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Directed evolution of glutathione transferases towards a selective glutathione-binding site and improved oxidative stability

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ABSTRACT

Background: Glutathione transferases (GSTs) are a family of detoxification enzymes that catalyze the conjugation of glutathione (GSH) to electrophilic compounds.
Methods: A library of alpha class GSTs was constructed by DNA shuffling using the DNA encoding the human glutathione transferase A1-1 (hGSTA1-1) and the rat glutathione transferase A1-1 (rGSTA1-1).
Results: Activity screening of the library allowed the selection of a chimeric enzyme variant (GSTD4) that displayed high affinity towards GSH and GSH-Sepharose affinity adsorbent, higher k_{cat}/K_m and improved thermal stability, compared to the parent enzymes. The crystal structures of the GSTD4 enzyme in free form and in complex with GSH were determined to 1.6 Å and 2.3 Å resolution, respectively. Analysis of the GSTD4 structure showed subtle conformational changes in the GSH-binding site and in electron-sharing network that may contribute to the increased GSH affinity. The shuffled variant GSTD4 was further optimized for improved oxidative stability employing site-saturation mutagenesis. The Cys112Ser mutation confers optimal oxidative stability and kinetic properties in the GSTD4 enzyme.
Conclusions: DNA shuffling allowed the creation of a chimeric enzyme variant with improved properties, compared to the parent enzymes. X-ray crystallography shed light on how recombination of a specific segment from homologous GSTA1-1 together with point mutations gives rise to a new functionally competent enzyme with improved binding, catalytic properties and stability.
General significance: Such an engineered GST would be useful in biotechnology as affinity tool in affinity chromatography as well as a biocatalytic matrix for the construction of biochips or enzyme biosensors.
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1. Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) comprise a large family of detoxifying enzymes that catalyze the conjugation of glutathione (GSH, γ-glutamyl-L-cysteinyl-L-glycine) with a diverse range of mainly hydrophobic electrophilic substrates [1,2]. GSTs are dimers of 26–29 kDa subunits. Each subunit consists of two structurally distinct domains: a thioredoxin-like domain 1 with βαβαββα folding topology (N-terminal domain) and an all α-helical domain 2 (C-terminal domain) [3,4]. The active site is located at the domain interface on each subunit [4]. GSTs are involved in redox signaling and antioxidant cellular mechanism and in the detoxification of carcinogens, and may be linked to carcinogenesis [4–6].

The multifunctional and wide catalytic capabilities of GSTs along with their good structural stability and ease of expression and purification have stimulated their exploitation in biotechnology. Today, the pGEX system is used widely for the overexpression in *E. coli* and affinity purification of a wide variety of proteins fused to the C-terminus of the GST isoenzyme from *Schistosoma japonicum* (*Sj*26GST) [7]. Other examples include the use of selected GST isoenzymes in the construction of enzyme biosensors for the direct monitoring of drugs, pollutants and pesticides in environmental and biological samples [8–11]. For example, a GST isoenzyme immobilized on carbon electrodes has been recently used for the detection and quantification of anticancer drugs (e.g. cisplatin) [10] and the thiocarbamate herbicide molinate [11]. In addition, due to their unique catalytic properties, GSTs are gaining increased attention in the field of green organic synthesis and nanobiotechnology for the construction of biochips [12–14] as well as nanowires and nanorings [15,16]. For example, the enzyme *Sj*26GST-2His was self-assembled in a fixed bending manner to form highly ordered protein nanorings, providing a de novo design strategy for the construction of novel protein superstructures [16]. In all these applications, the desired

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; GST, glutathione transferase; hGSTA1-1, human glutathione transferase A1-1; rGSTA1-1, rat glutathione transferase A1-1; *Sj*26GST, the GST isoenzyme from *Schistosoma japonicum*.

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requirements, concerning the catalytic and binding properties of a GST, are the high affinity and catalytic efficiency towards GSH as well as long-term stability. Research over the last years aiming at discovering natural GST isoenzyme from diverse arrays of organisms (plant, microbial, mammals, insects, etc.) showed that in the vast majority of studied isoenzymes their K_m for GSH is relative high (0.2–5 mM) [17–19]. Therefore, protein engineering efforts need to be undertaken for the design of new enzymes with improved GSH binding properties.

A protein design experiment requires a suitable protein scaffold. Robustness, tolerance towards mutations, ease of expression and purification are all very desirable properties. GSTs are good model systems for engineering studies of catalytic and binding properties, due to their modular feature with two binding sites: the GSH-binding site (G-site) and the hydrophobic site (H-site) for binding non-polar electrophilic substrates. Regarding specificity, the G-site is relatively stringent whereas the H-site is promiscuous [1–4]. During the last decade, delineation of important structural characteristics has laid the groundwork for experiments involving directed evolution of GSTs aiming at creating new forms with improved specificities. So far, several successful approaches have been undertaken to engineer the catalytic and binding properties of several GSTs. For example, Runarsdottir and Mannervik reported the creation of novel quasi-species of glutathione transferase with high activity towards naturally occurring isothiocyanates achieved by DNA shuffling from promiscuous low-activity variants from GST M1-1 and GST M2-2 [20]. In another work, stochastic recombination of fragments of cDNA encoding human GST T1-1 and rat GST T2-2 led to the isolation of a clone with 1700% increased activity with 1-menaphthyl sulfate and a 60% decreased activity with 4-nitrophenethyl bromide [21]. Similarly, Dixon et al., using reconstructive PCR, a series of mutant GSTUs were generated from in vitro recombination and mutagenesis of the maize sequences *Zm*GSTU1 and *Zm*GSTU2 [22]. Following screening, an enzyme with 19-fold higher catalytic efficiency towards fluorodifen was identified. In another work, a library of alpha class glutathione transferases (GSTs) composed of chimeric enzymes derived from human (A1-1, A2-2 and A3-3), bovine (A1-1) and rat (A2-2 and A3-3) cDNA sequences was constructed by the method of DNA shuffling. The study of sequence diversity of this library in combination with activity measurements allowed the identification of structural determinants of GSTs with azathioprine activity [23]. More recently, the generation of an engineered GSTU enzyme through DNA shuffling of three GSTU homologues from *Glycine max* (*Gm*GSTU2-2, *Gm*GSTU4-4, and *Gm*GSTU10-10) has produced a chimeric clone with enhanced detoxifying potential towards the nitrodiphenyl ether herbicide fluorodifen and unusual allosteric kinetics [24]. So far, in all reported directed evolution efforts of GSTs, the main scope was the design of an engineered enzyme with altered catalytic and binding properties towards the xenobiotic substrate, whereas, to the best of our knowledge, there is no published directed evolution study aiming at the GSH-binding properties.

In the present work, a library of GSTAs was constructed using DNA shuffling. Activity screening allowed the identification of a chimeric enzyme variant (GSTD4) that displayed improved kinetic properties and higher thermal stability, compared to the parent enzymes. This variant was further optimized by site-saturation mutagenesis for improved oxidative stability. Kinetic and structural analysis by X-ray crystallography shed light on how recombination of a specific segment from homologous GSTA1-1 together with point mutations gives rise to a new functionally competent enzyme with improved binding, catalytic properties and stability. All these properties make the GSTD4 enzyme suitable candidate for the development of new biotechnological applications.

2. Materials and methods

2.1. Materials

The TOPO plasmid pET101/D-TOPO and all other molecular biology reagents were obtained from Invitrogen (USA). The pGEX-3× vector was from Pharmacia (Sweden). Taq DNA polymerase and *Pfu* DNA polymerase were purchased from Promega. DyNAzyme EXT polymerase was obtained from Thermo Scientific (USA). Gel Extraction Kit and Mini Prep Kit were from Qiagen (Germany). Salts and buffers were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). The cloned rGSTA1-1 in pKKGTB vector was a much appreciated gift from Prof. W.M. Atkins, Department of Medicinal Chemistry, University of Washington.

2.2. Methods

2.2.1. DNA shuffling and screening

The plasmids pET101-GSTA1-1 [25] and pKKGTB [26] which contained the genes of hGSTA1-1 and rGSTA1-1, respectively, were used as templates in two separate PCR reactions. The reactions contained 10 ng/mL of each template, 0.2 mM of each dNTP, 10× *Pfu* buffer, 2.5 U *Pfu* DNA polymerase and 8 pmol of each primer. The primers used for the hGSTA1-1 were: the FGSTh (5′-CACCATGGCAGA GAAGCCCAAGCTCCAC-3′) as forward, and the RGSTh (5′-TTAAAACC TGAAAATCTTCCTTGCTTC-3′) as reverse. For the rGSTA1-1, the primers were: rFA (5′-ATGTCTGGGAAGCCAGTGCTTCAC-3′) as forward, and the rRA (5′-CTAAAACTTGAAAATCTTCCTTGC-3′) as reverse. Initial denaturation was achieved at 95 °C for 2 min. A total of 35 cycles of denaturation at 95 °C for 2 min, annealing at 55 °C for 2 min and polymerization at 72 °C for 2 min were followed by 10 min at 72 °C. The PCR products were run on a 1.2% (w/v) agarose gel, excised and purified from gel with QIAquick kit (Qiagen). For DNA fragmentation, equal proportions of the two purified PCR products were mixed and equilibrated at 15 °C. Digestion buffer (50 mM Tris-HCl pH 7.4, equilibrated at 15 °C), was mixed with $MnCl_2$ (10 mM, final concentration) and Dnase I (0.5 U, Mg^{2+} was removed from its buffer using mini gel filtration column). The digestion was performed at 15 °C. At different time-points, small aliquots were taken out and run on a 2% (w/v) agarose gel to check the progress of the fragmentation. The reaction was stopped (15 min) by addition of EDTA (50 mM). DNA fragments (approximately 50–100 bp) were recovered from the gel using the QIAquick kit (Qiagen). The recovered DNA fragments were subjected to PCR in the presence of 0.2 mM of each dNTP, 10× *Taq* buffer and 2.5 U *Taq* polymerase. Initial denaturation was achieved at 94 °C for 3 min. The PCR comprised of 50 cycles of denaturation at 94 °C for 30 s, annealing at 45 °C for 2 min and polymerization at 72 °C for 2 min, followed by 10 min at 72 °C. To amplify the reassembled products, 20 μL of the reassembly PCR was used as template in a PCR with primers FGSTh and RGSTh. This PCR contained 0.2 mM of each dNTP, 10× *Pfu* buffer, 8 pmol of each primer and *Pfu* DNA polymerase. Initial denaturation was achieved at 95 °C for 2 min. The PCR comprised of 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min and polymerization at 72 °C for 1 min, followed by 10 min at 72 °C. The PCR product was TOPO ligated to the expression vector pET101/D-TOPO. The ligated products were used to transform *E. coli* TOP10 cells, which were plated on LB agar plate containing ampicillin (100 μg/mL). For screening of shuffled library, hundreds of transformants were grown at 37 °C in LB medium (10 mL) containing ampicillin (100 μg/mL) and the enzymatic activities in crude cell free extracts were determined using the CDNB/GSH substrate system as described previously [25]. The activity screening was carried out using low GSH concentration in the assay (0.01 mM, instead of 2.5 mM in the normal assays) for the isolation of clones with low K_m towards GSH. A promising clone (GSTD4) showing increased activity, compared to the wild-type enzymes, was selected and sequenced. The corresponding enzyme was subsequently purified and characterized.

2.2.2. Site-saturation mutagenesis at position 112

Saturation mutagenesis at amino acid position 112 of the GSTD4 enzyme was performed by overlap extension using the polymerase chain reaction (PCR). The mutations were introduced using a set of degenerate

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