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Rational redesign of the active site of selenosubtilisin with strongly enhanced glutathione peroxidase activity

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ABSTRACT

The search for a perfect model to mimic the properties of the selenoenzyme glutathione peroxidase (GPx) has inspired great interest. Rational design and redesign of the structure–function relationship has become an indispensable technique. In this report, the active site of selenosubtilisin was successfully rebuilt by transferring the catalytically essential residue selenocysteine (Sec) to the edge of the substrate-binding pocket of the enzyme by artificial manipulation. Founding on computer-aided molecular simulation, the amino acid residue at position 63 (Ser in the wild-type enzyme) was selectively replaced with Sec using a cysteine auxotrophic expression system. The novel seleno^{63}-subtilisin E gave a prominent 100-fold higher efficiency than the original seleno^{221}-subtilisin E for GPx activity. Moreover, this seleno^{63}-subtilisin E also had efficient hydrolase activity.

1. Introduction

Enzyme redesign to optimize the catalytic properties and understanding of directed evolution are a promising and challenging investigation [1]. It includes changes in substrate specificity, introducing new catalytic functions into the same active site, and conversion of ligand-binding sites into catalytic centers [2,3]. With the advent of tools for enzyme engineering, such as site-directed mutagenesis and three-dimensional structure prediction by computer-aided molecular simulation, active site redesign is gaining importance as a promising starting point for the rational redesign of enzymes [4].

Glutathione peroxidase (GPx, EC1. 11.1.9) is a structurally and functionally well studied selenoenzyme that catalyzes the reduction of hydroperoxides (ROOH) by glutathione (GSH) [5,6]. It is thought to be an important antioxidant enzyme in the prevention of lipid peroxidation and the corresponding disruption of membrane function. Enormous effort has been made in the development of artificial GPx models to explore its structure–function relationships [7,8], for example, introducing a catalytic center into an existing or artificially generated substrate-binding scaffold by chemical or genetic strategies [9]. However, single chemical modification of the enzyme's primary catalytic group or alteration of some vicinal amino acid residues of the essential active site usually having some shortcomings resulting in not fulfilling perfectly efficient enzyme models.

Selenosubtilisin is the first GPx-like selenoenzyme obtained by chemical conversion of catalytic Ser221 to selenocysteine (Sec) to mimic GPx [10,11]. Our group has also generated it successfully using a cysteine auxotrophic expression system and has achieved high yield and catalytic efficiency [12]. The selenium side chain of Sec221 in selenosubtilisin is buried in a much deeper pocket and is presumably less accessible than its natural counterpart to hydroperoxides. Thus, this selenium-containing enzyme could not adopt the natural counterpart GSH of GPx but employs the aromatic donor 3-carboxy-4-nitroben-zenethethiol (ArSH) as ta reduced substrate. The GPx activity for enzyme-catalyzed reduction of H_2O_2 by ArSH is 4 μmol min^{-1} $μmol^{-1}$, which is much lower than the native GPx of 5780 μmol min^{-1} $μmol^{-1}$ [13].

Based on the principle of rational enzyme redesign, combined with computer-aided molecular simulation, we provide insight into biological function and mechanism in this investigation via free artificial manipulation of the essential catalytic group in the active site of enzymes to enhance the enzyme activity [14]. We redesigned active-site-directed mutation by first transferring the catalytically essential Sec to the edge of the substrate-binding pocket of the enzyme [15]. Depending on automated molecular docking by computer and the principle of energy minimization, the Ser63 was selectively replaced with Sec to be a novel GPx

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mimic seleno^{63}-subtilisin E [16]. Indeed, this alteration would allow the other substrate molecule to touch the catalytic site more easily and consequently enhance the catalytic efficiency. To this end, the novel seleno^{63}-subtilisin E was generated using a cysteine auxotrophic expression system. Its GPx activity was substantially increased in comparison to that of seleno^{221}-subtilisin E, and it retained efficient hydrolase activity.

2. Materials and methods

2.1. Materials

Plasmids and strains used in this study are listed in Table 1. Plasmid pSUB1 was kindly provided by Masayori Inouye of the University of Medicine and Dentistry of New Jersey, and the strains *Escherichia coli* DH5α and *E. coli* BL21DE3 were kept by our laboratory. The cysteine auxotrophic strain *E. coli* BL21cysE51 was a generous gift from August Bock of the Lehrstuhl fur Mikrobiologie der Universität München. Site-directed mutagenesis was carried out directly on the plasmid pET11a-prosubtilisin E, and the mutations were confirmed by DNA sequencing. A synthetic oligonucleotide (5′-CTTATAACGGAACGTGCA TGGCGACTCCTCA-3′) has been employed as two primers to introduce an active-site (Ser221 → Cys221) mutation into the wild-type subtilisin E gene. The other synthetic oligonucleotides, 5′-ACCAGGACGGCAGTTGTCACGGTAC GCATGT-3′, were for mutating Ser63 to Cys63. All chemical reagents and solvents were obtained from Sigma-Aldrich.

The DNA sequence of subtilisinE(S221C) is as follows:

1 GCGCAAAGCT TTCCTTATGG CATTTCTCAA ATTAAAGCGC CGGCTCTTCA
51 CTCTCAAGGC TACACAGGCT CTAACGTAAA AGTAGCTGTT ATCGACAGCG
101 GAATTGACTC TTCTCATCCT GACTTAAACG TCAGAGGCGG AGCTAGCTTC
151 GTACCTTCTG AAACAAACCC ATACCAGGAC GGCAGTTCTC ACGGTACGCA
201 TGTAGCCGGT ACGATTGCCG CTCTTAATAA CTCAATCGGT GTTCTGGGCG
251 TAGCGCCAAG CGCATCATTA TATGCAGTAA AAGTGCTTGA TTCAACAGGA
301 AGCGGCCAAT ATAGCTGGAT TATTAACGGC ATTGAGTGGG CCATTTCCAA
351 CAATATGGAT GTTATCAACA TGAGCCTTGG CGGACCTACT GGTTCTACAG
401 CGCTGAAAA AGTCGTTGAC AAAGCCGTTT CCAGCGGTAT CGTCGTTGCT
451 GCCGCAGCCG GAAACGAAGG TTCATCCGGA AGCACAAGCA CAGTCGGCTA
501 CCCTGCAAAA TATCCTTCTA CTATTGCAGT AGGTGCGGTA AACAGCAGCA
551 ACCAAAGAGC TTCATTCTCC AGCGCAGGTT CTGAGCTTGA CATGATGGCT
601 CCTGGCGTGT CCATCCAAAG CACACTTCCT GGAGGCACAA ACGGCGCTTA
651 TAACGGAACG TGCATGGCGA CTCCTCACGT TGCCGGAGCA GCAGCGTTAA
701 TTCTTTCTAA GCACCCGACT TGGACAAACG CGCAAGTCCG TGATCGTTTA
801 AATCAACGTA CAAGCAGCTG CACAA

Table 1
Plasmids and strains.

Plasmids	
pET11a	T7 expression vector, Apr
pSUB2	pET11a carrying wild-type subtilisin E
Strains	
TG1	SupE hsdΔ5 thia Δ(lac-pro AB) F′[tra D36 pro AB + lac I q lac ZΔM 5]
DH5α	SupE44 ΔlaC U169 (φ80 lac ZΔM 15) hsdR17 recA1 endA1 gyrA96 thi-1 rel1
BL21(DE3)	hsdS gal (lcIts857 indI sam7 nin5 lacUV5-T7 gene 1)
BL21cysE51	BL21(DE3) selB: kan cysE51

2.2. Rational enzyme redesign by computational analysis

All studies were performed on an SGI O3800 workstation. They consisted of a binding-site module, flexible docking, and a three-step Ludi scores. In this study, identification of enzyme active sites and binding sites used ActiveSite-Search by locating cavities in the subtilisin E structure. When the search was completed, the largest site was automatically displayed on the structure using Asite-Display. The results were used to guide the protein–ligand docking experiments. By means of the 3D structures of subtilisin E and ArSH, which are built and optimized through the InsightII/Builder program, automated molecular docking was performed using the docking program Affinity. The potential function of the complexes was assigned using a consistent-valence force field (CVFF) and nonbonding interaction was dealt with by the cell multipole approach. To account for the solvent effect, the centered enzyme–ligand complexes were solvated in a sphere of TIP3P water molecules with radius 10 Å. Finally, the docked complex of the receptor with the ligand was selected by the criteria of interacting energy and geometrical matching quality. The Ludi method screens a large number of compounds and analyzes the geometrical fit of given chemicals into the binding site. It also can determine other good binding properties such as hydrogen bonds, lipophilic interactions, ionic interactions, and acyclic interactions. In general, a higher Ludi score represents a higher affinity and stronger binding of a ligand to the receptor.

2.3. Expression and purification of two selenosubtilisin E

Plasmid I (pET11a carrying mutant gene S221C) and plasmid II (pET11a carrying mutant gene S63C) were respectively transformed into strain *BL21cysE51*. Overexpression of seleno^{221}-subtilisin E and seleno^{63}-subtilisin E in the presence of selenocysteine was performed as already described for $(Se)_2$ –thioredoxin. The proteins, which were produced as inclusion bodies, were also purified on a CM-Sephadex-50 cation exchange column and renatured by dialyzing stepwise as described previously.

2.4. Expression and purification of wild-type subtilisin E and two thiolsubtilisin E

Plasmid I (S221C), plasmid II (S63C), and plasmid III (wild type) were transferred to *E. coli* strain BL21DE3. The three proteins thiol^{221}-subtilisin E, thiol^{63}-subtilisin E, and wild-type subtilisin E were expressed and purified according to the method of Li and Inouye.

2.5. Refolding of proteins

Purified proteins were dissolved in PBS buffer and dialyzed stepwise against a refolding buffer (50 mM Tris-HCl, pH 7.0, 1 mM $CaCl_2$, 0.5 M $(NH_4)_2SO_4$) containing decreasing amounts of urea (4, 2, 1, 0.5, and 0 M). After about 1 week incubation at 4 °C, the final refolding protein with no urea was applied to a Sephadex G-100 column to separate it from the unfolding protein.