol–disulfide exchange makes it unlikely that attack of GSH on the glutathionyl adduct of selenoglutaredoxin to give the reduced enzyme plus GSSG is the limiting step in the peroxidase catalytic cycle (Scheme 1). Instead, oxidation of the selenol by the hydroperoxide substrate must be substantially slower than in Gpx.



Scheme 3. Disulfide exchange.

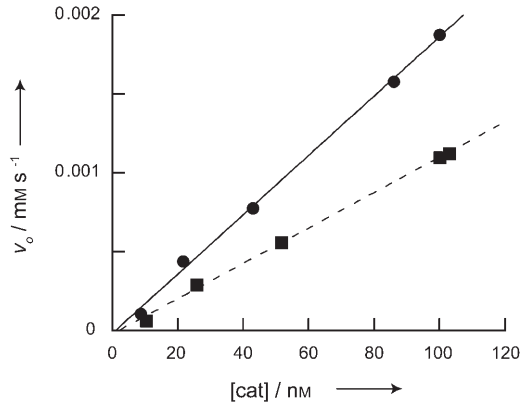


Figure 3. GSH disulfide oxidoreductase activity of selenoglutaredoxin (——) and C14S Grx1 (-----). The reduction of the mixed disulfide that was formed by premixing β-hydroxyethyl disulfide and glutathione was carried out at 28 °C in 100 mM Tris buffer (pH 8.0) that contained 2 mM EDTA, 0.1% BSA, 400 μM NADPH, 50 nM glutathione reductase (GR), and 10–100 nM C14S Grx1 or selenoglutaredoxin.

Reaction with glutathione reductase

Glutathione reductase, which was used in the coupled peroxidase and Grx assays, is an NADPH-dependent flavoprotein that regulates the oxidation state of glutathione in vivo. In control experiments, we found that this enzyme unexpectedly catalyzes the direct reduction of the glutathione adducts of selenoglutaredoxin and C14S Grx1. At pH 7.0 and 25 °C, the apparent biomolecular rate constants k_{cat}/K_m that were observed in reactions with 100 μM NADPH were $2.9\times10^5\ M^{-1}s^{-1}$ and $1.3\times10^5\ M^{-1}s^{-1}$ for the selenosulfide and disulfide, respectively. For comparison, k_{cat}/K_m for the reduction of glutathione is $1.3\times10^7\ M^{-1}s^{-1}$, which is approximately two orders of magnitude higher.[40] The reaction of the glutathione adduct of C14S Grx1 was examined in greater detail (Figure 4). Steady-state parameters $k_{cat}=150\pm20\ s^{-1}$ and $K_m=1.2\pm0.3$ mM were obtained. Comparison with the analogous values of glutathione ($k_{cat}=450\pm10\ s^{-1}$ and $K_m=0.039\pm0.003$ mM) shows that the decreased activity with the mixed Grx1 disulfide is largely a consequence of a substantially larger K_m value for this substrate.

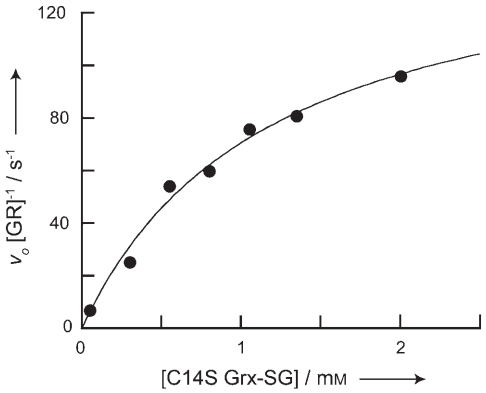


Figure 4. Catalytic reduction of the mixed disulfide between glutathione and C14S Grx1 (C14S Grx-SG) by glutathione reductase. Reactions were carried out with 2.0 nM glutathione reductase (GR) at 25 °C in 50 mM phosphate buffer (pH 7.0) that contained 100 mM NaCl, 1 mM EDTA, 0.1% BSA, and 100 μM NADPH. Initial rates were fitted to the Michaelis–Menten equation.

Discussion

Glutathione peroxidases are extraordinarily efficient enzymes. They catalyze the reduction of hydrogen peroxide and organic hydroperoxides at close to the diffusion limit.[19] As a consequence, there has been considerable interest in mimicking their antioxidant activity with selenium-containing proteins and small molecules, with an eye toward therapeutic applications.

A variety of successful Gpx mimics have been described.[10,21,50–52] The high peroxidase activity reported for a selenium-containing variant of glutathione-S-transferase (seleno-LuGST1-1)[22] is notable in this context (although a reinvestigation is potentially warranted given the lack of activity reported for another, similarly modified GST scaffold[53]). In general, though, Gpx mimics are much less active than the natural enzyme. This holds for selenoglutaredoxin as well. Despite the overall similarity of the Gpx and Grx1 folds and the presence of a well-defined glutathione-binding site in both, selenoglutaredoxin is a comparatively poor peroxidase. Its activity is four orders of magnitude lower than that of bovine Gpx, underscoring the remarkable efficiency of the natural enzymes. In fact, the Grx1 protein scaffold offers only a slight advantage over a simple 15-amino-acid-long peptide that contains a C-terminal selenocysteine residue[54] with H_2O_2 as an oxidant (data not shown).

Comparison of selenoglutaredoxin with C14S Grx1 shows that the selenol provides a 30-fold advantage over the thiol, but no more. Similarly, in several other systems, four to 100-fold increases in activity have been observed upon replacement of cysteines with selenocysteines in proteins.[31,55] In contrast, mutating selenocysteines to cysteine in highly evolved selenoenzymes typically leads to 100 to 1000-fold decreases in activity.[39,56–62] For example, the Sec46Cys mutant of phospholipid Gpx is 1000 times less active than the wild-type enzyme.[62] The 30-fold difference in activity for the glutaredoxin variants thus likely reflects the intrinsic difference in reactivity of a selenol and a thiol. The active-site microenvironment of natural Gpx apparently enhances this difference significantly and, at the same time, greatly increases overall reactivity. The $>10^4$-fold activation of selenocysteine in Gpx compared to selenoglutaredoxin or simple selenopeptides is similar in magnitude to the increase in reactivity that is seen for the active-site serine in serine proteases.[63]

What is special about glutathione peroxidase? Stabilization of the selenol form of the prosthetic group via hydrogen-bonding interactions with a conserved glutamine and tryptophan residues in the active site is certainly important. Mutation of these two residues in phospholipid Gpx causes a further 10^2 to 10^3-fold decrease in activity over the Sec46Cys substitution.[62] Such interactions might be crucial for shifting the equilibrium between the selenosulfide (ESeSG) and selenolate (ESe^-) forms of the enzyme in favor of the latter, which can then be rapidly oxidized by a proximally bound peroxide to complete the catalytic cycle (Scheme 1). The efficacy of selenosubtilisin compared to low-molecular-weight selenium-containing compounds has been similarly attributed to the unusually low pK_a of its active-site selenol.[15] An extensive hydrogen-bonding network stabilizes the selenolate and facilitates the attack of thiols on the selenosulfide intermediate.

In C14S Grx1, the interchange of the thiol–disulfide forms of the enzyme is quite facile, and can be further enhanced by a factor of approximately two by replacing Cys11 by selenocysteine; this is presumably because the catalytic thiol/selenol is located proximal to a glutathione binding site. As a consequence, this step is unlikely to limit the semisynthetic selenoenzyme's peroxidase activity. Instead, peroxide-mediated oxidation of the selenol to regenerate the selenosulfide (via a highly reactive selenenic acid intermediate, ESeOH, Scheme 1) appears to be unexpectedly inefficient. How natural Gpx activates hydrogen peroxide and alkyl hydroperoxides for reaction with the selenol is unknown, but its tetrameric structure might provide specific binding interactions with the oxidant and also sterically prevent formation of undesired oxidized forms of the catalyst, such as the diselenide dimer. The Grx1 scaffold, which is small and monomeric cannot exploit such mechanisms.

The ability of selenoglutaredoxin to efficiently catalyze the reduction of glutathionyl mixed disulfides is notable in light of current interest in naturally occurring monothiol glutaredoxins.[64] The latter enzymes exhibit a range of important redox activities, including disulfide reduction, disulfide isomerization, and glutathionylation reactions involving glutathionylated enzyme intermediates. While selenocysteine-containing analogues have not (yet) been found in nature, it is clear that selenium could provide a catalytic advantage to such systems.

A related but unexpected finding from our study is the ability of glutathione reductase to accept the glutathione adducts of selenoglutaredoxin and C14S Grx1 as substrates. Although they are processed two orders of magnitude less efficiently than oxidized glutathione itself, given the high k_{cat}/K_m value for the natural substrate, these activities are substantial. Indeed, this activity must be taken into account when interpreting the results of the coupled peroxidase assay, so as not to be misled. Structural studies of glutathione reductase have shown that the glutathione-binding site is located at the dimer interface.[65–68] Interactions with the reductase are focused primarily on one tripeptide unit of the dimeric substrate, so that mixed disulfides can be accommodated without problem. When the substrate is a mixed disulfide (selenosulfide) between glutathione and a relatively bulky (seleno)cysteine-containing protein like Grx1, some steric clashes inevitably ensue, which readily account for the 30-times-larger K_m that is observed. As seen in the thiol–disulfide exchange assay, selenium provides a modest (twofold) advantage over sulfur. In contrast, selenoglutathione is a ten-fold poorer substrate than conventional glutathione,[40] and the native disulfide in Grx3 is reduced by thioredoxin two- to fivefold more efficiently than either of the isomeric selenosulfide mutants.[37]

Our study highlights the challenge of generating high peroxidase activity in a generic protein scaffold. Selenium compounds have an intrinsic advantage over the analogous sulfur derivatives, but this advantage accounts for a relatively small fraction of the extraordinary effects that are achieved by natural Gpx. The availability of a well-defined binding site for one of the thiol donors is not sufficient in and of itself. Additional factors are needed to ensure an efficient reaction of the selenium prosthetic group with the oxidant. Elucidation of the structure–activity relationships in this simple system might ultimately help to improve our understanding of natural peroxidases.

Experimental Section

Materials: All chemicals were purchased from Sigma–Aldrich, Fluka, or Acros. Oligonucleotides were custom-synthesized and purified by Microsynth (Balgach, Switzerland). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Glutathione reductase (GR) and glutathione peroxidase from bovine erythrocytes (Gpx) were obtained from Sigma–Aldrich. *E. coli* strain BL21(DE3) cysE51[30] was provided by Professor A. Böck (Institute of Genetics and Microbiology, University of Munich, Germany).

Plasmids: Plasmid pRK793 for the production of S219P TEV protease[69] was obtained from Dr. D. S. Waugh (National Cancer Institute at Frederick, Maryland, USA). Plasmid pETGrxC14S, which encodes *E. coli* C14S Grx1 was constructed by Eric Peterson[70] from plasmid pAHOB1,[71] which was provided by Professor A. Holmgren (Medical Nobel Institute for Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institute, Sweden). Plasmid pMG211-NcoI-41–2–2 was provided by Adrian Hugenmatter and is a derivative of pMG211.[72] It contained a modified polylinker that was inserted into the XbaI-SpeI sites of pMG211, which encoded

an N-terminal $(His)_{10}$ tag that was fused in frame with the *folA* gene: 5′-TCTAGAAATAAT-TTTGTTTAACTTTAAGAAGGAGATATACCA-TGGCCCATCATCATCATCATCATCATCATCATGCATATG-*folA*-3′ (the XbaI and NdeI restriction sites used for cloning are underlined).

General methods: RP-HPLC was performed on a Waters HPLC system that was equipped with a UV detector. For analytical runs, a C_8 column (Macherey–Nagel; 250 mm × 4.6 mm × 300 Å, 5 μ) at a flow rate of 1 mL min^{-1}, or a Waters Atlantis (Dublin, Ireland) dC_{18}-3 (3×100 mm column) at a flow rate of 1.2 mL min^{-1}, were used. Peptides were eluted with linear gradients of solvents A and B (A = 0.05% TFA in acetonitrile, B = 0.1% TFA in H_2O). Preparative RP-HPLC separations were performed by using linear gradients of A and B on a C_8 column (Macherey–Nagel 250 mm×21 mm× 300 Å, 7 μ) or on a C_{18} column (Macherey–Nagel 250 mm× 21 mm×100 Å, 7 μ) at a flow rate of 10 mL min^{-1}. Protein production was carried out in *E. coli* strain KA13,[73] which is a derivative of KA12 that carried the DE3 phage in its chromosome;[74] this allowed IPTG-inducible expression of genes under the control of the T7 promoter. All nucleic acid manipulations were carried out according to standard procedures.[75] Polymerase chain reactions (PCRs) were performed by using Taq polymerase (Qiagen). All PCR-amplified portions of the constructed plasmids were confirmed by DNA sequencing on an Applied Biosystems PRISM 310 Automated DNA Sequencer by using the Terminator Ready Reaction Mix (BigDyeTM, PE Applied Biosystems) for chain termination chemistry.[76] DNA was prepared for sequencing by using a QIAGEN Mini-prep kit. Protein concentrations were determined by the Coomassie plus assay (Better Bradford Assay, Pierce).

C14S Grx1: BL21(DE3) cysE51 cells were transformed with plasmid pETGrxC14S, and the C14S variant of Grx1 was produced at 37 °C in LB medium that contained 150 μg mL^{-1} ampicillin and 30 μg mL^{-1} kanamycin (LB/Amp^{150}/Kan^{30}). An overnight culture (5 mL) was used to inoculate LB/Amp^{150}/Kan^{30} (1.25 L). After vigorous shaking for 4 h an OD of 0.8 was reached, and protein synthesis was induced with IPTG (1 mM). Cells were harvested by centrifugation 6 h after induction. Cell pellets were suspended in ice-cold cell lysis buffer (50 mL; 50 mM Tris pH 8, 1 mM EDTA, 1 mM PEFA-Block) and then sonicated. After removal of cell debris by centrifugation, a 7% streptomycin sulfate solution (5 mL) was added to the clarified lysate over 10 min. After incubation for 20 min at 4 °C, the suspension was centrifuged and the supernatant was extensively dialyzed against low-salt buffer (buffer A: 50 mM Tris, 1 mM EDTA, pH 8). The Grx1 variant was purified by ion-exchange chromatography (DEAE cellulose, SIGMA, 70 mL column volume) by eluting with a linear salt gradient from 0 to 250 mM NaCl in buffer A. The fractions that contained the desired protein were pooled, concentrated to ca. 25 mL (Centriprep Ultracel YM-3 with a 3 kDa cutoff), and purified by size exclusion on a Superdex 75 HiLoad (26/60) Prep Grade FPLC column (Amersham Pharmacia, Uppsala, Sweden) with buffer A that contained 100 mM NaCl. The overall yield of C14S Grx1 was ca. 100 mg/L culture. The purified protein was oxidized to a mixed disulfide with glutathione as previously described.[27] (Analytical RP-HPLC (C_8, 5 to 60% A in B over 45 min): t_R = 34.4 min. MALDI-TOF: *m/z* calcd for $C_{439}H_{678}N_{119}O_{141}S_3$: 9975.0 $[M+H]^+$, found 9973.0 ± 1.

***Nα-tert*-Butoxycarbonyl-Se-*p*-methoxybenzyl-L-selenocysteine (Boc-Sec(Mob)-OH)**. *p*-Methoxy- benzyl-L-selenocysteine (H-Sec-(Mob)-OH)[77] was converted to the Boc derivative as previously described.[78] Briefly, *tert*-butyl pyrocarbonate (1.44 g, 6.6 mmol) was added to a solution of H-Sec(Mob)-OH (1.96 g, 6.0 mmol) in 1 N NaOH (7.3 mL), H_2O (2 mL) and dioxane (1.5 mL) at 4 °C. After stirring for 15 min the ice bath was removed, and stirring was continued for an additional hour. The mixture was diluted with H_2O (10 mL), washed with excess Et_2O (70 mL) and then hexane (30 mL). EtOAc (40 mL) was added, and the pH of the aqueous layer was adjusted to pH 2–3 with 1 N HCl. The EtOAc layer was separated and washed with H_2O (30 mL) and brine (20 mL). After drying ($MgSO_4$), the solvent was removed in vacuo to give Boc-Sec-(Mob)-OH (1.51 g, 3.9 mmol, 65%) as a colorless oil. $[\alpha]_D = -3°$ (c = 1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.45 (s, 9H), 2.93 (d, J = 4.7 Hz, 2H), 3.71 (s, 2H), 3.78 (s, 3H), 4.60 (m, 1H), 5.30 (d, J = 7.5 Hz, 1H; NH), 6.83 (d, J = 8.7 Hz, 2H), 7.18 ppm (d, J = 8.7 Hz, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 25.55, 27.54, 28.37, 53.27, 55.23, 66.91, 80.41, 113.90, 129.88, 130.52, 155.29, 158.27, 174.84 ppm; HRMS (MALDI): *m/z* calcd for $C_{16}H_{23}NNaO_5Se$: 412.0639 $[M+Na]^+$, found 412.0630.

***Nα-tert*-Butoxycarbonyl-Se-*p*-methoxybenzyl-L-selenocysteine pentafluorophenyl ester (Boc-Sec(Mob)-OPfp)**: Boc-Sec(Mob)-OH was converted to a pentafluorophenyl ester as described.[79] Briefly, Boc-Sec(Mob)-OH (1.8 g, 3.7 mmol) was dissolved in DMF (40 mL, 0.09 M) under a N_2 atmosphere. The mixture was cooled to 0 °C, and pentafluorophenol (0.607 g, 3.3 mmol) was added. After complete solubilization, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC; 0.633 g, 3.3 mmol) was added and the reaction was warmed to room temperature and stirred overnight. The solution was then concentrated to a fifth of its initial volume, and extracted with EtOAc (150 mL). The organic layer was washed with H_2O (3×90 mL), 1 N HCl (2×90 mL), H_2O (1×90 mL), sat. $NaHCO_3$ (2×90 mL), brine (1×90 mL), and finally dried ($MgSO_4$). After concentration in vacuo the Boc-Sec(Mob)-OPfp solidified. A portion of the product was purified by preparative RP-HPLC (C_{18}, 30 to 95% A in B over 110 min). mp 106.6–107.2 °C; $[\alpha]_D = -30°$ (c = 1, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$): δ = 1.47 (s, 9H), 2.99 (d, J = 5.6 Hz, 2H), 3.79 (s, 3H), 3.84 (s, 2H), 4.86 (m, 1H), 5.25 (d, J = 7.8 Hz, 1H; NH), 6.85 (d, J = 8.7 Hz, 2H), 7.21 ppm (8.7 Hz); ^{13}C NMR (75 MHz, $CDCl_3$): δ = 24.86, 27.89, 28.35, 53.57, 55.31, 80.82, 114.00, 124.62, 129.90, 137.72 (d, J = 239.2 Hz, CF), 139.63 (d, J = 252.0 Hz, CF), 140.99 (d, J = 251.2 Hz, CF), 154.80, 158.57, 167.54 ppm. HRMS (ESI): *m/z* calcd for $C_{22}H_{22}F_5NNaO_5Se$: 578.0476 $[M+Na]^+$; found 578.0480 (based on the sixth isotopic peak on a full width at half maximum resolution of 30 000).

Solid-phase peptide synthesis: Peptides were synthesized in a stepwise fashion on an ABI 433 A peptide synthesizer. Modified versions of the standard HBTU/HOBt activation protocols for Fmoc chemistry (FastMoc® protocol, Applied Biosystems)[80] were used. Amino acid side chains were protected as follows: Arg(Pbf), Asp-(O*t*Bu), Gln(Trt), Lys(Trt), Sec(Mob), Ser(O*t*Bu), Thr(O*t*Bu), and Tyr-(O*t*Bu). After drying under high vacuum, the resin was stored at −20 °C until the peptides were cleaved. Following cleavage from the support, peptides were precipitated with cold Et_2O. After centrifugation at 4000 *g* for 20 min at 4 °C, the Et_2O was decanted and the trituration procedure repeated twice.

H-Grx1(1–10)-SEt 1: Peptide synthesis was performed on a PAM resin preloaded with glycine (obtained by treating commercial Boc-Gly-PAM resin with TFA) on a 0.25 mmol scale. The desired thioester was generated by the procedure of Swinnen et al.[43] by using resin (224 mg, 0.08 mmol). Protecting groups were removed with TFA/H_2O/EtSH/PhOH/thioanisole-/triisopropylsilane (9 mL; 88.5:4:4:2:1:0.5) for 2 h. The crude thioester was purified by preparative RP-HPLC (C_{18}, 20 to 50% A in B over 50 min) to give compound **1** (45 mg, 41% yield based on initial resin loading). Analytical RP-HPLC (C_{18}, 5 to 60% A in B over 45 min): t_R = 31.3 min. HRMS (ESI): *m/z* calcd for $C_{49}H_{82}N_{14}O_{13}S_2$: 1138.563 $[M]^+$, found: 1138.564.

C11U(Mob)/C14S H-Grx1(11–21)-SAr 2: Peptide synthesis was carried out on a 0.25-mmol scale on 2-Cl-trityl resin that was preloaded with alanine (Fluka). Boc-Sec(Mob)-OPfp was coupled by using the HOBt-catalyzed, base-free coupling procedure.[34] The thioester was prepared by the procedure of von Eggelkraut-Gottanka[44] by using resin (0.25 mmol), and subsequently deprotected with TFA/EDT/H_2O/TIPS (10 mL; 94:2.5:2.5:1) for 2 h at room temperature. Purification by preparative RP-HPLC (C_{18} column, eluting with a linear gradient of 5 to 50% A in B over 110 min) afforded compound **2** (90 mg, 20% yield based on initial resin loading). Analytical RP-HPLC (C_{18}, eluting with a linear gradient of 5 to 60% A in B over 25 min): $t_R = 15.3$ min. HRMS (ESI): calcd for $C_{69}H_{102}N_{16}O_{17}SSe$: 1538.651 $[M+H]^+$, found: 1538.650 (based on the sixth isotopic peak on a full width at half maximum resolution of 30,000).

Construction of plasmid pMG211-Grx1(22–85): The *Grx1* gene was amplified from plasmid pAHOB1[71] by using primers GRX1(22–85) (5′-GGAATTCCATATGTACGATATCCCAACGACCGAAAACCTGTATTTTCAG**TGC**AAATTGAGCAATGAACGCGATGAT) and reverse complement (5′-GGACTAGTTTATTAGGCGTCCAGATTTTCTTTCAC). The codon that corresponds to the E22C mutation is boldfaced, and the TEV cleavage site is underlined. The 255 bp PCR product was digested with NdeI-SpeI, and the 239 bp fragment was ligated to the 4565 bp NdeI-SpeI fragment of pMG211-NcoI-41–2–2, to give pMG211-Grx-22–85 (4804 bp). The construct was verified by DNA sequencing by using T7fw (TAATACGACTCACTATAGGG) and T7rw (TTACCACTCCCTATCAGTGA) as sense and antisense primers, respectively.

E22C Grx1(22–85) 3: Glycerol stocks of *E. coli* strain KA13 that had been transformed with pMG211-Grx(22–85) were plated on LB/Amp^{150} agar. A single colony that was obtained after 18 h at 37 °C was used to inoculate 1 L of LB/Amp^{150}. The culture was grown at 30 °C. When an OD_{600} of ≈0.6 was reached (after ca. 18 h), protein production was induced with 0.1 M IPTG to a final concentration of 0.5 mM. Cells were incubated for an additional 20 h at 30 °C and then harvested by centrifugation. The supernatant was discarded and the pellets were frozen at −80 °C prior to purification. Cell pellets (ca. 3 g) that were collected from 1 L of culture were resuspended in buffer B (20 mL; 20 mM Tris–HCl, 0.5 M NaCl, 8 M urea, 10 mM β-mercaptoethanol, 10 mM imidazole, pH 7.9) and sonicated. Cell debris was removed by centrifugation, and the supernatant was incubated for 1 h at room temperature with Ni-NTA beads that had been equilibrated with buffer B. The beads were poured into a short column and washed with ten column volumes of buffer C (20 mM Tris-HC1, 150 mM NaCl, 1 mM β-mercaptoethanol, pH 7.9) that contained 20 mM imidazole, followed by 10 column volumes of the same buffer with 40 mM imidazole. The desired protein fragment was eluted with buffer C that contained 250 mM imidazole. The appropriate fractions were pooled and dialyzed against 20 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 2 mM DTT, pH 8 at 4 °C. Yield: 6–8 mg/L culture; ESI-MS: *m/z* calcd for $C_{465}H_{679}N_{133}O_{139}S_2$: 10420.3 $[M+H]^+$; found: 10421.0 ± 6.

The sample was concentrated to ca. 1–2 mg mL^{-1} and digested with freshly produced S219P Tev protease.[69] Protease (ca. 3 mg in 1.4 mL of 25 mM phosphate buffer at pH 8, 10% glycerol, 200 mM NaCl, 2 mM EDTA and 10 mM DTT) was added to a 3.5 MWCO dialysis bag that contained the His-tagged E22C Grx1(22–85) fragment (ca. 60 mg, 5.7 μmol, 20 mL) in 20 mM Tris, 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT at pH 8.0 (2 L). After 2 h at room temperature, 50–70% of the recombinant protein was cleaved to give E22C Grx1-(22–85), as judged by SDS-PAGE and LC–MS. After 30 min dialysis against 20 mM Tris–HCl, 150 mM NaCl, pH 7.9 (2 L), the protease and His-tagged contaminants were removed by incubating the solution with Ni-NTA beads (4 mL) that had been equilibrated with the same buffer. The peptide that did not bind to the beads was collected, concentrated to 3 mL by ultracentrifugation (Centriprep Ultracel YM-3 with a 3 kDa cutoff), and purified by RP-HPLC on a C_8 column by eluting with a linear gradient of 5–50% A in B over 110 min. Typically, 1–3 mg of E22C Grx1(22–85) was obtained from 1 L of culture. Analytical RP-HPLC (C_8 column, 5 to 60% A in B over 45 min): $t_R = 34.2$ min. ESI-MS: *m/z* calcd for $C_{327}H_{499}N_{87}O_{105}S$: 7361.1 $[M+H]^+$, found 7361.0 ± 5.

C11U(Mob)/C14S/E22C Grx1(11–85): C11U(Mob)/C14S Grx1(12–21)-SAr (**2**) (2.1 μmol, 1.1 equiv) and E22C Grx1 (22–85) (**3**) (1.9 μmol, 1.0 equiv) were ligated[45] in degassed 100 mM phosphate buffer, pH 7.5 (1.9 mL) that contained 6 M GdmCl and 5% PhSH (95 μL) under N_2. After 16 h, the reaction was complete as judged by LC–MS and RP-HPLC. The peptide was precipitated by the addition of ice cold 25% aq. TFA (1.5 mL). Thiophenol was extracted with Et_2O, and the solution was allowed to stand on ice for an additional hour to complete precipitation. The peptide was collected by centrifugation and washed with ice-cold EtOH to remove residual TFA. Analytical RP-HPLC (C_8, 5 to 60% A in B over 45 min): $t_R = 35.1$ min. ESI-MS: *m/z* calcd for $C_{388}H_{592}N_{102}O_{121}SSe$: 8732.5 $[M+H]^+$, found 8732.0 ± 5.

C11U(Mob)/C14S/E22CmC Grx1(11–85): The procedure for cysteine alkylation was adapted from ref. [81]. Crude C11U(Mob)/C14S/E22C Grx1 (11–85) (22 mg, 2.2 μmol) was dissolved under N_2 in degassed buffer (500 mM Tris, 2 mM EDTA, 6 M GdmCl, 21 mM DTT, pH 8.5; 2.6 mL). Iodoacetic acid was added (0.67 mL of a 75 mM solution, which was prepared in the dark by dissolving iodoacetic acid (14 mg, 75 μmol) in degassed ultrapure water (1 mL)), and the mixture was allowed to stir in the dark for 1 h. The reaction, which was judged to be complete by LC–MS, was quenched with 25% aq AcOH (1.5 mL). The product (9 mg, 47% yield based on **3**) was purified by preparative RP-HPLC (C_8, 5 to 50% A in B over 110 min); it eluted with a retention time of approx 89 min. Analytical RP-HPLC (C_8, 5 to 60% A in B over 45 min): $t_R = 34.6$ min. ESI-MS: *m/z* calcd for $C_{390}H_{594}N_{102}O_{123}SSe$: 8790.5 $[M+H]^+$, found 8791.0 ± 5.

[C11U/C14S/E22CmC Grx1(11–85)]$_2$: Removal of the Mob protecting group was achieved by a literature procedure.[77] C11U(Mob)/C14S/E22CmC Grx1 (11–85) (10 mg, 0.98 μmol) was dissolved in a premixed solution (0.52 mL) that contained 82% TFA, thioanisole (590 μmol), and *m*-cresol (245 μmol) under a N_2 atmosphere. The mixture was cooled to 0 °C in an ice bath, and trimethylsilyl bromide (TMS-Br, 90 μL, 680 μmol, ca. 1 M) was added. After stirring at 0 °C for ca. 30 min, an orange precipitate appeared.[82] After an additional hour the solvent was evaporated, and the residual peptide was resuspended in H_2O, extracted with Et_2O, and isolated by lyophilization. The diselenide dimer of the deprotected C11U/C14S/E22CmC Grx1(11–85) fragment was the main component of the crude mixture (65%), as judged by LC–MS. ESI-MS: *m/z* calcd for $C_{764}H_{1170}N_{204}O_{244}S_2Se_2$: 17338.8 $[2M+H]^+$, found 17340.0 ± 8.

[C11U/C14S/E22CmC Grx1(1–85)]$_2$ (4): H-Grx1(1–10)-SEt (**1**; 2.2 mg, 1.5 μmol, 1.5 equiv) and [C11U C14S E22CmC Grx1 (11–85)]$_2$ (0.98 μmol, 1.0 equiv) were ligated under N_2 in degassed 100 mM phosphate buffer, pH 7.5 (0.98 mL) that contained 6 M GdmCl and 5% PhSH (49 μL). Because the starting diselenide was still present after 15 h, additional **1** (0.5 mg, 0.34 μmol) was added and allowed to react for 2 more hours. The product was precipitated by addition of ice-cold 25% aq. TFA (2.5 mL). Thiophenol was extracted with Et_2O. The solution was cooled on ice for 1 h to complete precipitation. The target peptide was isolated by centrifuga-

tion and washed with ice-cold EtOH to remove residual TFA. The main product that was detected by HPLC and LC–MS (70%) was the mixed selenosulfide between *C11U/C14S/E22CmC Grx1 (1–85)* and thiophenol (**4b**): ESI-MS: *m/z* calcd for $C_{435}H_{667}N_{116}O_{135}S_3Se$: 9857.8 $[M+H]^+$, found 9855.0±5. Minor amounts of the diselenide dimer (**4c**; 30%) were also observed: ESI-MS: *m/z* calcd for $C_{858}H_{1320}N_{232}O_{270}S_4Se_2$: 19490.3 $[2M+H]^+$, found 19493.0±9.

Preparation of the mixed selenosulfide of C11U/C14S/E22CmC Grx1(1–85) and glutathione: Peptides **4b** and **4c** (0.9 μmol) were dissolved in degassed 100 mM phosphate buffer, pH 7.5 (0.9 mL) that contained 6 M GdmCl and 40 mM DTT. After 30 min, oxidized glutathione (155 mg, 250 μmol) was added to oxidize the free selenol. When judged to be complete by LC–MS, the reaction was quenched with 25% aq. AcOH (0.5 mL). The desired adduct (3 mg, 14% yield based on E22C Grx1(22–85)) was purified by preparative RP-HPLC (C_8, 5 to 50% A in B over 110 min), and eluted with a retention time of approximately 86 min. Analytical RP-HPLC (C_8, 5 to 60% A in B over 45 min): t_R=32.5 min; MALDI-MS: *m/z* calcd for $C_{439}H_{678}N_{119}O_{141}S_3Se$: 10054.0 $[M+H]^+$, found 10054.4±5. The enzyme was folded by dialyzing a 1 mM solution in Tris (50 mM; pH 8.0), that contained EDTA (1 mM) and GdmCl (3.5 M), against Tris–HCl (50 mM; pH 8.0), which contained EDTA (1 mM), as previously described,[46] or by directly dissolving the lyophilized sample in phosphate buffer (50 mM; pH 7.0) that contained NaCl (100 mM) and EDTA (1 mM) to a concentration of 0.09 mM immediately prior to characterization. The dilute protein solutions were subsequently concentrated by ultrafiltration (Centriprep Ultracel YM-3 with a 3 kDa cutoff). Folding yields were determined by Bradford assay[83] and by CD spectroscopy.

Circular dichroism spectroscopy: CD spectra were recorded by using an Aviv Model 202 spectropolarimeter. Measurements were performed with 10–20 μM of the desired protein in 50 mM phosphate buffer (pH 7.0) that contained 100 mM NaCl, 1 mM EDTA at 25 °C. Spectra were recorded ten times in 1 nm steps with a 1 s averaging time. They were corrected for the corresponding solvent background, and normalized for protein concentration and number of residues.

Peroxidase activity: Reduction of *tert*-butylhydroperoxide by glutathione was measured according to the method of Wilson et al.[84] in 50 mM phosphate buffer that contained 100 mM NaCl, 1 mM EDTA, 210 μM NADPH, and 5 U mL^{-1} of glutathione reductase, at pH 7 and 25 °C. Reaction was initiated by the addition of 100 μM *t*BuOOH to 0.01–21 mM glutathione in the presence of either 30 μM selenoglutaredoxin, 100 μM C14S Grx1 or 1.5–6 nM Gpx. The disappearance of NADPH was monitored spectrophotometrically at 340 nm ($\Delta\varepsilon$=6220 M^{-1}cm^{-1}). Initial rates were corrected for the background reaction in the absence of protein. Experiments with DL-dihydrolipoic acid as an alternative reductant were performed analogously by directly monitoring the generation of DL-lipoic acid at 330 nm ($\Delta\varepsilon$=120 M^{-1}cm^{-1}).

Glutathione disulfide oxidoreductase activity: Thiol–disulfide exchange was assayed according to a procedure that was adapted from Holmgren et al.[48,49] Briefly, 0.1% BSA, 0.4 mM NADPH and 50 nM GR were mixed with the glutathione adducts of C14S Grx1 or selenoglutaredoxin (10 to 100 nM) in 0.1 mM Tris buffer (pH 8.0) that contained 2 mM EDTA at pH 8 and 28 °C. After incubating this mixture for 5 min, reactions were initiated by the addition of a mixture of GSH and β-hydroxyethylene disulfide (HED), which had also been preincubated for 5 min, to a final concentration of 1 mM and 0.7 mM, respectively. The disappearance of NADPH was monitored spectrophotometrically at 340 nm ($\Delta\varepsilon$=6220 M^{-1}cm^{-1}). The initial rates were corrected for the spontaneous background reaction. Specific activity in this system is defined as the consumption of 1 μmol of NADPH per minute (=1 unit) per mg enzyme.

Glutathione reductase activity: Glutathione reductase was assayed with the glutathione adducts of selenoglutaredoxin and C14S Grx1 according to the method of Carlberg and Mannervik.[85] Glutathione reductase (2 nM) was added to a solution of the protein disulfides (or selenosulfide) (50 μM to 2 mM C14S Grx1 or 50 μM to 100 μM selenoglutaredoxin) and 100 μM NADPH in 50 mM phosphate buffer, 100 mM NaCl, 1 mM EDTA, pH 7.0 and 25 °C, that contained 0.1% BSA. Initial velocities were determined by monitoring the absorbance change at 340 nm and corrected for background.

Abbreviations: DTT, dithiothreitol; Gpx, glutathione peroxidase; GSH, glutathione; GST, glutathione-S-transferase; GR, glutathione reductase; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotrizole; IPTG, isopropyl-1-thio-β-D-galactopyranoside; NADPH, β-nicotinamide adenine dinucleotide phosphate; PhSH, thiophenol; Sec, selenocysteine; TFA, trifluoroacetic acid; TIPS, triisopropylsilane.

Acknowledgements

We thank the ETH Zürich for financial support, Dr. K. Woycechowsky for helpful discussions, and the reviewers for valuable suggestions. G.C. is grateful to the Roche Research Foundation for a doctoral fellowship and G.R. gratefully acknowledges a postdoctoral fellowship from the Netherlands Organisation for Scientific Research (NWO).

Keywords: glutaredoxin · native chemical ligation · redox chemistry · selenium · selenoenzymes

[1] J. W. Forstrom, J. J. Zakowski, A. L. Tappel, *Biochemistry* **1978**, *17*, 2639.
[2] F. Ursini, M. Maiorino, R. Brigelius-Flohé, K. D. Aumann, A. Roveri, D. Schomburg, L. Flohé, *Methods Enzymol.* **1995**, *252*, 38.
[3] L. V. Papp, J. Lu, A. Holmgren, K. K. Khanna, *Antioxid. Redox Signaling* **2007**, *9*, 775.
[4] B. Ren, W. Huang, B. Åkesson, R. Ladenstein, *J. Mol. Biol.* **1997**, *268*, 869.
[5] O. Epp, R. Ladenstein, A. Wendel, *Eur. J. Biochem.* **1983**, *133*, 51.
[6] J. L. Martin, *Structure* **1995**, *3*, 245.
[7] H. E. Ganther, R. J. Kraus, *Methods Enzymol.* **1984**, *107*, 593.
[8] A. Wendel in *Enzymatic Basis of Detoxication, Vol. 1* (Ed.: W. B. Jakoby), Academic Press, New York, **1980**, pp. 333.
[9] Z. P. Wu, D. Hilvert, *J. Am. Chem. Soc.* **1989**, *111*, 4513.
[10] Z. P. Wu, D. Hilvert, *J. Am. Chem. Soc.* **1990**, *112*, 5647.
[11] I. M. Bell, D. Hilvert, *Biochemistry* **1993**, *32*, 13969.
[12] I. M. Bell, M. L. Fisher, Z. P. Wu, D. Hilvert, *Biochemistry* **1993**, *32*, 3754.
[13] E. B. Peterson, D. Hilvert, *Biochemistry* **1995**, *34*, 6616.
[14] E. B. Peterson, D. Hilvert, *Tetrahedron* **1997**, *53*, 12311.
[15] K. L. House, R. B. Dunlap, J. D. Odom, Z. P. Wu, D. Hilvert, *J. Am. Chem. Soc.* **1992**, *114*, 8573.
[16] K. L. House, A. R. Garber, R. B. Dunlap, J. D. Odom, D. Hilvert, *Biochemistry* **1993**, *32*, 3468.
[17] R. Syed, Z. P. Wu, J. M. Hogle, D. Hilvert, *Biochemistry* **1993**, *32*, 6157.
[18] D. Häring, E. Schüler, W. Adam, C. R. Saha-Möller, P. Schreier, *J. Org. Chem.* **1999**, *64*, 832.
[19] L. Flohé, G. Loschen, W. A. Günzler, E. Eichele, *Hoppe-Seyler's Z. Physiol. Chem.* **1972**, *353*, 987.
[20] J. Q. Liu, M. S. Jiang, G. M. Luo, G. L. Yan, J. C. Shen, *Biotechnol. Lett.* **1998**, *20*, 693.
[21] S. Boschi-Muller, S. Muller, A. Van Dorsselaer, A. Böck, G. Branlant, *FEBS Lett.* **1998**, *439*, 241.

[22] H. J. Yu, J. Q. Liu, A. Böck, J. Li, G. M. Luo, J. C. Shen, *J. Biol. Chem.* **2005**, *280*, 11930.
[23] L. Ding, Z. Liu, Z. Q. Zhu, G. M. Luo, D. Q. Zhao, J. Z. Ni, *Biochem. J.* **1998**, *332*, 251.
[24] A. Holmgren, *J. Biol. Chem.* **1989**, *264*, 13963.
[25] P. Sodano, T. H. Xia, J. H. Bushweller, O. Björnberg, A. Holmgren, M. Billeter, K. Wüthrich, *J. Mol. Biol.* **1991**, *221*, 1311.
[26] F. K. Gleason, A. Holmgren, *FEMS Microbiol. Rev.* **1988**, *54*, 271.
[27] J. H. Bushweller, F. Åslund, K. Wüthrich, A. Holmgren, *Biochemistry* **1992**, *31*, 9288.
[28] J. H. Bushweller, M. Billeter, A. Holmgren, K. Wüthrich, *J. Mol. Biol.* **1994**, *235*, 1585.
[29] E. S. J. Arnér, H. Sarioglu, F. Lottspeich, A. Holmgren, A. Böck, *J. Mol. Biol.* **1999**, *292*, 1003.
[30] S. Müller, H. Senn, B. Gsell, W. Vetter, C. Baron, A. Böck, *Biochemistry* **1994**, *33*, 3404.
[31] S. Hazebrouck, L. Camoin, Z. Faltin, A. D. Strosberg, Y. Eshdat, *J. Biol. Chem.* **2000**, *275*, 28715.
[32] R. J. Hondal, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2001**, *123*, 5140.
[33] R. Quaderer, A. Sewing, D. Hilvert, *Helv. Chim. Acta* **2001**, *84*, 1197.
[34] D. Besse, L. Moroder, *J. Pept. Sci.* **1997**, *3*, 442.
[35] M. Ralle, S. M. Berry, M. J. Nilges, M. D. Gieselman, W. A. van der Donk, Y. Lu, N. J. Blackburn, *J. Am. Chem. Soc.* **2004**, *126*, 7244.
[36] M. D. Gieselman, L. Xie, W. A. van der Donk, *Org. Lett.* **2001**, *3*, 1331.
[37] N. Metanis, E. Keinan, P. E. Dawson, *J. Am. Chem. Soc.* **2006**, *128*, 16684.
[38] S. Pegoraro, S. Fiori, S. Rudolph-Böhner, T. X. Watanabe, L. Moroder, *J. Mol. Biol.* **1998**, *284*, 779.
[39] B. Eckenroth, K. Harris, A. A. Turanov, V. N. Gladyshev, R. T. Raines, R. J. Hondal, *Biochemistry* **2006**, *45*, 5158.
[40] J. Beld, K. J. Woycechowsky, D. Hilvert, *Biochemistry* **2007**, *46*, 5382.
[41] S. Mathys, T. C. Evans, Jr, I. C. Chute, H. Wu, S. R. Chong, J. Benner, X. Q. Liu, M. Q. Xu, *Gene* **1999**, *231*, 1.
[42] G. G. Kochendoerfer, S. Y. Chen, F. Mao, S. Cressman, S. Traviglia, H. Shao, C. L. Hunter, D. W. Low, E. N. Cagle, M. Carnevali, V. Gueriguian, P. J. Keogh, H. Porter, S. M. Stratton, M. C. Wiedeke, J. Wilken, J. Tang, J. J. Levy, L. P. Miranda, M. M. Crnogorac, S. Kalbag, P. Botti, J. Schindler-Horvat, L. Savatski, J. W. Adamson, A. Kung, S. B. H. Kent, J. A. Bradburne, *Science* **2003**, *299*, 884.
[43] D. Swinnen, D. Hilvert, *Org. Lett.* **2000**, *2*, 2439.
[44] R. von Eggelkraut-Gottanka, A. Klose, A. G. Beck-Sickinger, M. Beyermann, *Tetrahedron Lett.* **2003**, *44*, 3551.
[45] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776.
[46] V. A. Sandberg, B. Kren, J. A. Fuchs, C. Woodward, *Biochemistry* **1991**, *30*, 5475.
[47] A. Wendel, *Methods Enzymol.* **1981**, *77*, 325.
[48] A. Holmgren, *J. Biol. Chem.* **1979**, *254*, 3664.
[49] A. Holmgren, F. Åslund, *Methods Enzymol.* **1995**, *252*, 283.
[50] G. Mugesh, W. W. du Mont, H. Sies, *Chem. Rev.* **2001**, *101*, 2125.
[51] G. Mugesh, H. B. Singh, *Chem. Soc. Rev.* **2000**, *29*, 347.
[52] H. Sies, H. Masumoto, *Adv. Pharmacol.* **1997**, *38*, 229.
[53] Z. H. Jiang, E. S. J. Arnér, Y. Mu, L. Johansson, J. M. Shi, S. Q. Zhao, S. J. Liu, R. Y. Wang, T. Z. Zhang, G. L. Yan, J. Q. Liu, J. C. Shen, G. M. Luo, *Biochem. Biophys. Res. Commun.* **2004**, *321*, 94.
[54] G. Casi, D. Hilvert, *J. Biol. Chem.* **2007**, *282*, 30518.
[55] H.-Y. Kim, V. N. Gladyshev, *PLoS Biol.* **2005**, *3*, 2080.
[56] L. W. Zhong, A. Holmgren, *J. Biol. Chem.* **2000**, *275*, 18121.
[57] L. W. Zhong, E. S. J. Arnér, A. Holmgren, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 5854.
[58] H. Sztajer, B. Gamain, K. D. Aumann, C. Slomianny, K. Becker, R. Brigelius-Flohé, L. Flohé, *J. Biol. Chem.* **2001**, *276*, 7397.
[59] S. R. Lee, S. Bar-Noy, J. Kwon, R. L. Levine, T. C. Stadtman, S. G. Rhee, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 2521.
[60] T. C. Stadtman, *Annu. Rev. Biochem.* **1996**, *65*, 83.
[61] C. Rocher, J. L. Lalanne, J. Chaudière, *Eur. J. Biochem.* **1992**, *205*, 955.
[62] M. Maiorino, K. D. Aumann, R. Brigelius-Flohé, D. Doria, J. Van den Heuvel, J. McCarthy, A. Roveri, F. Ursini, L. Flohé, *Biol. Chem. Hoppe-Seyler* **1995**, *376*, 651.
[63] P. Carter, J. A. Wells, *Nature* **1988**, *332*, 564.
[64] E. Herrero, M. A. de La Torre-Ruiz, *Cell. Mol. Life Sci.* **2007**, *64*, 1518.
[65] P. A. Karplus, E. F. Pai, G. E. Schulz, *Eur. J. Biochem.* **1989**, *178*, 693.
[66] P. A. Karplus, G. E. Schulz, *J. Mol. Biol.* **1987**, *195*, 701.
[67] R. L. Krauth-Siegel, R. Blatterspiel, M. Saleh, E. Schiltz, R. H. Schirmer, R. Untucht-Grau, *Eur. J. Biochem.* **1982**, *121*, 259.
[68] R. Thieme, E. F. Pai, R. H. Schirmer, G. E. Schulz, *J. Mol. Biol.* **1981**, *152*, 763.
[69] R. B. Kapust, J. Tözsér, J. D. Fox, D. E. Anderson, S. Cherry, T. D. Copeland, D. S. Waugh, *Protein Eng.* **2001**, *14*, 993.
[70] E. B. Peterson, PhD Thesis, The Scripps Research Institute (USA), **1998**.
[71] O. Björnberg, A. Holmgren, *Protein Expression Purif.* **1991**, *2*, 287.
[72] S. Sasso, C. Ramakrishnan, M. Gamper, D. Hilvert, P. Kast, *FEBS J.* **2005**, *272*, 375.
[73] P. Kast, C. Grisostomi, I. A. Chen, S. L. Li, U. Krengel, Y. F. Xue, D. Hilvert, *J. Biol. Chem.* **2000**, *275*, 36832.
[74] P. Kast, M. Asif-Ullah, N. Jiang, D. Hilvert, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 5043.
[75] J. Sambrook, E. F. Fritsch, T. Maniatis, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, New York, **1989**.
[76] F. Sanger, S. Nicklen, A. R. Coulson, *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 5463.
[77] T. Koide, H. Itoh, A. Otaka, H. Yasui, M. Kuroda, N. Esaki, K. Soda, N. Fujii, *Chem. Pharm. Bull.* **1993**, *41*, 502.
[78] M. Bodanszky, A. Bodanszky, *The Practice of Peptide Synthesis*, Springer, Berlin, **1994**.
[79] L. Kisfaludy, I. Schön, *Synthesis* **1983**, 325.
[80] R. Knorr, A. Trzeciak, W. Bannwarth, D. Gillessen, *Tetrahedron Lett.* **1989**, *30*, 1927.
[81] A. M. Crestfield, S. Moore, W. H. Stein, *J. Biol. Chem.* **1963**, *238*, 622.
[82] K. M. Harris, S. Flemer, Jr, R. J. Hondal, *J. Pept. Sci.* **2007**, *13*, 81.
[83] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248.
[84] S. R. Wilson, P. A. Zucker, R. R. C. Huang, A. Spector, *J. Am. Chem. Soc.* **1989**, *111*, 5936.
[85] I. Carlberg, B. Mannervik, *Methods Enzymol.* **1985**, *113*, 484.

Received: December 11, 2007
Published online on June 11, 2008

