A Novel Cyclodextrin-Derived Tellurium Compound with Glutathione Peroxidase Activity

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A novel dicyclodextrinyl ditelluride (2-TeCD) compound was devised as a functional mimic of the glutathione peroxidase (GPX) enzymes that normally remove hydroperoxides from the cell. The GPX activity of the mimic was found to be 46.7 U μM^{-1}, which is 46 times as active as Ebselen, a well-known GPX mimic. A detailed steady-state kinetic study was undertaken to probe the reason for the high catalytic efficiency of 2-TeCD. This high efficiency can be explained based on both the binding of the substrate to the cyclodextrin and the catalytic mechanism of 2-TeCD, which is different from that of diselenide compounds. 2-TeCD exhibits good water solubility and is chemically and biologically stable. The biological effect of 2-TeCD was evaluated by its ability to protect mitochondria from oxidative damage. 2-TeCD exhibited excellent antioxidant capacity in comparison with Ebselen.

KEYWORDS:

artificial enzymes · cyclodextrins · glutathione peroxidase · kinetics · tellurium

Introduction

Reactive oxygen species (ROS) are products of the normal metabolic activities of aerobic living organisms and are produced in response to various stimuli. Under normal conditions, there is a balance between the production of ROS and their destruction. In certain pathogenic states the production of ROS is enhanced and the excess ROS damage biomacromolecules such as DNA, lipids, proteins, and sugars;[1] this results in ROS-mediated diseases.[2] To prevent undesired ROS-induced damage, the organism is equipped with several antioxidant lines of defense. These antioxidants use either a nonenzymatic action (vitamin E, ascorbate, glutathione, and uric acid) or an enzymatic action (superoxide dismutase, catalase, and glutathione peroxidase (GPX)). GPX [EC 1.11.1.9] is a mammalian selenoenzyme that catalyzes the reduction of hydroperoxides by glutathione (GSH).[3] It was observed that GPX is substantially more efficient on a molar basis than the other enzymatic antioxidant defense systems.[4]

Enzyme therapies possess some limitations, such as solution instability, limited cellular accessibility, short half-lives, high production costs, and vulnerability to proteolytic digestion. Considerable efforts have therefore been made to find compounds that could mimic the properties of GPX. Ebselen (2-phenyl-1,2-benziososelenazol-3(2*H*)-one) was the first compound found to have this capacity.[5] Although extensive research has been done on this interesting molecule, from studies of its ability to scavenge ROS to clinical trials,[6] it has some drawbacks, such as low GPX activity and water insolubility. Many compounds with GPX activity have been prepared and these compounds are well-reviewed by Mugesh and co-workers.[7] Selenium and tellurium exhibit similar redox properties and thus some aryl tellurium compounds have been prepared to mimic GPX.[8] These compounds were insoluble in water, therefore the GPX activity was followed by using other thiol compounds instead of GSH.

Another potential use of redox enzymes is as bioelectronic devices, for example, amperometric biosensors, sensoric arrays, logic gates, optical memories, and biofuel cells.[9] Willner and co-workers showed that extracellular oxidized GSH (GSSG) can be reduced to GSH by GSSG reductase assembled on electrodes.[10] GPX catalyzes the oxidation of GSH to GSSG. Therefore, GPX and GSSG reductase can be combined to form a system that could be applied in bioelectronic devices. GPX is not easily available by purification from living organisms or by protein engineering so a highly effective mimic of GPX would be a good alternative.

The general principle of enzymatic models is that efficient catalysis involves binding of the substrate and subsequent intracomplex catalysis.[11] Cyclodextrins (CDs) have been extensively exploited in the past as enzyme models and molecular receptors because of their capacity to accommodate various guest molecules in their hydrophobic cavities through host–guest chemistry.[12] Here we report a novel GPX mimic (2,2′-tellurium-bridged β-cyclodextrin (2-TeCD)) that catalyzes the

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decomposition of hydroperoxides with a remarkably high activity that exceeds many small molecular mimics of GPX. The biological effect of 2-TeCD was evaluated by its ability to protect mitochondria against oxidative damage, and it was found to be a better antioxidant than Ebselen. 2-TeCD exhibited high GPX activity and water solubility. These properties may be useful in pharmacological application and bioelectronic devices.

Results

Synthesis and characterization of 2-TeCD

The route used to synthesize 2-TeCD is shown in Figure 1. Selective monotosylation of the secondary 2-hydroxy group of β-CD was carried out to synthesize 2-OTs-β-CD **2** as described previously.[13] The tellurol group was incorporated at the 2-position of β-CD by nucleophilic substitution of the

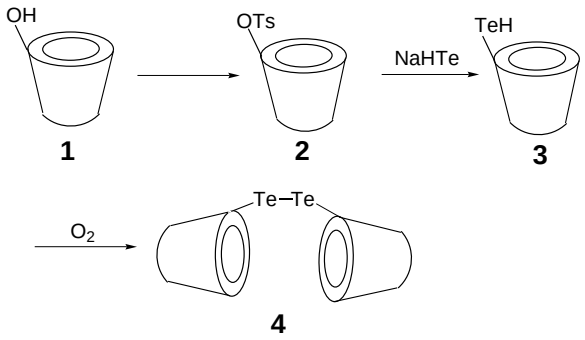
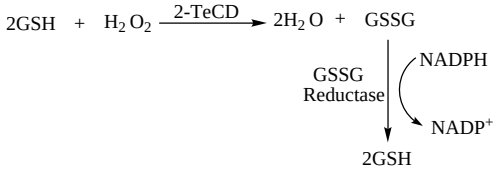


Figure 1. *Synthesis of 2-TeCD. Ts = tosyl = p-toluenesulfonyl.*

sulfate ester group of 2-OTs-β-CD to give telluroyl-β-CD **3**. Oxidation of **3** in air gave 2-TeCD **4**. 2-TeCD was characterized by elemental analysis, IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. The tellurium content and the valency of the tellurium in 2-TeCD were measured by X-ray photoelectron spectroscopy. The $Te3d_{5/2}$ electronic binding energy of 2-TeCD is 574.2 eV, which approaches the binding energy of diphenyl ditelluride (573.9 eV) and indicates that the tellurium in 2-TeCD is present in the -1 oxidation state (ditellurium bridge, Te—Te). The experiment also gave the C:Te ratio (43.5:1; calculated as 42:1), which indicates that the mimic contains 2 molar equivalents of tellurium per mole of mimic.

Estimation of GPX-like activity

GPX activity may be indirectly determined if the reaction is performed in the presence of GSSG reductase and β-nicotinamide adenine dinucleatide phosphate, reduced form (NADPH).[14] The progress of the reaction may be conveniently followed spectrophotometrically by observation of the decrease in NADPH absorbance at 340 nm (Scheme 1). The GPX activities measured in this way are shown in Table 1. The activity of 2-TeCD for the reduction of H_2O_2 by GSH was determined to be 46.7 U μM^{-1}, a catalytic efficiency remarkably higher than those of Ebselen,[14] diphenyl diselenide (PhSeSePh),[14] and 2-SeCD.[13]



Scheme 1. *The presence of GSSG reductase and NADPH allows the progress of the 2-TeCD-catalyzed reduction reaction to be spectrophotometrically followed by observation of NADPH absorption at 340 nm.*

Table 1. *Comparison of GPX activity of 2-TeCD and other GPX mimics.[a]*

GPX mimic	Hydroperoxide	Activity [U μM^{-1}][b]
Ebselen	H_2O_2	0.99
PhSeSePh	H_2O_2	1.95
2-SeCD	H_2O_2	7.4
2-TeCD[a]	H_2O_2	46.7 (1.2)
	*t*BuOOH	32.3 (0.8)
	CuOOH	87.3 (0.7)

[a] Reactions were carried out in potassium phosphate buffer (50 mM, pH 7.0) at 37 °C with GSH (1 mM) and the hydroperoxide (0.5 mM). [b] One unit of enzyme activity is defined as the amount of mimic that utilizes 1 μmol of NADPH per minute. All values are means of at least five measurements and standard deviations are shown in parentheses.

2-TeCD also catalyzes the reduction of a variety of structurally distinct hydroperoxides which range from the hydrophilic H_2O_2 to the bulky aromatic cumenyl hydroperoxide (CuOOH). The GPX activities of 2-TeCD for catalytic reduction of *tert*-butylhydroperoxide (*t*BuOOH) and CuOOH by GSH were determined to be 32.3 and 87.3 U μM^{-1}, respectively. These results show that 2-TeCD is substrate specific and the preferred substrate is CuOOH. To gauge the catalytic efficiency of 2-TeCD, we compared it with the model compound Ebselen, a well-studied GPX mimic. At 37.0 °C and pH 7.0, the initial rate of reduction of H_2O_2 (0.5 mM) by GSH (1 mM) in the presence of 2-TeCD (1.2 μM) is 3.2×10^{-5} M min^{-1}. When 1.2 μM of Ebselen was used as the catalyst under the same conditions, the initial rate was only 6.9×10^{-7} M min^{-1}. These data indicate that 2-TeCD is at least 46 times more efficient than Ebselen.

In order to gauge further the GPX-like activity of 2-TeCD, a direct assay was carried out by using PhSH as a GSH alternative, according to the procedure of Iwaoka and Tomoda.[15] The initial rate of the 2-TeCD-catalyzed reduction of H_2O_2 by PhSH was followed by observation of the UV absorption increase at 305 nm caused by diphenyl disulfide formation. At 1 mM PhSH and 0.5 mM H_2O_2, the initial rate of the reduction reaction is 51 μM min^{-1} in the presence of 1.2 μM 2-TeCD. In the presence of 1.2 μM Ebselen under the same conditions, the rate of reaction between H_2O_2 and PhSH is only 0.61 μM min^{-1}. These data show that the 2-TeCD-catalyzed reduction of H_2O_2 by PhSH is 83 times as efficient as the Ebselen-catalyzed reaction.

Kinetics of the 2-TeCD-catalyzed reduction of peroxides by GSH

Detailed kinetic studies were undertaken to probe the mechanism by which 2-TeCD catalyzes the reduction of hydroperoxides

by GSH (for graphs of the kinetics, see the Supporting Information). Plots of initial reaction velocities versus the 2-TeCD concentration gave a straight line, which shows that the mimic activity is proportional to its concentration. When the concentration of 2-TeCD was kept constant while substrate concentration was increased, a rapid increase of velocity was observed in the initial phase; however, when the substrate concentration was increased further, the rate became constant. When the concentration of 2-TeCD was increased at the same time as that of the substrate, the velocities became very high for higher substrate concentrations. Saturation kinetics were observed for the enzymatic peroxidase reaction at all the individual concentrations of GSH and *t*BuOOH, H_2O_2, or CuOOH investigated. Double-reciprocal plots of initial velocity versus substrate concentration were linear and revealed the characteristic parallel lines of a ping-pong mechanism. The apparent second-order rate constants of the enzymatic reactions between 2-TeCD and the hydroperoxide substrates *t*-BuOOH, H_2O_2, and CuOOH are listed in Table 2. These values were deduced from a fit of the

Table 2. *Kinetic parameters for the 2-TeCD-catalyzed reduction of hydroperoxides by GSH.*[a]

Hydroperoxides	k_{max}/K_{ROOH} [$M^{-1}\,min^{-1}$]	k_{max}/K_{GSH} [$M^{-1}\,min^{-1}$]
*t*BuOOH	$(5.24 \pm 0.32) \times 10^4$	$(6.26 \pm 0.27) \times 10^4$
H_2O_2	$(7.99 \pm 0.51) \times 10^4$	$(6.28 \pm 0.32) \times 10^4$
CuOOH	$(2.71 \pm 0.21) \times 10^5$	$(6.86 \pm 0.83) \times 10^4$

[a] Reactions were carried out in potassium phosphate buffer (50 mM, pH 7.0) at 37 °C. The experimental data were fit to a ping-pong mechanism to obtain the parameters shown. For detailed graphs of the kinetics, see the Supporting Information.

experimental data to a ping-pong kinetic mechanism [Eq. (1)]. In this model, v_0 is the initial reaction velocity, $[E]_0$ is the initial enzyme mimic concentration, k_{max} is the maximum rate of reaction, K_{GSH} is the Michaelis-Menten constant for GSH, and $K_{H_2O_2}$ is the Michaelis-Menten constant for H_2O_2.

$$\frac{v_0}{[E]_0} = \frac{k_{max}[GSH][H_2O_2]}{K_{GSH}[H_2O_2] + K_{H_2O_2}[GSH] + [H_2O_2][GSH]} \quad (1)$$

The data did not fit well to other models such as sequential or equilibrium-ordered mechanisms. In addition to the parameters in Table 2, the following parameters were also determined for *t*BuOOH: $k_{max} = 35 \pm 2\ min^{-1}$, K_{tBuOOH} (the Michaelis-Menten constant for *t*BuOOH) $= 0.67 \pm 0.02$ mM, and $K_{GSH} = 0.56 \pm 0.07$ mM; for H_2O_2: $k_{max} = 61 \pm 2\ min^{-1}$, $K_{H_2O_2} = 0.75 \pm 0.05$ mM, and $K_{GSH} = 0.97 \pm 0.07$ mM; for CuOOH: $k_{max} = 122 \pm 8\ min^{-1}$, K_{CuOOH} (the Michaelis-Menten constant for CuOOH) $= 0.45 \pm 0.08$ mM, and $K_{GSH} = 1.48 \pm 0.27$ mM.

Initial velocities for the spontaneous reaction between hydroperoxides and GSH were found to be linearly proportional to the concentrations of *t*BuOOH, H_2O_2, and CuOOH. A small deviation from this behavior was observed for *t*BuOOH and CuOOH at high concentration (*t*BuOOH > 0.4 M, CuOOH > 10 mM) for which the velocity was lower than that expected at the limit of solubility of the hydroperoxides in aqueous buffer. At 1 mM GSH, the pseudo-first-order rate constants for these background reactions were as follows: *t*BuOOH, $k_1 = (2.3 \pm 0.4) \times 10^{-2}\ min^{-1}$; CuOOH, $k_1 = (3.2 \pm 0.2) \times 10^{-2}\ min^{-1}$; H_2O_2, $k_1 = (7.0 \pm 0.3) \times 10^{-2}\ min^{-1}$.

The spontaneous reaction between GSH and *t*BuOOH was investigated in the presence and absence of the radical trap 2,6-di-*tert*-butyl-4-methylphenol (BHT). The GSH concentration was kept constant while the *t*BuOOH concentration was varied. In the presence of 50 μM BHT, the spontaneous reaction velocity was apparently inhibited, as evidenced by Figure 2 A. In contrast, in the presence of 1.1 μM 2-TeCD no inhibition of the enzymatic reaction by BHT (50 μM) was observed within experimental error (Figure 2 B). This result indicates that the 2-TeCD-catalyzed reaction does not involve the radical reaction, while the spontaneous reduction reaction does involve this radical process.

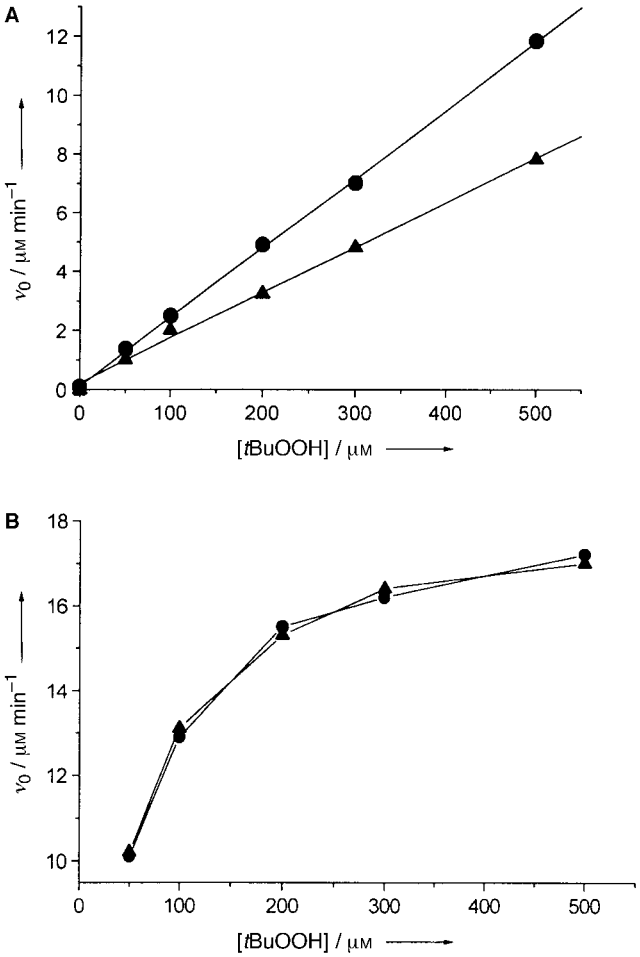


Figure 2. *Plots of v_0 against t*BuOOH *concentration for 1 mM GSH in potassium phosphate buffer (50 mM, pH 7.4) at 37 °C with [BHT] = 0 μM (●) and 50 μM (▲). A) [2-TeCD] = 0 μM. B) [2-TeCD] = 1.1 μM.*

Effect of the GPX mimic on swelling of damaged mitochondria

Mitochondrial swelling can be correlated with changes in light scattering (see Materials and Methods). Thus, Figure 3 A shows that the mitochondria were greatly swelled by ferrous sulfate/ascorbate-induced damage and that this swelling was decreased by the addition of 2-TeCD. The absorbance at 520 nm for the

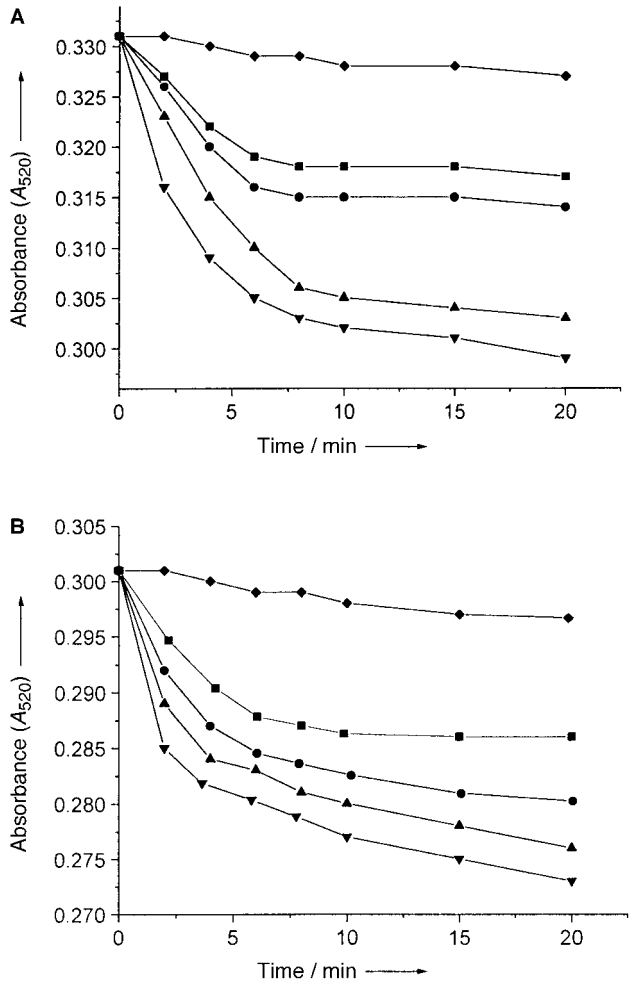


Figure 3. *A) Effect of concentration of 2-TeCD on the swelling of mitochondria. ◆ = control; ■ = damage + 16 μM 2-TeCD; ● = damage + 8 μM 2-TeCD; ▲ = damage + 2 μM 2-TeCD; ▼ = damage. B) Effect of the different GPX mimics on the swelling of mitochondria. ◆ = control; ■ = damage + 8 μM 2-TeCD; ● = damage + 8 μM 2-SeCD; ▲ = damage + 8 μM Ebselen; ▼ = damage. For damage conditions, see Materials and Methods section. The ordinate axes have been interrupted in both (A) and (B).*

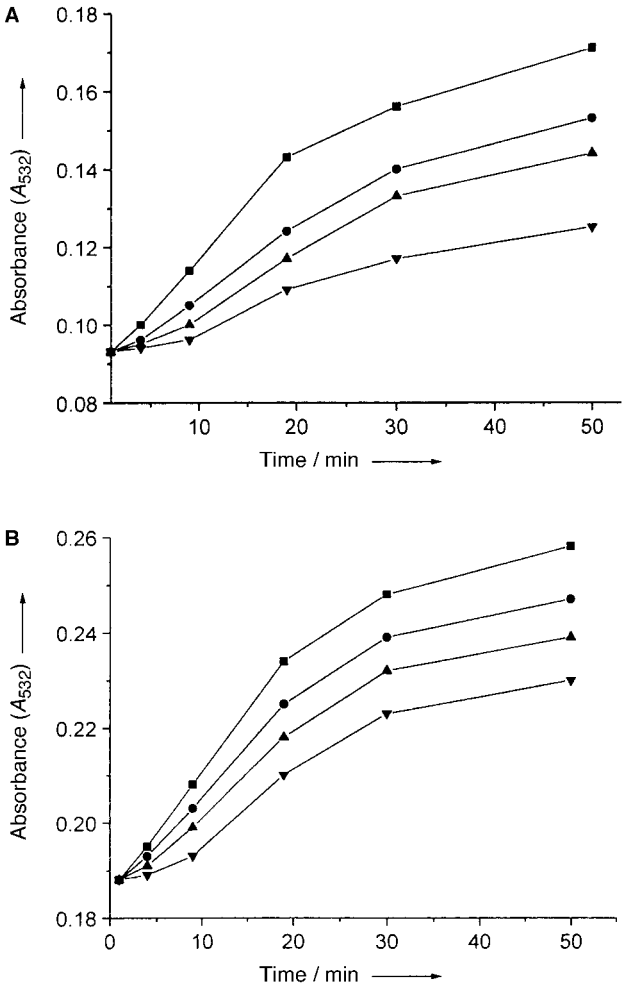


Figure 4. *A) Dependence of extent of MDA accumulation on concentration of 2-TeCD. ■ = damage; ● = damage + 2 μM 2-TeCD; ▲ = damage + 8 μM 2-TeCD; ▼ = damage + 16 μM 2-TeCD. B) Effect of different GPX mimics on the MDA accumulated during damage of mitochondria. ■ = damage; ● = damage + 8 μM Ebselen; ▲ = damage + 8 μM 2-SeCD; ▼ = damage + 8 μM 2-TeCD. For damage conditions, see Materials and Methods section. The optical density (absorbance) values represent MDA equivalents and each is the mean of three determinations. The ordinate axes have been interrupted in both (A) and (B).*

control group was basically constant, whereas the absorbance for the damaged group decreased considerably with time which shows that the mitochondrial swelling was considerably increased. However, the swelling was apparently inhibited for the protected group, which contained a known concentration of 2-TeCD, and this swelling of the mitochondria decreased further as 2-TeCD concentrations were increased. The GPX mimics 2-TeCD, 2-SeCD, and Ebselen displayed different levels of ability to inhibit the swelling of mitochondria. As evidenced by Figure 3B, 2-TeCD was the best ROS scavenger among those studied. This is in agreement with the H_2O_2 removal activities of these GPX mimics.

Inhibition of lipid peroxidation of mitochondria by 2-TeCD

Figure 4A shows the extent of protection afforded by 2-TeCD. The amount of malondialdehyde (MDA) accumulated whilst the mitochondria were damaged was considerably reduced in the presence of 2-TeCD and this decrease in the amount of MDA became greater as the concentration of 2-TeCD was increased. When the 2-TeCD concentration was 16 μM, after 50 min only 35.8% of the lipids in the damaged group had been converted into MDA, therefore 64.2% of MDA production was inhibited. To gauge the capacity of the three GPX mimics, 2-TeCD, 2-SeCD, and Ebselen to inhibit MDA accumulation, their antioxidant activities were determined under identical conditions. As evidenced by Figure 4B, the capacity of 2-TeCD to decrease the MDA accumulation was greater than that of 2-SeCD and Ebselen.

Protection of CCO activity in damaged mitochondria by 2-TeCD

Cytochrome *c* oxidase (CCO) is one of the key redox enzymes in the electron transport chain of mitochondria and is also the

marker enzyme of mitochondria. The integrity of the mitochondrion lipid membrane is important for the activity of this enzyme. Figure 5 shows that the CCO activity was greatly decreased by ferrous sulfate/ascorbate-induced mitochondrion damage. After 60 min, the CCO activity of the damaged group was 59% of that of the control group, whereas 88% of CCO activity was retained in the presence of 8 μM 2-TeCD. After 60 min under identical conditions, the CCO activities retained in the presence of 8 μM of 2-SeCD or Ebselen were 79% and 71% of the control group activity, respectively. This results indicates that 2-TeCD was the most effective GPX mimic among those tested.

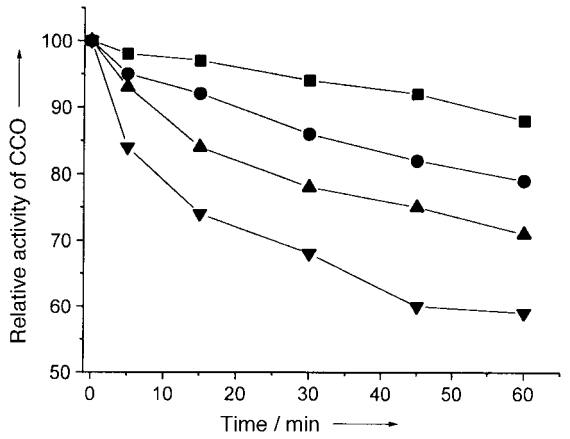


Figure 5. *Effect of different GPX mimics on CCO activity in damaged mitochondria. Activity of CCO in the control group is defined as 100%. ■ = damage + 8 μM 2-TeCD; ● = damage + 8 μM 2-SeCD; ▲ = damage + 8 μM Ebselen; ▼ = damage. For damage conditions, see Material and Methods section. Values are mean values from three sets of experiments. The ordinate axis has been interrupted.*

Discussion

Two systems were used to assess the GPX activity of the 2-TeCD-catalyzed reduction of hydroperoxides by thiols. The catalytic efficiency of 2-TeCD was compared with that of the model compound Ebselen and the indirect enzymatic assay system found that the 2-TeCD-catalyzed reduction of H_2O_2 by GSH is 46-fold more efficient than that with Ebselen. However, the direct assay system showed the catalytic efficiency of the 2-TeCD-catalyzed reduction of H_2O_2 by PhSH to be 83-fold more efficient than that with Ebselen. Clearly, the capacity to bind this thiol substrate is essential for the enzymatic activity. An enzyme binds its substrate and then stabilizes the transition state for a particular reaction. Enzymes must first recognize and bind their substrate to set up the correct geometry. The binding process is of key importance in the development of an enzyme mimic.[11] Cyclodextrin seems to have a preference for the hydrophobic compound PhSH (the aromatic group in PhSH) rather than the hydrophilic compound GSH.[16] This observation allows the difference between the activities seen in the two assay systems to be explained and suggests that attempts to enhance the catalytic activity of 2-TeCD should focus on the binding of the thiol substrate by redesign of the cyclodextrin to incorporate the binding group and thus facilitate binding of the thiol substrate.

The rate constants of the spontaneous reaction between hydroperoxides and GSH vary in magnitude in the order $k(H_2O_2) > k(CuOOH) > k(t\text{BuOOH})$. In contrast, the analogous bimolecular rate constants for 2-TeCD (k_{max}/K_{ROOH}) vary as $k(CuOOH) > k(H_2O_2) > k(t\text{BuOOH})$. It is possible that the first order reflects the intrinsic rate of reaction between the hydroperoxides and thiols in the absence of any significant binding effects, while the latter order indicates that CuOOH could have some binding advantage in the hydrophobic cavity of the cyclodextrin and hence be able to raise its k_{max}/K_{ROOH} value above that of H_2O_2 and *t*BuOOH by lowering K_{CuOOH}. Cyclodextrin is known to favor aromatic groups in the cavity, but further work will be necessary to clarify this assumption.

The values of k_{max}/K_{ROOH} provide a measurement of the rate of reaction between the free enzyme and hydroperoxides. For native GPX, the equivalent bimolecular rate constants approach the diffusion limit. This is believed to reflect the fact that in the native enzyme the catalytic group selenocysteine is located in a shallow depression on the surface of the protein and may react with any approaching hydroperoxides.[17] The bimolecular rate constants for the reaction between GSH and the hydroperoxides are: $k_{+1}(t\text{BuOOH}) = 4.5 \pm 10^8\,\text{M}^{-1}\,\text{min}^{-1}$, $k_{+1}(CuOOH) = 7.7 \times 10^8\,\text{M}^{-1}\,\text{min}^{-1}$, and $k_{+1}(H_2O_2) = 3.5 \times 10^9\,\text{M}^{-1}\,\text{min}^{-1}$, as determined previously.[17] Under similar conditions, these values for 2-TeCD are: $k_{+1}(t\text{BuOOH}) = 5.24 \times 10^4\,\text{M}^{-1}\,\text{min}^{-1}$, $k_{+1}(CuOOH) = 2.71 \times 10^5\,\text{M}^{-1}\,\text{min}^{-1}$, and $k_{+1}(H_2O_2) = 7.99 \times 10^4\,\text{M}^{-1}\,\text{min}^{-1}$. The bimolecular rate constants for the reactions between 2-TeCD and these hydroperoxides are approximately 4–5 orders of magnitude lower than those for the native GPX. The catalytic efficiency of the 2-TeCD-catalyzed reduction of hydroperoxides by GSH is higher than that of any other cyclodextrin-derived GPX mimics, as evidenced by the comparison of kinetic data. For example, the apparent second-order rate constant $k_{max}/K_m(H_2O_2)$ for 2-SeCD was determined to be $5.16 \times 10^4\,\text{M}^{-1}\,\text{min}^{-1}$,[13] but, under identical conditions, the equivalent parameter for 2-TeCD is $7.99 \times 10^4\,\text{M}^{-1}\,\text{min}^{-1}$. This may be caused in part by the fact that the catalytic mechanisms of 2-TeCD and 2-SeCD are different, thus it is necessary to characterize the catalytic intermediates.

As discussed above, 2-TeCD accepts a variety of structurally distinct thiol compounds and hydroperoxides as substrate, and the catalytic activity is dependent upon the nature of both the thiol compound and the hydroperoxide. These observations offer mechanistic insights, which may facilitate the rational improvement of the efficiency of the enzyme. Our enzyme model, which only has a hydrophobic environment for the substrate, is simpler than the native enzyme, which has not only a hydrophobic environment (Phe and Trp) but also two Arg residues and one Gln residue that form a salt bridge and hydrogen bond with GSH to help bind it.[18] It appears possible to obtain strong substrate binding by modification of cyclodextrin, based on molecular design. Kinetic experiments with the radical trap BHT suggest that, while the spontaneous reduction of *t*BuOOH by GSH involves free radicals, the same reaction catalyzed by 2-TeCD does not. These investigations help to define the mechanism of the peroxidase activity of 2-TeCD, many

aspects of which are very similar to that of the natural GPX, which does not involve free radicals.[19]

Mitochondria are one of the major sources of endogenous ROS in the cell, which include superoxide anions, hydrogen peroxides, and hydroxyl radicals.[20] Under normal conditions, there is a balance between the production of ROS and their destruction. When antioxidant defense system is overwhelmed, the accumulation of ROS results in injury to mitochondria. Moreover, it has recently been revealed that ROS of mitochondria regulate the physiological state of the cell and influence cell death.[2, 21] Therefore, we chose the mitochondrion as the model for our oxidative damage experiments. Exposing mitochondria in vitro to redox active xenobiotics can mimic the oxidative damage of mitochondria in vivo. The reactions involved in ferrous sulfate/ascorbate-induced mitochondrial damage can be proposed as in Equations (2), (3), and (4) in which H_2O_2 is produced by oxidation of ascorbic acid to dehydroascorbic acid, and then hydroxyl radicals are produced by the Fenton reaction.

$$\text{Ascorbic acid} + O_2 \rightarrow \text{Dehydroascorbic acid} + H_2O_2 \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (3)$$

$$\text{Ascorbic acid} + 2Fe^{3+} \rightarrow \text{Dehydroascorbic acid} + 2Fe^{2+} + 2H^+ \quad (4)$$

The biological molecules in the mitochondria were easily attacked by hydroxyl radicals, then changes in the mitochondrial composition, morphology, structure, integrity, and function took place. These changes were similar to the symptoms of mitochondrial disease. GPX and GPX mimics can scavenge hydroperoxides, block the production of hydroxyl radicals, and therefore protect mitochondria against oxidative damage.

In the ferrous sulfate/ascorbate-induced mitochondrion damage model system, the extent of swelling, MDA content, and CCO activity of the mitochondria were chosen as standards to be used to determine the extent of injury and protection of the mitochondria. 2-TeCD reduced the swelling of the mitochondria during damage and decreased the maximal level of MDA accumulation as well as the rate of accumulation in its rapid phase. The extent to which swelling of the mitochondria and MDA accumulation is decreased by 2-TeCD is dependent upon the dose of 2-TeCD. The reason that 2-TeCD inhibited MDA accumulation and decreased the mitochondrial swelling is that it acted as a GPX mimic and effectively scavenged hydroperoxides, which protected the mitochondria against oxidative damage.

In conclusion, we have prepared a novel class of GPX mimic. 2-TeCD is an excellent GPX mimic, as evidenced by its enzymatic properties. Studies of the kinetics of the 2-TeCD-catalyzed reduction of hydroperoxides by GSH show that it is important to consider the binding of the substrate in designing GPX mimics. The investigations of mitochondrial damage induced by ferrous sulfate/ascorbate reveal that 2-TeCD is a better antioxidant than other GPX mimics. We anticipate that 2-TeCD may have potential for the treatment of ROS-mediated diseases and also for use in bioelectronic devices.

Materials and Methods

Apparatus: Characterization of the mimic structure was performed with a Varian Unity-400 NMR spectrometer, a Bruker IFS-FT66V infrared spectrometer, and a Perkin-Elmer 240 DS elemental analyzer. The tellurium content and valency were determined by an ESCALAB MKII X-ray photoelectron spectrometer. The spectrometric measurements were carried out with a Shimadzu 3100 UV/Vis-near-IR spectrophotometer interfaced with a personal computer. Data were acquired and analyzed by using ultraviolet spectroscopy (UVS) software. The temperature for UV time course studies was controlled within (±)0.5 °C by use of a LAUDA compact low-temperature thermostat RC6 CP.

Materials: β-Cyclodextrin (β-CD) was purchased from Tianjin Chemical Plant, recrystallized twice from water, and dried for 12 hours at 120 °C in a vacuum. 2-SeCD was prepared as described previously.[13] *p*-Toluene sulfonylchloride (TsCl) and *t*BuOOH were also obtained from Tianjin Chemical Plant. Benzenethiol (PhSH) and benzoic acid were bought from Shanghai Chemical Plant. Tellurium powder, sodium borohydride, GSH, NADPH, CuOOH, Ebselen, and glutathione reductase (type III) were purchased from Sigma. Thiobarbituric acid, ferrous sulfate, and cytochrome *c* were obtained from Shanghai Second Reagent Plant. Ascorbic acid was purchased from Fluka. Sephadex G-25 was purchased from Amersham Pharmacia Biotech, Uppsala, Sweden. All other chemicals were of the highest purity commercially available and were used without further purification.

Synthesis of 2,2′-tellurium bridged β-cyclodextrin (2-TeCD): The regiospecific monotosylation of the 2-position hydroxy group of β-CD was carried out to prepare 2-OTs-2-deoxy-β-CD (2-OTs-β-CD) as described previously.[13] Finely ground elemental tellurium (1.27 g) and sodium borohydride (0.9 g) were heated in ethanol (20 mL) at reflux under nitrogen for 1 hour. After cooling to ambient temperature, acetic acid (1.2 mL) was added to the solution. Nitrogen was bubbled through 2-OTs-β-CD (2 g) dissolved in potassium phosphate buffer (50 mM, 20 mL; pH 7.0) for 30 min and this was then added to the above solution. The mixture was kept under nitrogen for 48 hours at 60 °C then oxidized in air and finally purified by centrifugation and Sephadex G-25 column chromatography ($\Phi 5 \times$ A80cm; $\lambda = 254$ nm) with distilled, deionized water as the eluent. The resulting solution was freeze-dried and the lyophilized powder provided the yellow product in 62% yield.

Characterization of 2-TeCD: The structure of 2-TeCD was analyzed by means of elemental analysis, IR, ^{1}H NMR, and ^{13}C NMR spectroscopies. ^{1}H NMR (400 MHz, D_2O, 25 °C): $\delta = 4.99$ (1-H), 4.0 – 3.65 (3-H, 5-H, 6-H), 3.65 – 3.28 (2-H, 4-H); ^{13}C NMR (400 MHz, D_2O, 25 °C): $\delta =$ 100.8 (1-C), 98.5 (1′-C), 80.1 (4-C), 75.9 (2′-C), 72.3 (2-C), 70.9 (3-C, 5-C, 5′-C), 68.3 (3′-C), 59.5 (6-C, 6′-C); IR (KBr): $\tilde{\nu} = 3367$ (OH), 2928 (CH, CH_2), 1630, 1154, 1083, 1027 (—O—); elemental analysis: calcd (%) for $C_{84}H_{138}O_{68}Te_2 \cdot 6H_2O$ (2599.2): C 38.32, H 5.58; found: C 37.87, H 5.65.

Determination of the tellurium content and valency in 2-TeCD: The content and valency of tellurium in the mimic were determined by X-ray photoelectron spectroscopy. The energy of the exciting X-ray was 1253.6 eV (Mg, Kα). $C_{1s} = 285.0$ eV served as the standard. The scans were performed 10 times.

Estimation of GPX-like activity: The GPX-like activity of the 2-TeCD-catalyzed reduction of hydroperoxide by thiols was assessed by using both an indirect enzymatic assay (coupled test procedure assay)[14] and a direct assay.[15]

Indirect enzymatic assay: GPX activities were examined by following the oxidation of NADPH in the presence of GSSG reductase. This reductase catalyzes the reduction of oxidized GSH formed by GPX

with a slight modification. The sample and control cuvettes both contained potassium phosphate buffer (50 mM, pH 7.0), EDTA (1 mM), sodium azide (1 mM), GSH (1 mM), and GSH reductase (1 unit) in a total volume of 0.5 mL. An aliquot of enzyme was added to the sample cuvette only. The reaction mixture was preincubated at 37 °C for 7 min, after which the reaction was started by the addition of hydroperoxide (0.5 mM) to both cuvettes. Progress of the reaction was followed by observation of the decrease of NADPH absorption at 340 nm. The activity was corrected for the value recorded in the control experiment. One unit of enzyme activity was defined as the amount of mimic that utilizes 1 μmol of NADPH per minute.

Direct assay: The reaction was carried out at 25 °C in 0.5 mL of a solution which contained the appropriate enzyme mimic and potassium phosphate buffer (50 mM, pH 7.0) and PhSH (1 mM) dissolved in methanol. The reaction was initiated by the addition of H_2O_2 (0.5 mM) and was monitored by UV spectroscopy at 305 nm. The molar extinction coefficient of PhSSPh ($\varepsilon_1 = 1.24 \times 10^3\,M^{-1}cm^{-1}$) at this wavelength is much larger than that of PhSH ($\varepsilon_2 = 9\,M^{-1}cm^{-1}$). The concentration of PhSH (*C*) was therefore calculated from the absorbance (*A*) according to Equation (5).

$$C = (\varepsilon_1 C_0 - 2A)/(\varepsilon_1 - 2\varepsilon_2) \quad (5)$$

The initial reduction rate of H_2O_2 (v_0) was then determined from plots of the reciprocal of the velocity versus the reciprocal of the PhSH concentration.

Assay of kinetics of 2-TeCD: The enzymatic mimic-catalyzed reduction and the nonenzymatic reduction of hydroperoxides (H_2O_2, *t*BuOOH, and CuOOH) by GSH were monitored by observation of the decrease in NADPH absorbance at 340 nm at 37 °C. To investigate the dependence of rate on substrate concentration, the initial velocities were determined at several concentrations of one substrate while the concentration of the other was kept constant. All kinetic experiments were performed in 0.5 mL of a solution containing potassium phosphate buffer (50 mM, pH 7.0), ethylendiaminetetraacetate (EDTA; 1 mM), sodium azide (1 mM), GSH (1 mM), GSH reductase (1 unit), and appropriate concentrations of GSH, hydroperoxides, and 2-TeCD. The reaction was initiated by the addition of the hydroperoxides. The enzymatic rates were corrected for the background (nonenzymatic) reaction between hydroperoxide and GSH. Lineweaver–Burk plots were obtained by using the Origin 6.0 (professional version) program. For each set of experiments a straight line was drawn with the best-fit method.

Preparation of mitochondria: Bovine heart mitochondria were isolated from fresh bovine heart as described in ref. [22], suspended in sucrose (0.25 M), EDTA (10 mM), and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES)/NaOH buffer (25 mM, pH 7.4), and maintained at 0 °C. The concentration of mitochondrial protein was determined by using Commassie brilliant blue[23] with bovine serum albumin as the standard.

Ferrous sulfate/ascorbate-induced mitochondria damage: The incubation mixture consisted of KCl (0.125 M), $MgCl_2$ (1 mM), glutamate (5 mM), mitochondria (0.5 mg protein mL^{-1}), GSH (1 μM), and the appropriate amount of enzyme mimic in potassium phosphate buffer (10 mM, pH 7.4) at 37 °C. MDA content and swelling of the mitochondria were determined at intervals after the addition of ascorbate (0.5 mM) and ferrous sulfate (12.5 μM). Damage experiments were also done without the enzyme mimic; control experiments were performed in the absence of enzyme mimic, ascorbate, and ferrous sulfate.

Measurement of malondialdehyde: The level of lipid peroxidation was determined as the formation of malondialdehyde (MDA), the final product of lipid peroxidation. MDA content in ferrous sulfate/ascorbate-treated mitochondria was analyzed by a thiobarbituric acid (TBA) assay. In this assay, TBA reacts with MDA and/or other carbonyl byproducts of free-radical-mediated lipid peroxidation to give a 2:1 molar ratio of colored conjugates.[24] The reaction was terminated by addition of trichloroacetic acid (0.1 mL, 0.5 % (w/w)). In plots of lipid peroxide formation, the absorbance reading is plotted directly rather than by means of a fixed conversion into MDA and/or other carbonyl byproduct equivalents.

Assays for mitochondria swelling: Swelling of mitochondria was assayed as described by Hunter et al.[25] Changes in light scattering are correlated with mitochondrial swelling. The swelling was measured as the decrease in turbidity of the reaction mixture at 520 nm. The decrease of the absorbance indicates an increase in the mitochondrial swelling and a decrease in mitochondrial integrity.

Assay of cytochrome *c* oxidase activity: An aliquot of incubation mixture was taken at different time intervals and centrifuged (10000 g, 4 °C, 2 min). The pellet was washed with potassium phosphate buffer (10 mM, pH 7.4) containing KCl (125 mM), MgCl (1 mM), and glutamate (5 mM), then suspended in a small amount of potassium phosphate buffer (100 mM, pH 7.0) and an aliquot taken to assay the cytochrome *c* oxidase (CCO) activity.[26] The CCO activity was measured in 2 mL of reaction system, in which the cytochrome *c* concentration was 15 μM. The absorbance decreased with oxidation of cytochrome *c* in the sample cell. $K_3Fe(CN)_6$ (5 μL of a 10 mM solution) was added to oxidize the cytochrome *c* thoroughly when the reaction was complete. The absorbance intensity at this time was recorded as A_8. A plot of $\ln(A_t - A_8)$ versus time was made (A_t = absorbance at time *t*). The absolute value of the line slope, K_{app}, was the apparent rate constant of cytochrome *c* oxidation and was used to express the COO activity.

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