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Reversible Ca^{2+} Switch of An Engineered Allosteric Antioxidant Selenoenzyme**

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Abstract: *A Ca^{2+}-responsive artificial selenoenzyme was constructed by computational design and engineering of recoverin with the active center of glutathione peroxidase (GPx). By combining the recognition capacity for the glutathione (GSH) substrate and the steric orientation of the catalytic selenium moiety, the engineered selenium-containing recoverin exhibits high GPx activity for the catalyzed reduction of H_2O_2 by glutathione (GSH). Moreover, the engineered selenoenzyme can be switched on/off by Ca^{2+}-induced allosterism of the protein recoverin. This artificial selenoenzyme also displays excellent antioxidant ability when it was evaluated using a mitochondrial oxidative damage model, showing great potential for controlled catalysis in biomedical applications.*

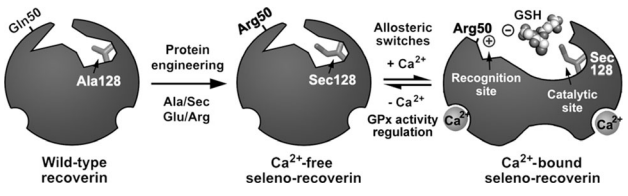
Many allosteric enzymes act as key regulators involved in the regulation of the rates of various metabolic pathways for signal transduction, cell growth, and survival.[1] Mimicking living systems through the design of artificial "switch" enzymes using stimuli-responsive materials by changing their structure and properties in response to external stimuli such as temperature,[2] pH,[3] light,[4] and small molecules,[5] has attracted increasing attention in recent years. Among these successful examples, the progress of naturally engineered allosteric proteins employed to dynamically modulate enzyme activity is still relatively slow.[6] The major challenges of this strategy include not only grafting new catalytic moieties (e.g., catalytic and recognition components) into an appropriate position within a substrate-binding cleft, but also utilizing the allosteric conformational change to precisely control the designed protein function.

Herein, we attempt to use an allosteric protein as a scaffold to design a smart artificial GPx enzyme that has the ability to spontaneously respond to the concentration change of reactive oxygen species (ROS). It is well known that ROS are normal products of aerobic metabolism for signal transduction, but excessive ROS also could oxidize biomacromolecules and ultimately lead to cell death.[7] This dual property of ROS depends on a fine regulation of its concentration. GPx is an antioxidant selenoenzyme that protects biomembranes and other cellular components against oxidative damage. Its efficient antioxidant activity mainly depends on the presence of the rare amino acid residue selenocysteine (Sec) that can catalyze the reduction of a variety of hydroperoxides (ROOH) by use of reduced glutathione (GSH).[8] In addition, some specific residues such as arginine are able to stabilize the binding between GSH and GPx to increase its catalytic efficiency through an intramolecular synergistic effect of catalysis and recognition. The catalytic reaction equation is as follows:

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

The mitochondrion is the main location of aerobic respiration and the transient storage site of Ca^{2+}.[9] When ROS attack the mitochondrial membrane, the ability of mitochondria to rapidly take in Ca^{2+} by a membrane-potential-dependent uniporter begins to decline, which ultimately triggers the release of Ca^{2+} back to the cytoplasm of the cell.[10,11] Thus, there is a direct correlation between ROS and cytosolic free calcium concentration. If the designed artificial GPx is sensitive to the Ca^{2+} concentration, it could also respond to the ROS concentration change and thereby remove the excess ROS intelligently.

In this work, a Ca^{2+}-binding protein, recoverin,[12] was chosen as a scaffold to construct a Ca^{2+}-responsive artificial GPx (Scheme 1). As reported by NMR structural studies,



Scheme 1. Schematic representation of the design of a Ca^{2+}-switched artificial GPx based on recoverin by incorporating a catalytic Sec residue and a recognition site (Arg50).

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recoverin undergoes a large conformational change upon Ca^{2+} binding, which mainly occurs in the N-terminal domain.[13] Using molecular docking, we determined the low-energy interaction mode between this region and the GSH substrate (Figure S1). The results showed that Ala128 is localized in a sterically favorable position with respect to the reactive sulfhydryl group of GSH, suggesting that Ala128 could be appropriately substituted by the catalytically competent Sec for the nucleophilic attack of GSH. As shown in Figure 1 A, the catalytic selenium center, Sec128, is surface-

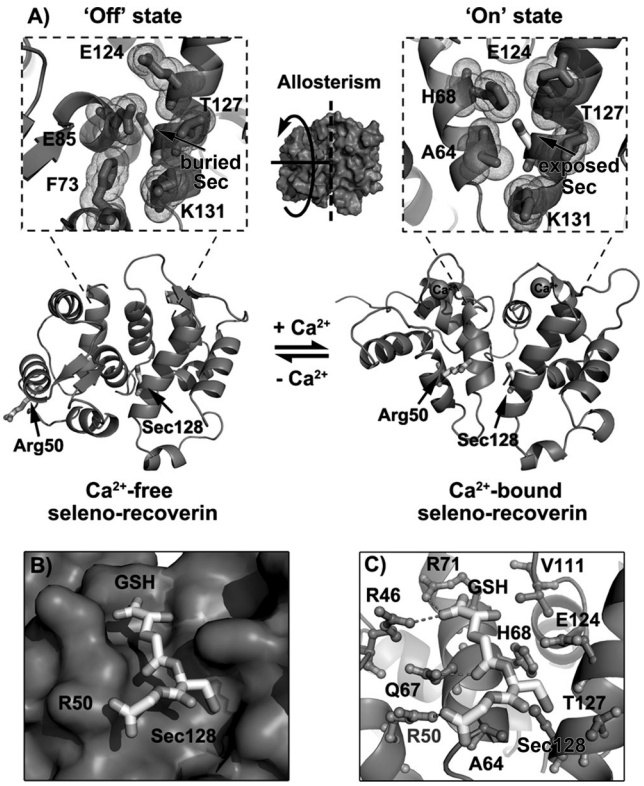

Figure 1. A) The structure and Sec-microenvironment of seleno-recoverin in the "off"- and "on"-states. B) The GSH-binding cleft and C) the detailed recognition mechanism between GSH and seleno-recoverin-Q50R simulated by molecular docking.

exposed in the Ca^{2+}-bound state of recoverin, whereas it would be buried in the Ca^{2+}-free state. This allosterism-induced conformational change could lead to a Ca^{2+}-switched regulation of this GPx enzymatic activity. Moreover, electrostatic interactions between both Arg46 and Arg71 and one carboxyl group of GSH have been observed in the docking complex model (Figure 1 C). To further enhance the recognition capacity of GSH when designing the binding site of this GPx mimic, glutamine at position 50 has been replaced by an arginine (Q50R) to increase the binding specificity of the other carboxyl group of GSH (Figure 1 B,C).

Based on the above-mentioned computational design, Cys39 located in a random coil of wild-type recoverin was first replaced by Ser (C39S) to eliminate its possible interference. Then, Ala128 was mutated to Cys (A128C) and the plasmid carrying the cDNA of the recoverin-C39S/A128C mutant was transformed in the *E. coli* derived strain BL21cysE51. We engineered Sec128 into the GSH-binding site of recoverin (seleno-recoverin) by a cysteine-auxotrophic system[14] and generated the Q50R mutant (seleno-recoverin-Q50R) using site-directed mutagenesis. The purified wild-type recoverin and its mutants were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Figure S2) and MALDI-TOF mass spectrometry, clearly demonstrating their purity and the successful incorporation of Sec (Figure S3). Furthermore, the circular dichroism (CD) spectra of seleno-recoverin-Q50R showed a striking increase in negative ellipticity at 222 nm after adding at least five equivalents of $CaCl_2$ to the Ca^{2+}-free solution (Figure S4), revealing a conformational rearrangement upon Ca^{2+} binding.[15] This result is consistent with the Ca^{2+}-induced conformational changes of wild-type recoverin observed by NMR analyses.[13]

The GPx activity of these newly designed proteins has been evaluated using a GSH reductase-reduced nicotinamide adenine dinucleotide phosphate (NADPH) coupled assay[16] and the control experiment indicated that calcium has no direct influence on the GPx activity. Like native GPxs, Ca^{2+}-bound seleno-recoverin catalyzes the reduction of H_2O_2 by GSH with an activity of 23.5 $\mu mol\,min^{-1}\,\mu mol^{-1}$ (Figures S5 and S6), whereas no GPx activity was observed with wild-type recoverin, recoverin-C39S/A128C, and Ca^{2+}-free seleno-recoverin (Table 1). Given that only the catalytic Sec site

Table 1: GPx activities of wild-type recoverin, recoverin mutants, and other catalysts.[a]

Catalyst	Substrate	Activity [$\mu mol\,min^{-1}\,\mu mol^{-1}$]
wild-type recoverin	H_2O_2	ND[b]
recoverin-C39S/A128C	H_2O_2	ND
Ca^{2+}-free seleno-recoverin	H_2O_2	ND
Ca^{2+}-bound seleno-recoverin	H_2O_2	23.5 ± 0.5
Ca^{2+}-bound seleno-recoverin-Q50R	H_2O_2	140 ± 3
ebselen[17]	H_2O_2	1
pGPx (human plasma)[18]	H_2O_2	302
native GPx (rabbit liver)[17]	H_2O_2	5780

[a] The GPx activities of all catalyst above were determined by the same method. The concentration of GSH and H_2O_2 were 1 and 0.5 mM, respectively (for more details, please see SI). [b] ND: no detectable GPx activity.

was incorporated into recoverin, the resulting GPx activity of seleno-recoverin was relatively low. However, a significant improvement has been achieved when the additional Q50R mutation was conducted in the seleno-recoverin. As shown in Table 1, Ca^{2+}-bound seleno-recoverin-Q50R was much more efficient than seleno-recoverin, with a high GPx activity of up to 140 $\mu mol\,min^{-1}\,\mu mol^{-1}$ (Figure S5), thus suggesting that the mutation Q50R highly improved GSH binding and, consequently, the catalytic efficiency of seleno-recoverin by promoting its substrate recognition capacity. Compared to ebselen,[19] a well-known GPx mimic without substrate binding site, a 140-fold enhanced activity was observed. Moreover, the activity of seleno-recoverin-Q50R was found to achieve the same level of magnitude as that of some native GPx, such

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as pGPx (Table 1).[19] These data suggest that a synergy most likely takes place between the catalytic and recognition site of seleno-recoverin-Q50R, analogous to that of native GPxs.

The Ca^{2+}-switched GPx activity of seleno-recoverin-Q50R was evaluated by the GSH assay system using H_2O_2 as a substrate. In the Ca^{2+}-free form of seleno-recoverin-Q50R, Sec128 is located in the interior of the protein and is therefore unable to attack the substrate GSH. Therefore, no GPx activity is displayed in these experiments. In contrast, upon binding Ca^{2+}, seleno-recoverin-Q50R undergoes a conformational change with Sec128 exposed on the surface of the protein, which is favorable to the catalytic reduction of H_2O_2 by GSH (Figure 2A). As a switchable GPx model, the reversibility of the catalytic activity of seleno-recoverin-Q50R was also tested. The results revealed that the catalytic activity of this enzyme is completely reversible after multiple Ca^{2+}-bound and Ca^{2+}-free cycles (Figure 2B).

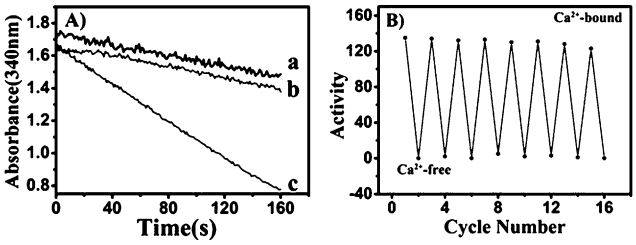

Figure 2. A) Catalytic curves of Ca^{2+}-free and Ca^{2+}-bound seleno-recoverin-Q50R. a) Ca^{2+}-free seleno-recoverin-Q50R; b) negative control containing no seleno-recoverin-Q50R; c) Ca^{2+}-bound seleno-recoverin-Q50R. B) Catalytic activity of seleno-recoverin-Q50R in the presence and absence of calcium ions.

To investigate the enzymatic kinetics, the initial rate of reduction of H_2O_2 catalyzed by Ca^{2+}-bound seleno-recoverin-Q50R was assayed as a function of the substrate concentration. Double-reciprocal plots of the initial velocity versus substrate concentration yielded a family of parallel lines for both substrates (Figure 3), which indicates a ping–pong kinetic mechanism. The apparent kinetic parameters are listed in Table S1. The second-order constant $k_{cat}/K_{M\,H_2O_2}$ and $k_{cat}/K_{M\,GSH}$ are $10^5\,M^{-1}min^{-1}$ and $10^6\,M^{-1}min^{-1}$, respectively. These kinetic constants are relatively high and are comparable to those of some GPx mimics such as Se-4 A4 and protein-imprinted GPx mimics,[20] although they are lower than those of some natural GPxs.[21] Furthermore, we investigated the dependence of this activity on pH and temperature (Figure S7). An optimal pH of 7.0 and optimal temperature of 45 °C were found for Ca^{2+}-bound seleno-recoverin-Q50R (the optimal pH and temperature are 8.8 and 50 °C, respectively, for the native GPx).[22]

The Ca^{2+}-switchable performance of seleno-recoverin-Q50R should be robust enough when it comes to maintaining beneficial antioxidant level in cells. Mitochondria were widely used to evaluate the protecting effects of cells against oxidative damage. Once mitochondria are damaged by oxidative stress, their integrity decreases and swelling occurs, leading to a decrease of the absorbance at 520 nm. Also, malondialdehyde (MDA) accumulates in this process. The swelling assay was measured by light scattering. When the mitochondria were incubated in the presence of oxidant (ferrous sulfate/ascorbate), damage was induced and a large swelling was observed (curve e, Figure 4A). When mitochondria were incubated with 0.90 μM Ca^{2+}-free seleno-recoverin-Q50R in the presence of oxidant, the absorbance was almost at the same level as that of the damaged mitochondria (compare curves d and e, Figure 4B). However, a smaller swelling was observed when mitochondria were incubated with 0.45 μM or 0.90 μM Ca^{2+}-bound seleno-recoverin-Q50R in the presence of the oxidant (curves c and b, Figure 4A). Moreover, it is noteworthy that the protecting effect of Ca^{2+}-bound seleno-recoverin-Q50R is much larger than that of the well-known GPx mimic, ebselen (curve d, Figure 4A).

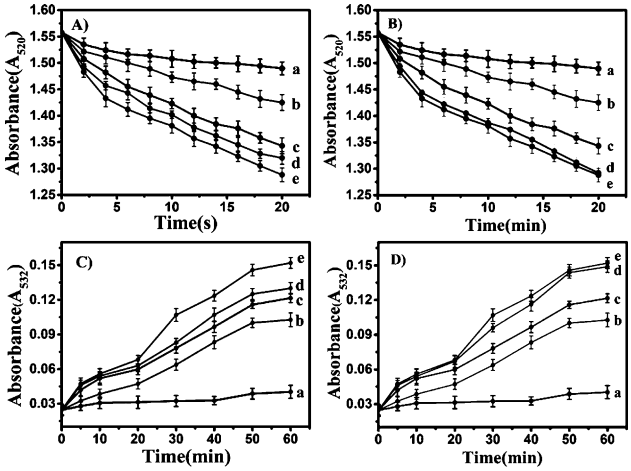

Figure 4. Determination of mitochondria swelling (A, B) and lipid peroxidation (C, D). A) control with no oxidant (a); 0.90 μM and 0.45 μM of Ca^{2+}-bound seleno-recoverin-Q50R (b, c); 8 μM ebselen (d); mitochondrial damage in the presence of ferrous sulfate/ascorbate and no GPx mimics (e). B) control with no oxidant (a); 0.90 μM and 0.45 μM of Ca^{2+}-bound seleno-recoverin-Q50R (b, c); 0.90 μM of Ca^{2+}-free seleno-recoverin-Q50R (d); mitochondrial damage in the presence of ferrous sulfate/ascorbate and no GPx mimics (e). The concentration of GPx mimics in (C) and (D) are the same as those in (A) and (B), respectively.

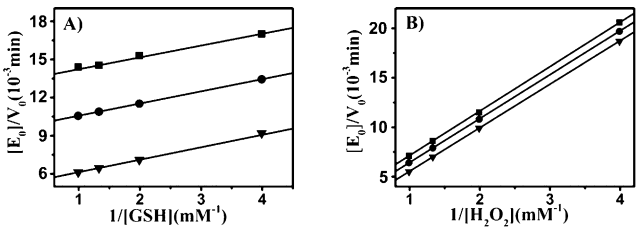

Figure 3. Double-reciprocal plots of the reduction of H_2O_2 by GSH under the catalysis of Ca^{2+}-bound seleno-recoverin-Q50R ($[E_0]$ = total enzyme concentration). A) $[E_0]/V_0$ versus 1/[GSH] (mM^{-1}) at $[H_2O_2]$ = 0.25 (■), 0.50 (●), and 1.00 mM (▼). B) $[E_0]/V_0$ versus $1/[H_2O_2]$ (mM^{-1}) at [GSH] = 0.25 (■), 0.5 (●), and 1.00 mM (▼).

The production of mitochondrial lipid peroxidation was determined using a thiobarbituric acid (TBA) assay as described previously.[23] MDA is known to react with TBA

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to form a colored conjugate with a 2:1 molar ratio.[24] As shown in Figure 4C, a large amount of accumulated MDA was observed when mitochondria were incubated in the presence of oxidant (ferrous sulfate/ascorbate) (curve e, Figure 4C), and the addition of 0.90 μM Ca^{2+}-free seleno-recoverin-Q50R did not result in any inhibitory effect (curve d, Figure 4D). However, MDA accumulation was obviously reduced in the presence of 0.90 μM Ca^{2+}-bound seleno-recoverin-Q50R (curve b, Figure 4C) and this inhibition was larger than that observed when using ebselen (curve d, Figure 4C). The results are thus showing that Ca^{2+}-bound seleno-recoverin-Q50R is able to reduce the swelling of mitochondria as a consequence of oxidative damage as well as to a decrease of the maximal level of MDA accumulation in its rapid phase of production. In contrast, Ca^{2+}-free seleno-recoverin-Q50R could not provide this protection because its active-site Sec is sequestered in the interior of the enzyme. Although the complexity of this system is far from that taking place in cells, to some extent, it simulates well the intracellular redox changes. This system could thus provide a basic concept for further designing more intelligent artificial enzymes based on natural protein scaffolds.

In conclusion, the protein recoverin was used as a scaffold to develop a Ca^{2+}-switched artificial selenoenzyme by introducing a GPx-like machinery. The catalytic behavior and biological effects of this GPx mimic clearly demonstrate that its activity is tightly controlled by its conformational changes upon Ca^{2+} binding. This strategy opens a new way in the design of efficient artificial enzymes with environmental responsiveness. It is anticipated that such smart artificial selenoenzymes could be controlled according to the needs of the human body in the future.

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Keywords: antioxidants · artificial glutathione peroxidase · cysteine-auxotrophic system · recoverin · selenocysteine

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