Engineering Glutathione Peroxidase to a Novel Glutathione Peroxidase Mimic With High Catalytic Efficiency

INCORPORATION OF SELENOCYSTEINE INTO A GLUTATHIONE-BINDING SCAFFOLD USING AN AUXOTROPHIC EXPRESSION SYSTEM*

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Glutathione peroxidase (GPx, EC 1.11.1.9) protects cells against oxidative damage by catalyzing the reduction of hydroperoxides with glutathione (GSH). Several attempts have been made to imitate its function for mechanical study and for its pharmacological development as an antioxidant. By replacing the active site serine 9 with a cysteine and then substituting it with selenocysteine in a cysteine auxotrophic system, catalytically essential residue selenocysteine was bioincorporated into GSH-specific binding scaffold, and thus, glutathione S-transferase (GST, EC 2.5.1.18) from Lucilia cuprina was converted into a selenium-containing enzyme, seleno-LuGST1-1, by genetic engineering. Taking advantage of the important structure similarities between seleno-LuGST1-1 and naturally occurring GPx in the specific GSH binding sites and the geometric conformation for the active selenocysteine in their common GSH binding domain-adopted thioredoxin fold, the as-generated selenoenzyme displayed a significantly high efficiency for catalyzing the reduction of hydrogen peroxide by glutathione, being comparable with those of natural GPxs. The catalytic behaviors of this engineered selenoenzyme were found to be similar to those of naturally occurring GPx. It exhibited pH and temperature-dependent catalytic activity and a typical ping-pong kinetic mechanism. Engineering GST into an efficient GPx-like biocatalyst provided new proof for the previous assumption that both GPx and GST were evolved from a common thioredoxin-like ancestor to accommodate different functions throughout evolution.

Glutathione peroxidase (GPx, EC 1.11.1.9) is a selenoenzyme that functions to catalyze the reduction of hydroperoxides using glutathione (GSH) as a reducing substrate (1). It therefore plays an important role in the organismal antioxidant defense mechanism protecting cells from oxidative stress, which is responsible for many diseases such as reperfusion injury, brain ischemia, tumor, catarract, inflammation, and physiological aging. Extensive investigations of the structure and the catalytic mechanism of GPx reveal that a selenocysteine (Sec) residue is in the enzyme active site and thus participates in GPx activity. In the catalytic cycle, the selenol in the reduced Sec residue is oxidized by hydroperoxide to produce the selenenic acid, which is further converted to the seleneny1 sulfide by the nucleophilic attack of GSH. Reaction of selenenyl sulfide with second equivalent of GSH regenerates the reduced selenol (2).

Because the Sec is encoded by a stop codon UGA, it is difficult to prepare GPx with traditional recombinant DNA technology. To imitate GPx properties, Sec has been incorporated into some natural enzymes by chemical modification or genetic engineering in an auxotrophic expression system (3, 4). The first semisynthetic GPx-like selenoenzyme is selenosubtilisin, which was generated by chemical conversion of the catalytically active residue serine (Ser-221) of subtilisin to Sec. Alternatively, the biosynthetic substitution of the catalytically essential residue cysteine (Cys-149) of phosphorylating glyceraldehyde-3-phosphate dehydrogenase by Sec led to selenoglyceraldehyde-3-phosphate dehydrogenase, which displayed the GPx-like properties. However, the mechanism that these two selenoenzymes applied to catalyze the reduction of hydroperoxide involves use of aryl thiols instead of GSH as reducing substrate due to the lack of a GSH-specific binding site in their active sites. To enhance the GPx activities, the GSH binding site has been successfully introduced into GPx mimics using monoclonal antibody (5) and bioimprinting techniques (6), and the as-synthesized selenoantibody and bioimprinted selenoprotein containing the GSH binding site exhibited high GPx activities. Besides the efficient binding of substrates, however, some other factors, such as the correct geometric conformation and the microenvironment of the active site, are also dominant for the enhancement of enzyme catalysis. Thus, it is challenging to generate novel models that are more suitable for GPx catalysis. Intensive studies of protein structures have revealed that the evolution of proteins for novel functions is largely based on the redesign of existing protein frameworks in nature (7, 8). This principle can be exploited in generation of novel GPx-like biocatalyst by redesigning existing protein scaffolds that are similar to that of natural GPx.

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional proteins capable of detoxifying endogenous and xenobiotic electrophiles by addition of GSH to the electro-
philes (9–11). Owing to their modular features, their stability, ease of purification, and the wealth of accumulated knowledge of their structure–activity relationship, GSTs have been used as ideal candidates to redesign novel binding properties and catalytic functions (9, 12, 13). GSTs are divided into different classes according to the sequence similarity, but all of their structures consist of two domains, a conserved GSH binding site at the N terminus and a hypervariable hydrophobic xenobiotic substrates binding site at the C terminus (9–11). GSTs and GPxs both belong to the thioredoxin superfamily (also including thioredoxin, glutaredoxin, and disulfide-bond formation catalyst) classified by the common glutathione binding domain-adopted thioredoxin fold (9, 14). Their active site residues (Tyr or Ser in GST and Sec in GPx) that interact with the thiol group of the substrate glutathione hold the similar positions in their protein structures (14). Moreover, several GSTs display GPx-like catalytic properties toward organic hydroperoxides, although they do not catalyze the reduction of hydrogen peroxide (15). Accordingly, GSTs appear to offer model protein scaffolds to confer GPx properties by engineering Sec residue into the GSH binding sites. Recently, we have successfully converted the rat θ-class glutathione transferase T2-2 into a selenoenzyme by chemically modifying the active site Ser to Sec (16). This novel selenium-containing enzyme displayed dramatically high GPx activities for catalyzing the reduction of hydrogen peroxide by GSH. However, because the chemical modification is incapable of specifically targeting amino acid residues in the active site, other hydroxyl groups in the protein are inevitably converted into selenols, which would hamper the further structure–function studies of this important selenoenzyme. In view of this, genetic engineering should provide a better and more suitable alternative to incorporate Sec into the defined GST binding site. Here, we report the conversion of Australian sheep blowfly (Lucilia cuprina) glutathione transferase (LuGST1-1) to a selenoenzyme (seleno-LuGST1-1) by means of genetic engineering. The serine 9 in the active site of the LuGST1-1 was mutated to cysteine and then biosynthetically substituted to selenocysteine in an auxotrophic expression system. The as-grown novel selenium-dependent enzyme exhibited high catalytic activity toward the reduction of H₂O₂ by GSH, which is in the same order of magnitude compared with natural GPx. For the first time, a selenium-containing enzyme with such remarkable GPx activity was generated by genetic engineering in bacteria.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media—Strains and plasmids used in this study are listed in Table I. The media employed in cultivation and expression experiments were two types of modifications of M9 minimal medium as described previously (17).

DNA Manipulations—All restriction enzymes and nucleic acid-modifying enzymes were obtained from TAKARA. Plasmid isolation, DNA restriction endonuclease analysis, ligation, and transformation were performed as described previously (18). The Qiagen miniprep DNA purification system and Biotech DNA clean-up system were used to prepare plasmid DNA for restriction enzyme digestion and recover DNA fragment from low melting agarose gels, respectively.

Oligonucleotide-directed Mutagenesis and Construction of Vectors—Mutations in GST-1 were introduced by overlap PCR. Plasmid pLUC1-EX was used as the template to obtain mutants. The mutagenic oligonucleotides used were as follows. The GST S9C was amplified using primers S9CF (5′-GCCCGCATATTGGATTTCTACTACTGCCGGTTGTTATCCACACTCAG-3′) and S9CR (5′-CCGGATCCCTTAAGATGGTTTCAGAGAAGAATTTC-3′). The amplified GST S9C fragment was used as a template for the next PCR reaction. C86S and C200S were introduced using primers as follows: FndI (5′-GCCCGCATATTGGATTTCTACTACT-3′), C86SF (5′-TCCCCAAGTCCCAAGAGG-3′), C86SR (5′-TTTCTTGGGGGACTTGGGGAAC-3′), RCbamHI (5′-CCGGATCCCTTAAGATGGTTTCAGAGAAGAATTTC-3′), and C200SFR (5′-CCGGATCCCTTAAGATGGTTTCAGAGAAGAATTTC-3′). Substitutions that resulted in mutation are underlined. The amplified fragments GST S9C and GST S9C/C86S/C200S were cloned into the NdeI-BamHI sites of pT7-7. The amplified GST S9C fragment was used as a template for the next PCR reaction. C86S and C200S were introduced using primers as follows: FndI (5′-GCCCGCATATTGGATTTCTACTACT-3′), C86SF (5′-TCCCCAAGTCCCAAGAGG-3′), C86SR (5′-TTTCTTGGGGGACTTGGGGAAC-3′), RCbamHI (5′-CCGGATCCCTTAAGATGGTTTCAGAGAAGAATTTC-3′), and C200SFR (5′-CCGGATCCCTTAAGATGGTTTCAGAGAAGAATTTC-3′). Substitutions that resulted in mutation are underlined. The amplified fragments GST S9C and GST S9C/C86S/C200S were cloned into the NdeI-BamHI sites of pT7-7. The amplified GST S9C fragment was used as a template for the next PCR reaction.

Plasmids

| Strain/plasmid | Genotype | Ref. |
|---------------|----------|-----|
| Strains       |          |     |
| TG1           | supE hsdΔ5 thiA Δlac-pro AB F’ (tra D36 pro AB’ lac F’ lac ZAM15) | 18 |
| DH5α          | SupE44 Δlac U169 (ось lac ZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA | 18 |
| BL21(DE3)     | hsdS gal (λcits857 ind1 am7 nin5 lacU5V-T7 gene 1) | 18 |
| BL21 cyS     | BL21(DE3) selB::kan cyS E51 | 17 |
| Plasmids      |          |     |
| pLUC1-EX      | gst1 expression vector, Ap’ | 19 |
| pT7-7         | Cloning and expression vector, Ap’ | 20 |
| pSM3          | Ndel-BamHI fragment containing the gst s9c c86/200s (codons for Ser-9 changed to Cys codon) cloned into the Ndel-BamHI sites of pT7-7 | This study |
| pSM4          | Ndel-BamHI fragment containing the gst s9c (codons for Ser-9 changed to Cys codon) cloned into the This study |

Overexpression and Purification of Wild Type LuGST1-1 and the Mutants GST(S9C) and GST(S9C/C86S/C200S)—Wild type LuGST1-1 was expressed and purified by affinity chromatography on glutathione-Sepharose 4B as described previously (19). Plasmids pSM4 and pSM3 were transferred into BL21(DE3), and the strains were grown in the presence of isopropyl thio-D-galactopyranoside to induce expression of mutant GST(S9C) and GST(S9C/C86S/C200S), respectively (21). With regard to the purification, the two mutants were expressed to the purity of 90% and passed through DE52 ion-exchange columns and then applied to glutathione-Sepharose 4B columns as described previously (22) with a slight modification.

Overexpression and Purification of Seleno-LuGST1-1—Plasmid pSM3 were transferred into strain BL21cyS E51. Overexpression of seleno-LuGST1-1 in the presence of selenocysteine was performed as already described for (Se)₂-thioredoxin (17). And the protein was also purified by affinity chromatography on glutathione-Sepharose 4B as described previously (19).

Determination of Protein Concentration—Protein concentration was determined by a Bio-Rad protein assay using bovine serum albumin as a standard (20).

Electrospray Mass Spectrometry Analysis—All MALDI-TOF mass spectra were acquired on a Voyager DE-STR Biospectrometry worksta (PerSeptive Biosystems, Framingham, MA) using a nitrogen laser (337 nm). The protein samples were purified by high-performance liquid chromatography and prepared using a conventional dried droplet protocol in which sinapinic acid was used as the matrix. The sinapinic acid matrix was prepared as a saturated, aqueous solution that contained 60% acetonitrile and 0.3% trifluoroacetic acid. An aliquot sample of 1 μl was mixed with 20 μl of sinapinic acid matrix before depositing 1.2 μl of the sample matrix mixture on the MALDI sample stage.

Binding Experiments—Binding of GSH to seleno-LuGST1-1 was studied as described previously (24).
Assay of Enzyme Activities—The activities of enzymes were measured using a UV-visible spectrophotometer (Shimadzu UV-3100). The GPx activities of enzymes were measured according to Wilson’s method (32). The reaction was carried out at 37 °C in 500 μl of solution containing 50 mM potassium phosphate buffer, pH 7.0, 0.5 mM GSH, 1 unit of GSH reductase, and 0.5–2.5 μg of enzyme. The mixture was preincubated for 7 min, and 0.25 mM NADPH solution was added. After the mixture was incubated for 3 min at 37 °C, the reaction was initiated by addition of 0.5 mM hydrogen peroxide. The activity was determined by the decrease of NADPH absorption at 340 nm. Background absorption was run without enzyme and was subtracted. The activity unit of enzyme is defined as the amount of enzyme that catalyzes the turnover of 1 μmol of NADPH per min. The specific activity is expressed in μmol min⁻¹ μmol⁻¹ of enzyme.

The GST activities of wild type LuGST1-1 and its various mutants were measured as described by Habig (26). The reaction was carried out at 30 °C in 1 ml of solution containing 100 mM, pH 6.5, sodium phosphate buffer, 1 mM GSH, and 0.5–5 μg of enzyme. After preincubation for 3 min, 1 mM 1-chloro-2,4-dinitrobenzene was added and then the absorbance was recorded at 340 nm for 3 min. Background absorption was run without enzyme and was subtracted. The activity unit of enzyme is defined as the amount of enzyme that catalyzes the turnover of 1 μmol of 1-chloro-2,4-dinitrobenzene per min. The specific activity is expressed in μmol min⁻¹μmol⁻¹ of enzyme.

Determination of Optimal pH and Temperature for Seleno-LuGST1-1 Catalysis—The initial rates were measured using 1 mM GSH and 0.5 mM hydrogen peroxide. The pH value of the buffer was changed from 6.0 to 10.0 to determine the initial rates of the reaction to obtain the optimal pH condition for seleno-LuGST1-1-catalyzed reaction. Similarly, a catalytic reaction was carried out at different temperatures from 10 to 45 °C to determine the optimal temperature for the seleno-LuGST1-1-catalyzed reduction of hydrogen peroxide.

Steady-state Kinetics of Seleno-LuGST1-1—The assay of kinetics of seleno-LuGST1-1 for the reduction of H₂O₂ by GSH was similar to that of selenium-containing catalytic antibody, Se-4A4 (5). The initial rates were measured by observing the decrease of NADPH absorption at 340 nm at several concentrations of one substrate while the concentration of the other substrate was kept constant. All kinetic experiments were performed in a total volume of 0.5 ml containing 50 mM potassium phosphate buffer, 1 mM EDTA, 1 unit of GSH reductase, 0.25 mM NADPH, and varying concentrations of GSH, H₂O₂, and seleno-LuGST1-1. After the enzyme was preincubated with GSH, NADPH, and GSH reductase, the reaction was then initiated by the addition of H₂O₂. Subtraction of the nonenzymatic background absorption gave the ratio of the enzyme-catalyzed reaction.

RESULTS AND DISCUSSION

Production and Isolation of Seleno-LuGST1-1—The method used was based on the assumption that an efficient charging of tRNAcys occurred with selenocysteine when cysteine was omitted (17). Thus, Cys could be substituted efficiently by Sec in a yeast auxotrophic strain when Cys was omitted in the growth medium. In the case of LuGST1-1 from the Australian sheep blowfly (L. cuprina), the active residue Ser-9 was mutated (17). Thus, Cys could be substituted efficiently by Sec in a yeast auxotrophic strain instead of the three mutations (S9C, C86S, and C200S). Seleno-LuGST1-1 was found to be much more efficient than most of other GPx mimics for the reduction of SeO₂⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻
The activities were determined as initial rates and corrected for the spontaneous reaction. All values are the means of at least five determinations, and the standard deviations are shown in parentheses. GPx activities were determined in 50 mM potassium phosphate buffer (pH 7.0) at 37 °C, and GST activities were determined in 100 mM potassium phosphate buffer (pH 6.5) at 30 °C. The concentration of GSH was 1 mM under all conditions investigated. The concentrations of H₂O₂ and CDNB were 0.5 mM and 1 mM, respectively.

### Table II

| Catalysts          | Substrate | Activity \( \mu \text{mol} \cdot \text{min}^{-1} \cdot \mu \text{mol}^{-1} \) |
|--------------------|-----------|----------------------------------------------------------|
| Wild type GST      | H₂O₂      | ND\*                                                      |
|                    | CDNB      | 235.3 (9.1)                                               |
| Seleno-LuGST1-1    | H₂O₂      | 2657 (30)                                                 |
|                    | CDNB      | 23.3 (3.1)                                                |
| GST mutant (SSC)   | H₂O₂      | ND\*                                                      |
|                    | CDNB      | ND\*                                                      |
| GST mutant (S9C)   | H₂O₂      | ND\*                                                      |
| (S9C C86/200S)     | CDNB      | ND\*                                                      |
| Selenosubtilisin   | H₂O₂      | 5406 (6)                                                 |
| Ebselen (27)       | H₂O₂      | 4.6 (0.2)                                                 |
| Native (GPx, rabbit liver) (15) | H₂O₂ | 0.99                                                      |
|                    | CDNB      | 5780                                                      |

* ND, no detectable GPx or GST activity.

\( ^{b} \) Calculated from the data in Ref. 16.

\( ^{c} \) The catalyst was prepared as described in Ref. 3, and the activity was detected in this study.

The effects of catalytic conditions on the GPX activity

![Figure 2](http://www.jbc.org/)

**Figure 2.** Effects of the catalytic conditions on the GPX activity of H₂O₂ by GSH catalyzed by seleno-LuGST1-1. A, relative GPX activity versus pH. B, relative GPX activity versus temperature. The activity was determined when the concentrations of GSH and H₂O₂ were 1 mM and 0.5 mM, respectively. The GPX activity was converted to the relative value, and the activity detected at 37 °C and pH 7.0 was defined to be 100%.

![Figure 3](http://www.jbc.org/)

**Figure 3.** Double-reciprocal plots for the reduction of H₂O₂ by GSH catalyzed by Seleno-LuGST1-1. A, [E]₀/E versus 1/[H₂O₂] (mm⁻¹) at [GSH] = 0.25 mM (■), 0.50 mM (○), and 2.00 mM (▲). B, [E]₀/E versus 1/(GSH) (mm⁻¹) at [H₂O₂] = 0.1 mM (■), 0.2 mM (○), 0.5 mM (▲), and 1.0 mM (▼).

Table III. These values were deduced from fitting the experimental data to a ping-pong kinetic scheme. The relevant steady-state rate equation is shown as Equation 1.

\[
\frac{v_0}{[E_0]} = \frac{K_{\text{H}_2\text{O}_2}[\text{GSH}]}{K_{\text{H}_2\text{O}_2} + K_{\text{GSH}}[\text{H}_2\text{O}_2] + [\text{GSH}][\text{H}_2\text{O}_2]} \quad (\text{Eq. 1})
\]

The first-order rate constant \( k_{\text{cat}}/[\text{H}_2\text{O}_2] \) and the apparent Michaelis constant \( K_{\text{m,H}_2\text{O}_2} \) at 1 mM GSH were determined to be \( 2100 \text{ min}^{-1} \) and \( 2.1 \times 10^{-4} \text{ M} \), respectively. At 0.5 mM H₂O₂, \( K_{\text{m,GSH}} \) was \( 1.7 \times 10^{-4} \text{ M} \). The apparent second-order rate constant \( k_{\text{cat}}/K_{\text{m,H}_2\text{O}_2} \) and \( k_{\text{cat}}/K_{\text{m,GSH}} \) provide measures of the rates of reactions between the free enzyme and the relative substrates (GSH and hydrogen peroxide, respectively). \( k_{\text{cat}}/K_{\text{m,GSH}} \) and \( k_{\text{cat}}/K_{\text{m,H}_2\text{O}_2} \) of seleno-LuGST1-1 were both \( \approx 10^7 \text{ M}^{-1} \text{ min}^{-1} \), i.e., \( k_{\text{cat}}/K_{\text{m,GSH}} \) of seleno-LuGST1-1 was in the same order of magnitude as that of natural GPx (33, 35), indicating...
that they had similar affinities to GSH. Although $k_{cat}/K_{M, GSH}$ of seleno-LuGST1-1 was still one order of magnitude lower than that of natural GPx ($10^6$ M$^{-1}$min$^{-1}$) (35), it was much higher than those of most GPx mimics (for example, $4.5 \times 10^5$ M$^{-1}$min$^{-1}$ for selenium-containing catalytic antibody Se-4A4 (5)). The remarkably high GPx activity of seleno-LuGST1-1 could be ascribed to its special enzyme scaffold. Studies on crystal structures of LuGST1-1 and natural GPx (9, 11, 14) reveal that there are some important structure similarities between these two enzymes. Both of the two enzymes include specific GSH binding sites (14) and thus could both strongly bind to their common specific substrate GSH, although their residues involved in the binding are different. As has been proved by binding experiments, the specific affinity of seleno-LuGST1-1 toward GSH was hardly weakened after the conversion of serine 9 to Sec-9 in its active site. Because the natural GPx couples the reduction of hydroperoxides with the oxidation of GSH in vivo, the ability to bind this thiol substrate is essential for GPx activity. This has been suggested by the catalytic efficiency difference among several GPx types. Cellular GPx contains a GSH binding site consisting of one lysine and four arginine residues, extracellular GPx lacks three of the arginine residues, and phospholipid hydroperoxide GPx lacks all of the five residues. As a result, cellular GPx, which has the strongest binding to GSH, exhibits the highest GPx activity (34). The favor for special thiol substrate of the first semisynthetic selenoenzyme, selenosubtilisin, which decomposes hydroperoxides with aryl thiols as reductant instead of GSH, is also due to the deficiency of the specific GSH binding site (35). Therefore, the specific GSH binding site of seleno-LuGST1-1 and its high GSH affinity should have contributed greatly to its significantly high GPx activity. Moreover, GPx and GST adopt a common thioredoxin fold, even though they share low sequence identity (9, 11, 14). Endowing GST with efficient GPx activity by the conversion of the key Ser in the active site to Sec provides a new proof for the previous assumption on their evolution and suggests that their active site residues would have evolved separately from their common thioredoxin-like ancestor in previous studies on the basis of the catalytic triad in the active site consisting of selenocysteine, glutamine, and tryptophan residues (38) for the perfect balance between stabilizing a high energy species and lowering its energy. Deletion of two of them in the seleno-LuGST1-1 model may also cause a decrease of its efficiency to some extent. However, this also promises seleno-LuGST1-1 high potential to be redesigned on the basis of modeling studies and to enhance its reactivity and specificity by rational mutation.

GST and GPx have been proposed to have a “glutathione-binding protein” ancestor in previous studies on the basis of the similarities in their overall structures and the positioning of their important active-site residues despite their functional differences and low sequence identity (9, 14). Endowing GST with efficient GPx activity by the conversion of the key Ser in the active site to Sec provides a new proof for the previous assumption on their evolution and suggests that their active-site residues would have evolved separately from their common thioredoxin-like ancestor to accommodate different functions along with evolution. This result leads further credence to the principles that the evolution of new catalytic activities involves the incorporation of new catalytic groups within an active site and the reservation of those groups necessary to catalyze the partial reaction common to all of them (7, 8). In addition, it supports the notion that the dominant factor in the evolution of new enzymatic activities is chemistry in nature rather than binding specificity. This could also be deduced from some other enzyme superfamilies within which their members share a common structural scaffold but catalyze different overall reactions (8).

In all organisms investigated to date, Sec is encoded by a UGA opal codon, usually a stop codon. However, the presence of a downstream mRNA stem-loop structure, designated as the Sec insertion sequence, precludes termination of the polypeptide biosynthesis and promotes Sec incorporation into the nascent protein (39). Consequently, expression of a eukaryotic

### Table III

#### Kinetic parameters for the GPx activity of seleno-LuGST1-1

| [GSH] | $k_{cat}$ (min$^{-1}$) | $K_{M, H_2O_2}$ (mM) | $k_{cat}/K_{M, H_2O_2}$ (min$^{-1}$) | $K_{M, GSH}$ (mM) | $k_{cat}/K_{M, GSH}$ (min$^{-1}$) |
|-------|----------------------|----------------------|-------------------------------------|-------------------|-----------------------------------|
| 0.25  | 1300 (20)            | 1.3 (0.2)            | 9.80 $\times 10^3$ (0.14)          | 0.1               | 0.7 (0.1)                         |
| 0.50  | 1740 (10)            | 1.7 (0.3)            | 1.00 $\times 10^4$ (0.04)          | 0.2               | 1.2 (0.3)                         |
| 1.00  | 2100 (30)            | 2.1 (0.1)            | 9.90 $\times 10^3$ (0.28)          | 0.5               | 1.7 (0.2)                         |
| 2.00  | 2350 (20)            | 2.5 (0.3)            | 1.00 $\times 10^2$ (0.02)          | 1.0               | 2.1 (0.3)                         |

Reactions were carried out in 50 mM potassium phosphate buffer (pH 7.0) at 37 °C. The data in the table were calculated from the plots in Fig. 3, and the standard deviations are shown in parentheses.
selenoprotein in transformed bacteria would require the presence of a bacterial-type Sec insertion sequence inside the open reading frame of the recombinant protein, which, however, could result in alteration of its amino acid sequence and may affect its function. So far, the only success of heterologous expression of genes coding for eukaryotic selenoproteins in bacteria is the expression of selenium-containing citrus phospholipid hydroperoxide glutathione peroxidase, but functional enzyme could not be purified to homogeneity (39). Chemical modification is an efficient method to incorporate Sec into protein. The successful chemical incorporation of functional Sec has been demonstrated by the first semisynthetic selenoenzyme, selenosubtilisin (3), and other high efficiency GPx-like biocatalysts, such as selenium-containing catalytic antibody (5) and imprinted proteins (6). But non-site-directed substitution hampers further structure-function characterization of as-generated proteins. In contrast, the method of using the auxotrophic expression system to incorporate Sec into protein could circumvent this problem efficiently. Mischarging of tRNA<sub>Cys</sub> with Sec in the cysteine auxotrophic strain when Cys was omitted from the growth medium allows the efficient biosynthetic substitution of cysteine residues by selenocysteine. 70–80% substitution ratios and high level yields were achieved for all the three proteins that have been successfully expressed using this method: (Se)<sub>2</sub>-thioredoxin, selenoglyceraldehyde-3-phosphate dehydrogenase, and seleno-LuGST1-1. Because of less structural modifications, the efficient substitution, and high product yields, this system is of high potential for performing mutational analysis that is unconceivable in animal cell system. It is also an excellent strategy either to yield and characterize other novel selenoproteins or to extend our understanding of the mechanisms and the evolution of selenoenzymes.

In conclusion, we have successfully engineered LuGST1-1 into seleno-LuGST1-1 by substitution of the catalytic residue Ser-9 by Sec using the auxotrophic expression system. Because of the important structure similarities in the specific GSH binding site and geometric conformation for the active Sec in thioredoxin fold between seleno-LuGST1-1 and naturally occurring GPx, the engineered selenoenzyme exhibited the high efficiency. Its GPx activity was in the same order of magnitude compared with that of naturally occurring GPx. This study provided a proof that both GST and GPx are evolved from a common “glutathione-binding protein” ancestor. We expect that the seleno-GST would offer a more suitable enzymatic model for a further understanding of the relationships between structure and function of GPx. In addition, this novel engineered selenoenzyme is also a prospective catalyst as an excellent antioxidant especially useful for both industrial and medical applications.

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REFERENCES
1. Cotegrave, I. A., Moldeus, P., Brattsand, R., Hallberg, A., Andersson, C. M., and Engman, L. (1992) Biochem. Pharmacol. 43, 783–882
2. Fleché, L. (1989) in Coenzymes and Cofactors: Glutathione Chemical, Biochemical, and Medical Aspects, pp. 644–731, John Wiley & Sons, New York
3. Wu, Z. P., and Hilvert, D. (1990) J. Am. Chem. Soc. 112, 5647–5648
4. Boschi-Muller, S., Muller, S., Dersselser, A. V., Böck, A., and Brandlant, G. (1998) FEBS Lett. 439, 241–245
5. Ding, L., Liu, Z., Zhu, Z. Q., Luo, G. M., Zhao, D. Q., and Ni, J. Z. (1998) Biochem. J. 332, 251–255
6. Liu, J. Q., Luo, G. M., Gao, S. J., Zhang, K., Chen, X. F., and Shen, J. C. (1999) Chem. Commun. 20, 199–200
7. Mannervik, B., Cameron, A. D., Fernandez, E., Gustafsson, A., Hansson, L. O., Jemth, P., Jiang, F., Jones, T. A., Larsson, A. K., Nilsson, L. O., Olin, B., Pettersson, P. L., Ritterström, M., Stenberg, G., and Widersten, M. (1998) Chem. Biol. Interact. 111–112, 15–21
8. Balbott, P. C., and Gerlt, J. A. (1997) J. Biol. Chem. 272, 30591–30594
9. Armstrong, R. N. (1997) Chem. Res. Toxicol. 10, 2–18
10. Durr, H. W., Reinemer, P., and Huber, R. (1994) Eur. J. Biochem. 220, 645–661
11. Wilce, M. C. J., Board, P. G., Feil, S. C., and Parker, M. W. (1995) EMBO J. 14, 2135–2143
12. Qi, D., Tann, C. M., Haring, D., and Di Stefano, M. D. (2001) Chem. Rev. 101, 3081–3111
13. De Santis, G., and Jones, J. B. (1999) Curr. Opin. Biotechnol. 10, 324–330
14. Martin, J. L. (1995) Structure 3, 245–250
15. Mannervik, B. (1985) Methods Enzymol. 113, 490–495
16. Ren, X., Jemth, P., Board, P. G., Luo, G., Mannervik, B., Liu, J., Zhang, K., and Shen, J. (2002) Chem. Biol. 9, 789–794
17. Muller, S., Senn, H., Geel, B., Vetter, W., Baron, C., and Böck, A. (1994) Biochemistry 33, 3404–3412
18. Shohukov, J., Fritzsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Board, P. G., Russel, R. J., Marano, R. J., and Oakeshott, J. G. (1994) Biochem. J. 299, 425–430
20. Tabor, S., and Richardson, C. C. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1014–1078
21. Board, P. G., and Pierce, K. (1987) Biochem. J. 248, 937–941
22. Board, P. G., Coggan, M., Wilce, M. C. J., and Parker, M. W. (1995) Biochem. J. 311, 247–250
23. Bradford, M. (1976) Anal. Biochem. 72, 248–254
24. Caccuri, A. M., Antonini, G., Nicotra, M., Battistoni, A., Bello, M. L., Board, P. G., Parker, M. W., and Ricci, G. (1997) J. Biol. Chem. 272, 29681–29686
25. Wilson, S. R., Zucker, P. A., Huang, R. R. C., and Spector, A. (1989) J. Am. Chem. Soc. 111, 5936–5946
26. Habig, W. H., and Jakoby, W. B. (1981) Methods Enzymol. 77, 385–405
27. Muge, G., and Singh, H. B. (2000) Chem. Soc. Rev. 29, 347–357
28. Ma, W., Luo, G., and Yang, T. (1993) Sheng Wu Hua Hsueh Tsa Chinh 9, 234–236
29. Chu, F. F., Doroshow, J. H., and Esworthy, R. S. (1993) J. Biol. Chem. 268, 2571–2576
30. Yamamoto, Y., and Takahashi, K. (1993) Arch. Biochem. Biophys. 305, 541–545
31. Fleché, L., Eisleb, B., and Wendel, A. (1971) Hoppe-Seyler’s Z. Physiol. Chem. 353, 151–158
32. Forstorn, J. W., Zakowski, J. J., and Tappel, A. L. (1978) Biochemistry 17, 2639–2644
33. Luo, G. M., Zhu, Z. Q., Ding, L., Gao, S. J., Sun, Q. A., Liu, Z., Yang, T. S., and Shen, J. C. (1994) Biochim. Biophys. Res. Commun. 196, 1240–1247
34. Takebe, G., Yarimizu, J., Saito, Y., Hayashi, T., Nakamura, H., Yodoi, J., Nagasawa, S., and Takahashi K. (2002) J. Biol. Chem. 277, 41245–41258
35. Bell, I. M., Fisher, M. L., Wu, Z. P., and Hilvert, D. (1995) Biochemistry 32, 3754–3762
36. Epp, O., Ladenstein, R., and Wendel, A. (1983) Eur. J. Biochem. 133, 51–69
37. Hol, W. G. J., van Duijn, P. T., and Berendsen, H. J. C. (1978) Nature (Lond.) 273, 443–446
38. Ursini, F., Maiorino, M., Brigeli-Flohé, R., Aumann, K. D., Reveri, A., Schomburg, D., and Flohé, L. (1995) Methods Enzymol. 252, 38–53
39. Hazebrouck, S., Camoun, L., Faltin, Z., Strobos, A. D., and Eshdat, Y. (2000) J. Biol. Chem. 275, 28715–28721
40. Amersham Pharmacia Biotech (1992–1993) Hoefer Scientific Instruments Manual, pp. 5–11, Hoefer Scientific, UK
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