Enzyme Design

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Incorporation of Tellurocysteine into Glutathione Transferase Generates High Glutathione Peroxidase Efficiency**

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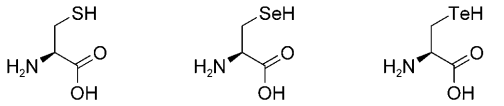
Oxidative stress is implicated, either directly or indirectly, in the pathology of a range of human diseases: An increase in intracellular concentrations of oxidizing species leads to the oxidation of membranes, proteins, DNA, and ultimately to cell death.[1] As a consequence, the development of efficient antioxidants for medical use has become increasingly important. Glutathione peroxidases (GPxs, EC 1.11.1.9) were first discovered in mammals as key enzymes involved in the scavenging of reactive oxygen species. Their efficient antioxidant activity depends on the presence of the rare amino acid residue selenocysteine (SeCys) at the catalytic site. This residue is successively oxidized and then reduced during catalytic cycles.[2] As intrinsic disadvantages of natural GPxs have limited their application, enormous efforts have been made to simulate the functions of GPx. For example, the low-molecular-weight GPx mimic 2-phenyl-1,2-benzoisoselenazol-3(2*H*)-one (ebselen) has already been evaluated clinically for the treatment of stroke,[3] and a series of low-molecular-weight selenium/tellurium-containing compounds and chemically modified selenium-containing biomacromolecules have been reported as GPx mimics.[3,4] However, because of the absence of the binding site for substrate glutathione (GSH), the mimics displayed limited activity when GSH was used as the reducing substrate. The GSH binding site was introduced successfully into GPx models by using monoclonal antibody and bioimprinting techniques,[5] and the resulting selenium-containing proteins exhibited high GPx activity. The striking overall structural similarity between the glutathione-binding-domain folds in glutathione S-transferase (GST, EC 2.5.1.18) and GPx, and the similarity in the orientation of their catalytic center, led to the selection of GST as an excellent scaffold for inducing GPx function.[6] The essential active-site residue Ser9 of GST from *Lucilia cuprina* (LuGST1-1) was replaced with an SeCys residue to give the GPx mimic seleno-LuGST1-1, which exhibits high activity with GSH as the reducing substrate.[7] In contrast to the vast body of knowledge with regard to selenium incorporation in biological systems, surprisingly little is known about the occurrence of tellurium.



L-cysteine L-selenocysteine L-tellurocysteine

Tellurocysteine (TeCys)[8] has an intrinsically lower redox potential (−850 mV versus Ag/AgCl) than that of SeCys (the catalytic center of GPx; −640 mV versus Ag/AgCl).[9] As a result, proteins containing TeCys residues can participate in unique and biologically fundamental redox reactions. Telluromethionine has been incorporated into proteins for structure analysis.[10] The chemical introduction of TeCys into subtilisin to form semisynthetic tellurosubtilisin is the only example of TeCys incorporation to have been reported.[4] However, chemical modification has an obvious disadvantage: Only the active serine residue (e.g. Ser221 in subtilisin) can be modified; other serine residues are not accessible. Thus, the development of a general strategy for the incorporation of TeCys into proteins to give telluroenzymes poses a great challenge. By using an auxotrophic expression system, we have successfully incorporated TeCys residue into a protein (Figure 1). The rationally designed telluroenzyme, which combines an existing GSH binding site with a catalytic TeCys residue, exhibits a remarkable GPx activity that rivals that of native GPxs.

Along with the successful incorporation of selenocysteine into LuGST1-1,[7] telluro-LuGST1-1 was prepared with modifications.[9] The cysteine-auxotrophic expression system used was derived from an efficient synthesis of $(Se)_2$-thioredoxin.[11] This synthesis was based on the assumption that an efficient charging of $tRNA^{Cys}$ occurs with SeCys when cysteine is omitted. However, TeCys may be on the borderline in terms of the substrate tolerance of cysteinyl-tRNA synthetase (CysRS) from *Escherichia coli*; it is also toxic to

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Supporting information for this article (experimental details of the preparation, characterization, and kinetic investigation of telluro-LuGST1-1) is available on the WWW under http://dx.doi.org/10.1002/anie.200805365.

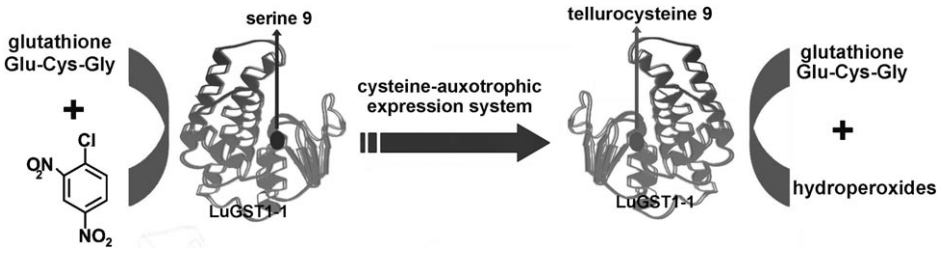

Figure 1. Transformation of wild-type LuGST1-1 into telluro-LuGST1-1 by incorporation of a TeCys residue. The single active-site mutation changed the mechanisms of enzyme catalysis. Wild-type LuGST1-1 promotes an aromatic substitution reaction by which the sulfur atom of glutathione replaces the chlorine atom of 1-chloro-2,4-dinitrobenzene. Telluro-LuGST1-1, on the other hand, showed strong peroxidase activity and catalyzed the reduction of a variety of hydroperoxides with glutathione.

cells. The aminoacylation of tRNA with TeCys was tested by using *E. coli* derived $tRNA^{Cys}$ containing ^{32}P-labeled A76. The results clearly showed that CysRS can aminoacylate tRNA with TeCys effectively, although the plateau value is lower than with cysteine. An inhibitory effect of TeCys on the aminoacylation of tRNA with ^{35}S-labeled cysteine by CysRS provided further evidence for the ability of CysRS to aminoacylate tRNA with TeCys.[9,12]

In the preparation of telluro-LuGST1-1, the active-site Ser9 residue of LuGST1-1 was first replaced with a cysteine residue and then substituted with the catalytically essential TeCys residue by using a cysteine-auxotrophic system. It was essential to optimize the conditions to achieve the efficient incorporation of TeCys into LuGST1-1. We used the bacterial growth rate as a measurement of the relative toxicity of cysteine/selenocysteine/tellurocysteine to *E. coli* derived BL21*cysE51* transformed with plasmid pSM3, which carries the gene for LuGST1-1. The periods of time before a stationary phase was reached reflected the toxicity trend tellurocystine > selenocystine > cysteine. The reduction of tellurocystine by GSH was confirmed by HPLC and UV/visible spectrometry.[9] The purity of the enzyme was determined by electrophoresis on sodium dodecyl sulfate (SDS) polyacrylamide gel (Figure 2).

The modified enzyme telluro-LuGST1-1 was characterized by polyacrylamide gel electrophoresis, ESIMS, tellurium-content analysis, and circular dichroism.[9] The mass difference between telluro-LuGST1-1 and wild type LuGST1-1 almost corresponds to S9C C86/200S plus the atomic-weight difference between tellurium and sulfur and the atomic weight of two oxygen atoms. This result indicated that telluro-LuGST1-1 was present in the oxidized form Enz-TeO_2H, as observed for tellurosubtilisin,[4] and clearly demonstrated that the TeCys residue was incorporated into the enzyme.[9] The determination of tellurium content showed the presence of 1.08 equivalents of tellurium per mole of telluro-LuGST1-1 and thus further proved the efficient incorporation of the TeCys residue.

Like natural GPxs, telluro-LuGST1-1 can catalyze the reduction of hydroperoxides by GSH. The GPx activity of telluro-LuGST1-1 was found to be 3872 U μmol^{-1}, whereas no GPx activity was observed with wild-type LuGST1-1 and GST mutants (Table 1). These results confirm that the GPx activity observed for the telluro isolate is derived from the TeCys residue introduced into the active site of LuGST1-1. The activity of 3872 U μmol^{-1} is remarkable: It is at the same level as that of some native GPxs (for example, 5780 U μmol^{-1} for rabbit-liver GPx)[13] and approximately 3900 times that of the well-studied GPx mimic ebselen.[14] Also, the activity of telluro-LuGST1-1 is higher than that of seleno-LuGST1-1.

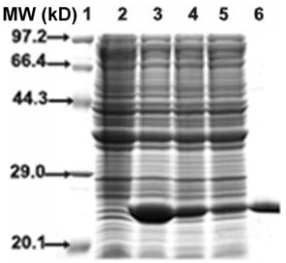

Figure 2. SDS-PAGE of purified telluro-LuGST1-1 and lysates of auxotrophic *E. coli* cells transformed with pSM3. Polyacrylamide gel (15%) was stained with Coomassie Brilliant Blue R-250. Lane 1: protein marker; lane 2: lysates of BL21 *cys* cells with pSM3 expressing telluro-LuGST1-1 in the absence of the inducer isopropylthio-β-D-galactopyranoside (IPTG); lane 3: lysates of BL21 *cys* cells with pSM3 expressing sulfur-LuGST1-1 in the presence of IPTG; lane 4: lysates of BL21 *cys* cells with pSM3 expressing seleno-LuGST1-1 in the presence of IPTG; lane 5: lysates of BL21 *cys* cells with pSM3 expressing telluro-LuGST1-1 in the presence of IPTG; lane 6: purified telluro-LuGST1-1.

Table 1: GPx activities of telluro-LuGST1-1 and other catalysts.[a]

Catalyst	Substrate	Activity [U μmol^{-1}]
wild-type GST	H_2O_2	ND
telluro-LuGST1-1	H_2O_2	3872 (46)
telluro-LuGST1-1	CUOOH	2645 (22)
telluro-LuGST1-1	*t*BuOOH	1389 (16)
GST mutant (S9C)	H_2O_2	ND
GST mutant (S9C C86/200S)	H_2O_2	ND
seleno-LuGST1-1[7]	H_2O_2	2957
ebselen[14]	H_2O_2	0.99
native GPx (rabbit liver)[13]	H_2O_2	5780

[a] The GPx activities were determined in potassium phosphate buffer (50 mM, pH 7.0) at 37 °C as initial rates and corrected for the spontaneous reaction. The standard deviations are shown in parentheses. The concentrations of GSH and H_2O_2 were 1 and 0.5 mM, respectively. ND: no detectable GPx activity.

Telluro-LuGST1-1 also exhibited high GPx activity, similar to that of seleno-LuGST1-1,[15] towards the structurally distinct peroxides *tert*-butyl peroxide (*t*BuOOH) (1389 U μmol^{-1}) and cumene peroxide (CUOOH) (2645 U μmol^{-1}). For telluro-LuGST1-1, the dissociation

constant of GSH at pH 6.5 is 102 μM, which is similar to that observed for wild-type LuGST1-1 (107 μM). This result suggests that the exchange of the Ser9 residue in LuGST1-1 for a TeCys residue did not affect the binding to substrate GSH. Therefore, the high GPx activity of telluro-LuGST1-1 relative to that of other GPx mimics may be ascribed to its strong binding to the specific substrate GSH (an interaction which facilitates the reaction)[7] and to the catalytic tellurium center. The optimal pH value for telluro-LuGST1-1 activity is 8.0, and the optimal temperature is 42.5 °C. These values are close to those for the native GPx (pH 8.8 and 50 °C).[16]

To investigate the dependency of rate on substrate concentration, a kinetic assay of telluro-LuGST1-1 for the reduction of H_2O_2 by GSH was carried out by varying the concentration of one substrate while keeping the concentration of the other constant. Double-reciprocal plots of the initial velocity versus substrate concentration yielded a series of intersecting linear plots (Figure 3), which indicate a sequential mechanism. The kinetic constants k_{max}/K_{GSH} and $k_{max}/K_{H_2O_2}$ (k_{max}: the maximal k_{cat}) for the telluro-LuGST1-1-catalyzed reduction of hydroperoxides by GSH were large: up to $1.46\times10^7\,M^{-1}min^{-1}$ and $7.75\times10^7\,M^{-1}min^{-1}$, respectively. These kinetic constants are high relative to those for seleno-LuGST1-1 and other GPx mimics, and are even comparable to those for the native GPx (Table 2).

In conclusion, we redesigned the active site of the GSH-binding domain of LuGST1-1 and made use of an auxotrophic expression system for the introduction of the catalytic residue TeCys to produce the telluroprotein. This first successful bioincorporation of TeCys into a protein to form an efficient GPx model can be viewed as a viable general route for the synthesis of telluroenzymes with GPx activity. We are now extending this approach to more tellurium-based proteins to gain access to additional models for the further investigation of tellurium chemistry in proteins. It is anticipated that telluroenzymes will be developed with more desirable catalytic performance. The study reported herein will continue to open new avenues for protein engineering, not only for structure–function studies, but also for medical applications.

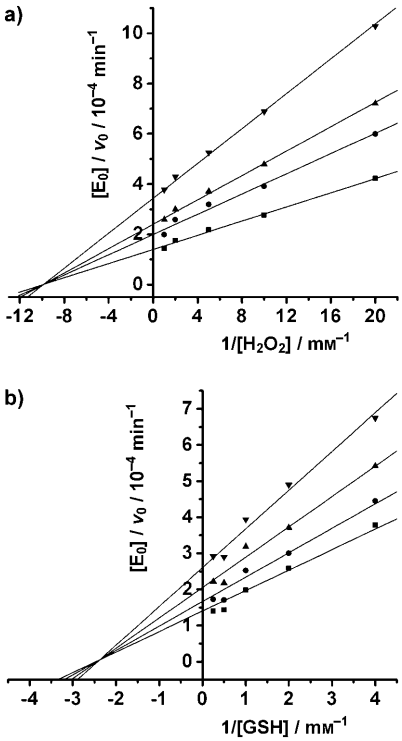

Figure 3. Double-reciprocal plots for the reduction of H_2O_2 by GSH under the catalysis of telluro-LuGST1-1 ($[E_0]$ = total enzyme concentration). a) $[E_0]/v_0$ versus $1/[H_2O_2]$ (mM^{-1}) at [GSH] = 0.25 (▼), 0.50 (▲), 1.00 (●), and 2.00 mM (■). b) $[E]_0/v_0$ versus 1/[GSH] (mM^{-1}) at $[H_2O_2]$ = 0.10 (▼), 0.20 (▲), 0.50 (●), and 1.00 mM (■).

Table 2: Kinetic parameters for the reduction of H_2O_2 by GSH under the catalysis of telluro-LuGST1-1 and other GPx mimics.[a]

	k_{max}/K_{GSH} [$M^{-1}min^{-1}$]	$k_{max}/K_{H_2O_2}$ [$M^{-1}min^{-1}$]
6-SeCD[17]	3.87×10^2	1.53×10^4
2-TeCD[18]	6.28×10^4	7.99×10^4
seleno-LuGST1-1[15]	9.60×10^6	9.80×10^6
telluro-LuGST1-1	1.46×10^7	7.75×10^7
native GPx[19]	5.60×10^7	5.78×10^8

[a] Reactions were carried out in potassium phosphate buffer (50 mM, pH 7.0) at 37 °C.

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