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A Semisynthetic Glutathione Peroxidase with High Catalytic Efficiency: Selenoglutathione Transferase

Xiaojun Ren,[1,2] Per Jemth,[3] Philip G. Board,[5] Guimin Luo,[2,6] Bengt Mannervik,[4] Junqiu Liu,[1] Kun Zhang,[2] and Jiacong Shen[1]

[1]Key Laboratory for Supramolecular Structure and Materials of Ministry of Education
[2]Key Laboratory of Molecular Enzymology and Engineering of Ministry of Education
Jilin University
Changchun 130023
China
[3]Department of Medical Biochemistry and Microbiology
[4]Department of Biochemistry
Uppsala University
Biomedical Center
SE-75123 Uppsala
Sweden
[5]Molecular Genetics Group
John Curtin School of Medical Research
Australian National University
Canberra
Australia

Summary

Glutathione peroxidase (GPX) protects cells against oxidative damage by catalyzing the reduction of hydroperoxides by glutathione (GSH). GPX therefore has potential therapeutic value as an antioxidant, but its pharmacological development has been limited because GPX uses a selenocysteine as its catalytic group and it is difficult to generate selenium-containing proteins with traditional recombinant DNA technology. Here, we show that naturally occurring proteins can be modified to generate GPX activity. The rat theta-class glutathione transferase T2-2 (rGST T2-2) presents an ideal scaffold for the design of a novel GPX catalyst because it already binds GSH and contains a serine close to the substrate binding site, which can be chemically modified to bind selenium. The modified Se-rGST T2-2 efficiently catalyzes the reduction of hydrogen peroxide, and the GPX activity surpasses the activities of some natural GPXs.

Introduction

Enzyme engineering has proven to be an invaluable tool for elucidating enzyme reaction mechanisms as well as for producing enzymes for industrial purposes. However, it is extremely difficult to produce selenium-containing proteins by these methods, and it is especially challenging to generate selenocysteine (Sec)-dependent enzymes in vitro.

Glutathione peroxidase (GPX, EC.1.11.1.9) is a selenium-containing enzyme in which Sec is the catalytically active residue. GPX catalyzes the reduction of hydroperoxide by glutathione (GSH) and thereby protects cells against oxidative damage. The enzyme has been well studied, but some uncertainties remain with respect to its reaction mechanism [1, 2]. Because GPX is a scavenger of reactive peroxides in aerobic organisms, considerable efforts have been made to find compounds that could imitate the properties of GPX [3–5]. The strategies employed in the generation of GPX-like catalysts include chemical synthesis of model systems, the production of catalytic antibodies, and mutation of naturally occurring enzymes by chemical or genetic means. To date, two different strategies have been tested to chemically synthesize GPX mimics [3]; one in which the selenium atom binds directly to a heteroatom such as nitrogen and generates the well-known enzyme, 2-phenyl-1,2-benzisoselenazol-3(2H)-one (Ebselen) **1** (Figure 1), and a second in which the selenium atom is not directly bound to the heteroatom (N or O), but instead is located in close proximity to it. Enzymes generated in this way include the catalysts of Spector **2**, Tomado **3**, and Singh **4** (Figure 1). Monoclonal antibodies could also be used as a basis for generating GPX catalysts (Figure 2) [4, 5] if a monoclonal antibody with a GSH substrate binding site were generated and then a catalytically active Sec were incorporated into the antibody's binding site by chemical mutation. So far, engineering existing enzymes by chemical or genetic means [4, 5] has resulted in the semisynthetic enzyme selenosubtilisin, studied by Hilvert and colleagues, that exhibits GPX-like properties [6] and a mutant version of glyceraldehyde-3-phosphte dehydrogenase that mimicked GPX activity when a Cys residue was replaced with selenocysteine [7]. However, because there are no GSH-specific binding sites in the active sites of these two enzymes, their mechanism of action of decomposing hydroperoxides involves aryl thiols as reductants instead of GSH.

Chemical methods provide a means for introducing into enzymes diverse functions that do not occur naturally and cannot be easily incorporated by genetic engineering. However, although many new enzymes have been generated via covalent modification of naturally occurring enzymes [8, 9], it appears to be difficult to obtain highly efficient semisynthetic enzymes. There are two principal phenomena that underlie the activity of enzymes: substrate binding and the subsequent reaction. In the active site of an enzyme, the bound substrate and the enzyme catalytic groups are positioned for efficient catalysis. Thus, for the creation of an efficient artificial enzyme, the affinity for the transition state of the enzyme-substrate complex must be reasonably high, and the catalytic groups should be juxtaposed to the reactive group of the substrate. On the basis of this principle, we set out to imitate the action of GPX by chemically modifying a naturally occurring glutathione transferase (GST, EC.2.5.1.18).

GSTs are a family of enzymes involved in cellular detoxification [10]. GSTs occur in most species and are divided into different classes based on sequence simi-

[6]Correspondence: gmluo@mail.jlu.edu.cn

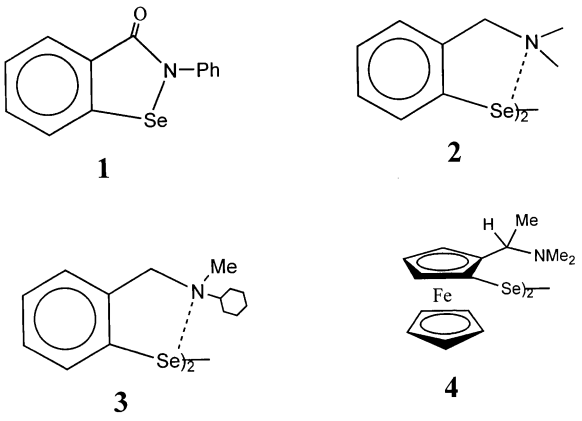

Figure 1. The Structure of Some Small GPX Mimics

larity [11]. For the theta-class of GSTs, catalysis is dependent on the presence of a conserved serine residue in the N-terminal region [12, 13]. The thiolate form of GSH is activated when the formation of a hydrogen bond between the hydroxyl group of this serine and the deprotonated sulfhydryl group facilitates catalysis [14]. Several GSTs, including those of the theta class, act as selenium-independent GPX that catalyze reduction of organic hydroperoxides but do not catalyze reduction of hydrogen peroxide (H_2O_2) [15–17]. Thus, theta-class GSTs appear to be excellent protein scaffolds for grafting GPX activity by engineering the N-terminal serine into selenocysteine.

Here, we report that the rat theta-class glutathione transferase T2-2 (rGST T2-2) was converted into a selenium-containing enzyme (Se-rGST T2-2) by chemical mutation. This novel selenium-dependent enzyme displays high catalytic activity toward H_2O_2 and hence justifies our approach to generating efficient biocatalysts.

Results and Discussion

Preparation of Selenium-Containing Proteins

The serine residues of the proteins studied were activated with phenylmethanesulfonyl fluoride (PMSF) to produce a sulfonylester of the serine hydroxyl group, then the sulfonate was replaced with hydrogen selenide to generate selenoproteins (Figure 3; [6]). To ensure the complete sulfonylation of the serines, we set the molar ratio between PMSF and protein at 50:1 and allowed the sulfonylation to proceed for 3 hr. To counteract the probable oxidation of hydrogen selenide by air, we used an amount of sodium hydrogen selenide that was in great excess over that of the sulfonate present. Carrying out the displacement reaction at 35°C rather than 40°C minimized the denaturation of the proteins. Wild-type and mutant rGST T2-2, egg albumin (OVA), and bovine serum albumin (BSA) were thus converted into selenium-containing proteins via this method. After purification, the yield of all selenium-containing proteins was about 30%-40%.

Selenium Content of Selenium-Containing Proteins

The selenium content of the proteins was determined by anaerobic reduction of the samples with sodium borohydride and subsequent titration with 5,5-dithiobis(2-nitrobenzoic acid). The selenium contents of Se-OVA, Se-BSA, Se-rGST T2-2, and Se-GST (S11A mutant) were determined to be 5.6, 5.2, 4.1, and 2.9 equivalents of selenium per mol of enzyme subunit, respectively. The selenium 3D electron signals determined by X-ray photoelectron spectroscopy of selenoproteins were essen-

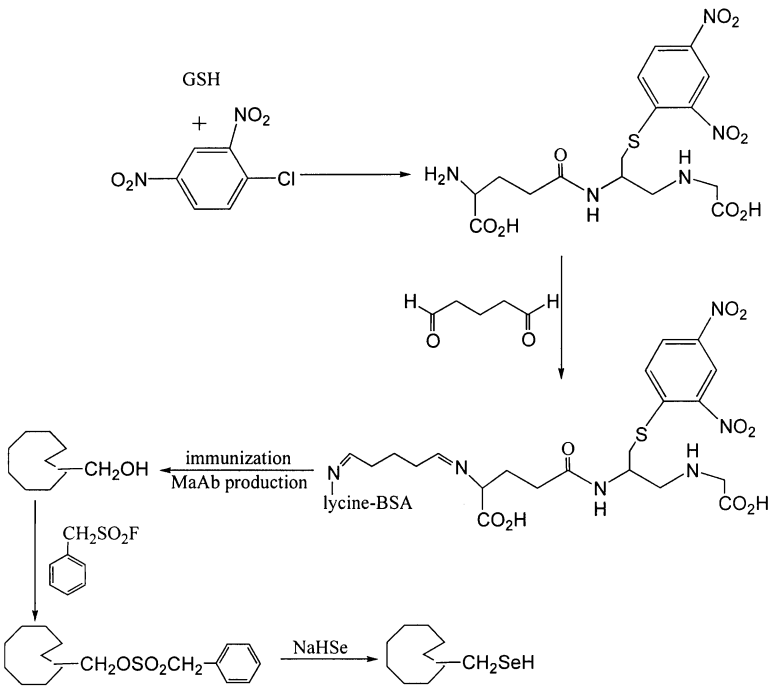

Figure 2. The Protocol of Production of Selenium-Containing Catalytic Antibody with GPX Activity

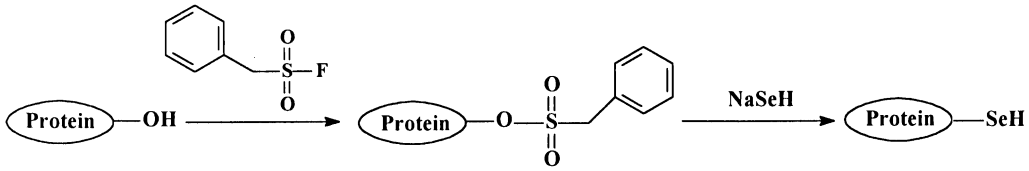

Figure 3. The Serine Residues of the Proteins Studied Were Activated with Phenylmethanesulfonyl Fluoride to Produce a Sulfonylester of the Serine Hydroxyl Group

The sulfonate was then replaced with hydrogen selenide to generate selenoproteins.

tially identical and emerged in the 55.3 ± 0.4 eV region assigned to the selenium of seleninic acid, suggesting that the selenium in the selenoproteins was bonded to oxygen.

The GPX Activity of Se-rGST T2-2

The GPX and GST activities of Se-rGST T2-2 and other catalysts are listed in Table 1. The GPX activity of Se-rGST T2-2 for the reduction of H_2O_2 by GSH was found to be 102 μmol·min^{-1}·mg^{-1}, indicating that Se-rGST T2-2 displays a significantly higher GPX activity than Se-OVA, Se-BSA, Se-GST (S11A mutant), and Se-4A4 [26]. The GPX activity of Se-rGST T2-2 is also higher than that of rabbit liver GPX [18], bovine liver GPX [19], human hepatoma HepG 2 cell giGPX [19], and human plasma pGPX [20]. To gauge the catalytic efficiency of Se-rGST T2-2, we compared the Se-rGST T2-2 with the model compound ebselen, a well-studied GPX mimic [3–5]. At 37.0°C and pH 7.0, the initial rate of the reduction of H_2O_2 (0.5 mM) by GSH (1 mM) in the presence of 0.02 μmol Se-rGST T2-2 is 2.2 ×10^{-4} M·min^{-1}, but under the same conditions when 2 μmol ebselen was used as the catalyst, the initial rate is only 4.1 ×10^{-6} M·min^{-1}. Assuming that the rate has a first-order dependence on GSH or H_2O_2 concentration, we used the background rate, determined to be 3.12 ×10^{-7} M·min^{-1}, to calculate the rate constant for the uncatalyzed reaction (k_{uncat} = 0.62 M^{-1}·min^{-1}). These data indicate that Se-rGST T2-2 is at least 5000-fold more efficient than ebselen.

The chemical modification does not allow specific targeting of amino acid residues in the active site; therefore, other hydroxyl groups in the proteins could be modified to selenols (SeH), which could contribute to GPX activity. However, these contributions are small as judged by the activities of Se-OVA and Se-BSA, in which no GSH binding sites are present. Se-GST (S11A mutant) and Se-4A4 have GSH binding sites, and indeed they display higher GPX activities than Se-OVA and Se-BSA, but still the GPX activities are much less than that of Se-rGST T2-2. Importantly, the much higher GPX activity of Se-rGST T2-2 as compared to Se-GST (S11A mutant), in which Ser-11 of wild-type rGST T2-2 had been replaced with alanine by site-directed mutagenesis [14] before the chemical modification, strongly suggests that most GPX activity of Se-rGST T2-2 is due to a Sec residue at position 11. This makes sense because the GSH thiol is within hydrogen bonding distance of Ser-11 in wild-type rGST T2-2 based on structural homology with human GST T2-2 [21]. The difference in selenium content, approximately one per subunit, between Se-rGST T2-2 and Se-GST (S11A mutant) further corroborates that it is the ideally positioned Sec-11 that promotes the high GPX activity (Table 1). On the other hand, in Se-GST (S11A mutant) and Se-4A4, the Sec residues are not in favorable positions with regard to the sulfhydryl group of GSH. Also, although the selenium contents of Se-OVA and Se-BSA are higher than that of Se-rGST T2-2, the GPX activities of the former are much lower

Table 1. The GPX and GST Activities of Se-rGST T2-2 and Other Catalysts

Catalyst	Substrate	Substrate Concentration (mM)	Activity[a] (μmol min^{-1} · mg^{-1})	Selenium (eq/mol of subunit)
Se-4A4	H_2O_2	0.50	6.9[b]	5.0
Se-OVA	H_2O_2	0.50	1.6 (0.2)	5.6
Se-BSA	H_2O_2	0.50	0.42 (0.03)	5.2
Se-GST (S11A mutant)	H_2O_2	0.50	3.2 (0.2)	2.9
Se-rGST T2-2	CDNB	0.50	ND[c]	4.1
	MS	0.075	ND[c]	4.1
	H_2O_2	0.50	102 (2)	4.1
	CuOOH	1.50	23 (3)	4.1
WT rGST T2-2	CDNB	0.50	0.9 (0.1)	0
	MS	0.075	4.0 (0.2)	0
	H_2O_2	0.50	ND[c]	0
	CuOOH	1.50	2.1 (0.2)	0
Ebselen	H_2O_2	0.50	3.61	1.0
GPX (erythrocytes)	H_2O_2	–	100	4.0[d]

[a] All values are the means of at least five determinations, and the standard deviations are shown in parentheses.
[b] From references [26, 35].
[c] No detectable GPX or GST activity.
[d] From reference [15].

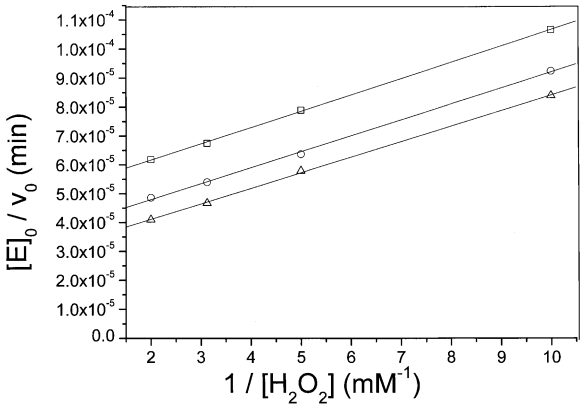

Figure 4. Double-Reciprocal Plots for the Reduction of H_2O_2 by GSH
The reaction was catalyzed by Se-rGST T2-2 in 50 mM potassium phosphate buffer (pH 7.0) at 37.0°C. The initial rates were measured at 1.0 mM (squares), 2.0 mM (circles), and 3.0 mM (triangles) GSH, respectively, by enzyme-coupled assay [34].

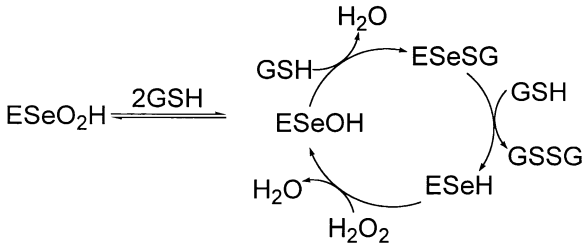

Figure 5. The Catalytic Mechanism of Native GPX
This figure was derived from [1].

than those of the latter, which again demonstrates that improperly positioned selenium atoms contribute little to the GPX activity.

The GPX activity of Se-rGST T2-2 was assayed in the absence of GSH, and the fact that no GPX activity was observed shows that contaminants from the reagents in the modification of the protein were not responsible for the GPX activity.

Steady-State Kinetics and Catalytic Mechanism of Se-rGST T2-2

As shown in Table 1, unmodified wild-type rGST T2-2 acts as a selenium-independent GPX by catalyzing decomposition of CuOOH with a specific activity of 2.1 ± 0.2 μmol min^{-1}·mg^{-1}. However, the enzyme does not reduce H_2O_2. After chemical modification of rGST T2-2, the resulting Se-rGST T2-2 has no GST activity when CDNB and MS are used as substrates. However, with CuOOH as a substrate, the GPX activity was increased by an order of magnitude in comparison with unmodified rGST T2-2 [22]. This indicates that the catalytic process by which Se-rGST T2-2 catalyzes the decomposition of organic peroxides differs from that of the unmodified rGST T2-2.

The initial velocities for H_2O_2 reduction catalyzed by Se-rGST T2-2 were determined as a function of substrate concentration at 37.0°C and pH 7.0; the concentration of one substrate was varied while the other was held fixed. Under all conditions investigated, Michaelis-Menten kinetics was observed for H_2O_2 at fixed GSH concentrations. Double reciprocal plots of the initial velocity versus the concentration of the substrates yielded a family of parallel lines (Figure 4), indicating a ping-pong mechanism with at least one covalent intermediate. To further investigate if the catalytic mechanism of Se-rGST T2-2 is similar to that of native GPX (Figure 5) [1], we treated the enzyme with excess iodoacetate in the presence of GSH and found complete loss of GPX activity; this finding indicates that enzyme bound selenol is present in the catalytic cycle. Hence, the catalytic mechanism of Se-rGST T2-2 may be similar to that of native GPX, but further characterization of the intermediates and the molecular mechanism is needed to fully establish this.

The kinetic constants for Se-rGST T2-2 further emphasize the high catalytic efficiency of this semisynthetic enzyme (Table 2). For example, the first-order rate constant $k_{cat}(H_2O_2)$ and the apparent Michaelis constant $K_m(H_2O_2)$ at 1 mM GSH were determined to be 2.0×10^4 min^{-1} and 1.1×10^{-4} M, respectively, and $k_{cat}/K_m(H_2O_2)$, the second-order rate constant for the reaction between the free enzyme and substrate, was determined to be 1.8×10^8 M^{-1}·min^{-1}. At 1 mM of GSH, the bimolecular rate constant for the reaction between Se-4A4 and H_2O_2 is only 4.5×10^3 M^{-1}·min^{-1} [23]. Thus, the catalytic efficiency of Se-rGST T2-2 is almost five orders of magnitude higher than that of Se-4A4 [26]. The second-order rate constant for the reaction of Se-rGST T2-2 with H_2O_2 was also higher than that for native GPX; for instance, the $k_{cat}/K_m(H_2O_2)$ value of phospholipid hydroperoxide glutathione peroxidase from *Schistosoma mansoni* was only 2.7×10^7 M^{-1}·min^{-1} [24].

In summary, this is the first example in which a semi-

Table 2. Kinetic Parameters for the GPX Activity of Se-rGST T2-2[a,b]

[GSH] (mM)	k_{cat}(min^{-1})	$K_m(H_2O_2)$ (10^{-4} M)	$k_{cat}/K_m(H_2O_2)$ (M^{-1} min^{-1})
1	20,000 ± 200	1.1 ± 0.2	(1.8 ± 0.23) × 10^8
2	27,000 ± 320	1.5 ± 0.3	(1.8 ± 0.17) × 10^8
3	33,000 ± 180	1.8 ± 0.2	(1.8 ± 0.12) × 10^8

[a] Reactions for H_2O_2 were carried out in 50 mM potassium phosphate buffer (pH 7.0).
The data in the table were obtained from the plots in Figure 4.
[b] The relevant steady-state rate equation is:

$$\frac{v_0}{[E]_o} = \frac{k_{cat}[GSH][H_2O_2]}{K_{GSH}[H_2O_2] + K_{H_2O_2}[GSH] + [H_2O_2][GSH]}$$

synthetic selenium-containing enzyme has been shown to catalyze the reduction of H_2O_2 by GSH with higher catalytic efficiency than some native GPX, and this is probably due to the specific GSH binding site and the adjacent serine residue utilized. Thus, we have succeeded in applying a general and simple principle for the preparation of highly efficient GPX enzyme. We believe that using the same approach on additional protein scaffolds could allow the generation of other efficient semisynthetic enzymes.

Significance

Previous attempts at generating semisynthetic selenoenzymes with peroxidase activity have not been able to match the catalytic efficiencies of naturally evolved GPX, presumably because they do not incorporate any specificity for the reducing equivalents of the donor. In this study, we have taken advantage of the highly specific GSH binding site of a GST scaffold in order to achieve both high thiol selectivity and high catalytic efficiency. The enzyme design involved the incorporation of selenium in juxtaposition to the electron-donating sulfur of active site bound GSH. The general principle of combining a functional group involved in catalysis with a specific binding site for the substrate is an approach that could be applied to the generation of other efficient semisynthetic biocatalysts. The success in converting GST to GPX will give us the opportunity to pursue detailed study, to search for a better enzyme model by chemically or genetically modifying naturally occurring proteins, and to understand the catalytic mechanism of enzymes and enzyme-like catalysts.

Experimental Procedures

Materials

Reduced glutathione (GSH) was obtained from Aldrich. Glutathione disulfide (GSSG) was obtained from Roche. Glutathione reductase (type III baker's yeast), reduced nicotinamide adenine dinucleotide phosphate (NADPH), cumene hydroperoxide (CuOOH), and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma. Hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide *(t*-BuOOH) were obtained from Beijing Chemical Factory, China. All the other reagents were of analytic grade. The concentrations of hydroperoxides were determined by an iodometric method [25]. 1-menaphthyl sulphate (MS) was a gift from Dr. Brian Gillham. The wild-type rGST T2-2 and the S11A mutant of rGST T2-2 were heterologously expressed in *E. coli* and purified as described previously [14, 22]. The catalytic antibody Se-4A4 with substrate GSH binding site was generated with a substrate analog as a hapten. Incorporation of catalytic selenocysteine residues into the substrate binding site followed [23, 26].

Preparation of Se-rGST T2-2

rGST T2-2 (2 mg) was dissolved in 50 mM PIPES and 10 mM $CaCl_2$ (pH 7.0) and was allowed to react at 25°C for 3 hr with 1 mg of PMSF dissolved in acetonitrile. The resulting solution was mixed with 200 μl of 1 M sodium hydrogen selenide solution prepared as described previously [27] and was then incubated at 35°C for 48 hr under pure nitrogen. The Se-rGST T2-2 was purified according to the procedure of Bell [28]. According to the above procedure, egg albumin (OVA) and bovine serum albumin (BSA) were also converted into selenium-containing proteins.

Determination of the Selenium Content of Selenoproteins

The selenium content of the selenoprotein was determined by titration of the sodium borohydride-reduced protein with 5,5-dithiobis (nitrobenzoic acid) as described [29]. Selenium 3d (3/2, 5/2) electron signals were studied by X-ray photoelectron spectroscopy with Alka as a target [30].

Determination of GST and GPX Activity

The specific activity of GST toward 1-chloro-2,4-dinitrobenzene (CDNB) was measured as described by Habig and Jakoby [31]. The reaction was carried out in 50 mM sodium phosphate solution (pH 6.5) containing 1 mM EDTA, 0.5 mM CDNB, and 1.0 mM GSH. The GST activity toward CuOOH was determined in 50 mM sodium phosphate (pH 7.0) containing 1 mM EDTA, 1.0 mM GSH, and 1.5 mM CuOOH [32]. The GST activity toward MS was measured in 50 mM sodium phosphate (pH 7.5) containing 1 mM EDTA, 0.075 mM MS, and 10 mM GSH [33].

The GPX activity was assayed with the coupled assay described by Wilson et al. [34]. The glutathione disulfide (GSSG) that formed in the first step was reduced to GSH by NADPH in a reaction catalyzed by GSSG reductase. After GSSG formation, NADH consumption caused the absorbance to decrease at 340 nm. At the NADPH concentration used, the coupled reaction was a zero-order reaction with respect to NADPH, and the oxidation of GSH was the rate-limiting step. The amount of GSH in the reaction was thus kept constant (Equations 1 and 2).

$$2GSH + ROOH \xrightarrow{\text{Enzyme}} GSSG + ROH + H_2O$$

$$R = H,\ t - \text{butyl, cumenyl}$$

$$GSSG + NADPH + H^+ \xrightarrow{\text{GSSG reductase}} 2GSH + NADP^+$$

The reaction was carried out at 37°C in 500 μl of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1 mM sodium azide, 1 mM GSH, 1 unit of GSSG reductase, and 10–50 nM of enzyme. The mixture was preincubated for 7 min. Then 0.25 mM NADPH solution was added, and the mixture was incubated for 3 min at 37°C. Thereafter, the reaction was initiated by the addition of 0.5 mM H_2O_2. The activity was determined from the decrease of NADPH absorption at 340 nm. Appropriate control of the nonenzymatic reaction was performed and subtracted from the catalyzed reaction. The activity unit is defined as the amount of the enzyme that catalyzes the turnover of 1 μmol of NADPH per min. The specific activity is expressed in $\mu mol \cdot min^{-1} \cdot mg^{-1}$.

Steady-State Kinetics of Se-rGST T2-2

The assay of kinetics of Se-rGST T2-2 for the reduction of H_2O_2 by GSH was similar to that of Se-4A4 [35]. 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 second substrate was kept constant. All kinetic experiments were performed in a total volume of 0.5 ml containing 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 unit of GSSG reductase, 0.25 mM NADPH, and varying concentrations of GSH, H_2O_2, and Se-rGST T2-2. The enzyme was preincubated with GSH, NADPH, and GSSG reductase. The reaction was then initiated by the addition of H_2O_2. Subtraction of the nonenzymatic reaction rate gave the rate of the enzyme-catalyzed reaction.

Acknowledgments

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