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Engineering the enantioselectivity of glutathione transferase by combined active-site mutations and chemical modifications

Ylva Ivarsson [a,1], Malena A. Norrgård [a], Ulf Hellman [b], Bengt Mannervik [a,*]

[a] *Department of Biochemistry and Organic Chemistry, Uppsala University, Biomedical Center, Box 576, SE-751 23 Uppsala, Sweden*
[b] *Ludwig Institute for Cancer Research, Uppsala University, Biomedical Center, Box 595, SE-751 24, Uppsala, Sweden*

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Abstract

Based on the crystal structure of human glutathione transferase M1-1, cysteine residues were introduced in the substrate-binding site of a Cys-free mutant of the enzyme, which were subsequently alkylated with 1-iodoalkanes. By different combinations of site-specific mutations and chemical modifications of the enzyme the enantioselectivity in the conjugation of glutathione with the epoxide-containing substrates 1-phenylpropylene oxide and styrene-7,8-oxide were enhanced up to 9- and 10-fold. The results also demonstrate that the enantioselectivity can be diminished, or even reversed, by suitable modifications, which can be valuable under some conditions. The redesign of the active-site structure for enhanced or diminished enantioselectivities have divergent requirements for different epoxides, calling for a combinatorial approach involving alternative mutations and chemical modifications to optimize the enantioselectivity for a targeted substrate. This approach outlines a general method of great potential for fine-tuning substrate specificity and tailoring stereoselectivity of recombinant enzymes.

Keywords: Enantioselectivity; Epoxide resolution; Glutathione transferase; Protein modification; Rational redesign

1. Introduction

Site-specific chemical modification is a method of great potential for generating enzyme variants with new or altered properties [1]. This expedient surpasses the limitations set by the genetic code and the canonical 20 amino acids, and it enables virtually unlimited variations of an amino acid side chain. Of the functional groups present in proteins, thiols groups, provided by cysteines, are particularly amenable to chemical modifications. If a protein is engineered to contain a single cysteine residue at a chosen site, the thiol group can be selectively chemically modified. The combination of site-directed mutagenesis and chemical modifications has, for example, been used to alter the substrate specificity of *Bacillus lentus* subtilisin [2,3]. Here we employ a similar approach to modify the enantioselectivity of human glutathione transferase M1-1 (GST M1-1, originally called GST μ) in the conjugation of the tripeptide glutathione (GSH) with alternative epoxide-containing substrates. GST M1-1 has relatively high catalytic activity with epoxides, but shows modest enantioselectivity with many of these substrates [4–6]. Epoxides are valuable building blocks in the production of chiral compounds, and racemic mixtures of epoxides can be resolved kinetically using epoxide hydrolases, as reviewed elsewhere [7–9].

Four cysteines are present in a monomer of GST M1-1 (Fig. 1A). Although evolutionarily largely conserved, these cysteines are not required for catalytic activity [10]. Cysteine-free GST M1-1 is hence a suitable scaffold into which novel cysteine residues can be introduced at selected sites and then posttranslationally modified by chemical reagents. After inspection of the crystal structures of GST M1-1 (PDB 1XW6) and of a T210S mutant of the closely related GST M2-2 in complex with a conjugation product between GSH and S-SO (PDB 2C4J), two amino-acid residues, I10 and G112, were selected for mutagenesis and chemical modifications. These non-catalytic residues are located

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; GSH, Glutathione; GST, Glutathione transferase; PPO, 1-phenylpropylene oxide; SO, Styrene-7,8-oxide; tSBO, *trans*-stilbene oxide

* Corresponding author. Fax: +46 18 558431.
E-mail address: Bengt.Mannervik@biorg.uu.se (B. Mannervik).
[1] Current address: Department of Biochemical Sciences "A. Rossi Fanelli", University of Rome "La Sapienza", Piazzale A. Moro 5, I-00185 Rome, Italy.

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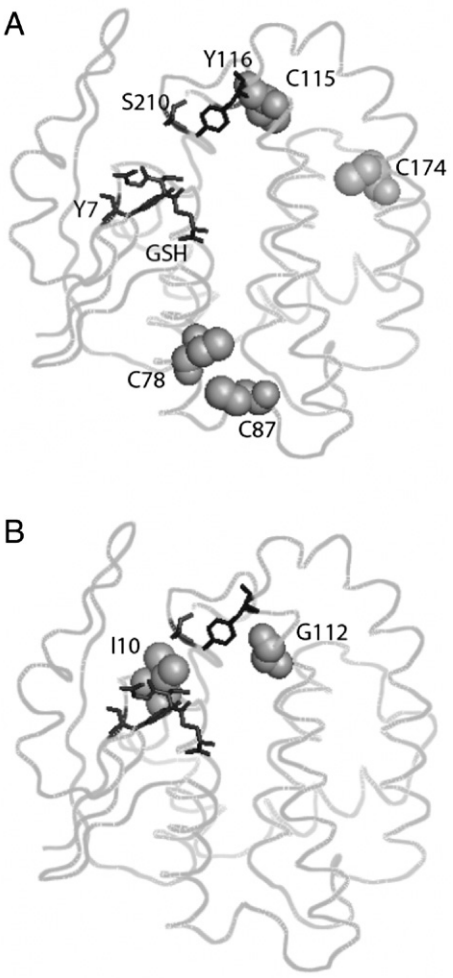

Fig. 1. Structure of human GST M1-1 (PDB 1XW6). One subunit of the homodimeric enzyme is shown. The active site is identified by Y7, Y116, S210 and GSH. (A) The four cysteine residues present in wild-type GST M1-1 are displayed in space-filling representation. (B) Residues I10 and G112 contribute to the hydrophobic binding pocket, and these positions were selected as targets for mutagenesis and subsequent chemical modifications.

in the walls of the hydrophobic substrate-binding pocket (Fig. 1B) and modifications of their side-chains were presumed to differentially alter the steric interactions with enantiomeric substrates, and thereby influence the enantioselectivity. The corresponding two positions in the homologous rat GSTs M1-1 and M2-2 have previously been shown to influence the stereoselectivities of these enzymes in epoxide conjugations [11]. The effects of the mutations and chemical modifications of human GST M1-1 were here investigated by analysis of the steady-state kinetics with alternative epoxide substrates, and we demonstrate that the enantioselectivity of GST M1-1 in epoxide conjugations can be enhanced by structure-based rational mutations and site-specific chemical modifications.

2. Materials and methods

2.1. Mutagenesis and cloning

The GST M1-1/C78A construct [10] in the pKKD vector [12] was available in the laboratory and was used as starting template for mutagenesis. Silent mutations were introduced in the 5′-end to improve the expression level [13] and restriction sites for *Eco*RI and *Sal*I were added simultaneously with these mutations. The PCR product was digested with *Eco*RI and *Sal*I and ligated into the pGEM vector. The remaining three cysteines (C87, C115 and C174) were replaced by alanines using inverted PCR and custom-made 5′-phosphorylated oligonucleotide primers as described in [14], but using an annealing temperature of 50°C. The sense and anti-sense primers used (with the mutations underlined) were: C87A, 5′-GCA AGC ACA ACC TGG CTG G-3′ and 5′-GGG CCA TGT AAG CCA AGA TGG-3′; C114A, 5′-GCA TGA TCG CTT ACA ATC CAG A-3′ and 5′-CCA GCT GCA TAT GGT TGT CC-3′; and C174A, 5′-CCC AAC GCT TTG GAC GCC-3′ and 5′-CTC AAA TAT ACG GTG GAG GTC AAG-3′. The I10C (5′-GCG CAC GCC ATC CGC CTG-3′ and 5′-CAG GCC ACG GCA GTC CCA GTA-3′), G112C (5′-CCT ACA ATC CAG AAT TTG AGA AAC TG-3′ and 5′-CGA TCA TGC ACA GCT GCA TAT GG-3′) and I10A (5′-CAG GCC ACG AGC GTC CCA GTA-3) mutations were added subsequently. The entire coding regions were subjected to DNA sequence analysis for confirmation of the expected primary structures. The DNA variants were transferred into the expression vector pKKD using the *Eco*RI and *Sal*I restriction sites. GST M1-1 variants were expressed and purified as described elsewhere [15], with the modification that elution from GSH-affinity columns was made with GSH (10 mM) in Tris–HCl (10 mM), pH 7.8. Enzyme concentrations were estimated by absorbance measurements at 280 nm, using the extinction coefficient and molecular mass of the wild-type GST M1 subunit (39,000 M^{-1} cm^{-1} and 25.7 kDa, respectively), and protein purity was confirmed by SDS-PAGE. Specific activities of the variant enzymes with the general GST substrate CDNB (1-chloro-2,4-dinitrobenzene) were determined under standard conditions [15].

2.2. Alkylation of thiols

Purified enzymes were incubated with alkylating agent (50–100 mM) in glycine–NaOH buffer (50 mM), pH 9, dithiothreitol (0.1 mM), EDTA (2.5 mM). The incubation time was 18 h for iodomethane, iodoethane, and 1-iodobutane, or 6 h for 2-iodoethanol and iodoacetamide [16]. Reagents were removed by gel filtration on PD10-columns (GE Healthcare, Uppsala, Sweden), followed by dialysis for 18 h against Tris–HCl buffer (10 mM), pH 7.8 and glycerol (20% v/v). Further extension of the incubation times resulted in decrease of catalytic activity. As a control, cysteine-free GST M1-1 was subjected to the alkylating reagents.

To verify that the alkylations were site-specific at the I10C and G112C positions, alkylated GST M1-1 variants were analyzed by mass spectrometry following SDS-PAGE, Coomassie staining, and in-gel tryptic digestion [17]. The tryptic peptides were analyzed by peptide mass fingerprinting using matrix assisted laser desorption/ionization time of flight mass spectrometry on a Bruker Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany). Matrices used were α-cyano 4-hydroxycinnamic acid or dihydrobenzoic acid, and the instrument was optimized according to the manufacturer's protocol. The instrument was externally calibrated using a mixture of nine peptides ranging from 757.399 to 3147.471 *m/z*. Prior to sequence analysis by Post Source Decay, the tryptic digests were sulfonated using the Ettan CAF MALDI Sequencing Kit (GE Healthcare, Uppsala, Sweden) [18].

All enzymes (wild-type, cysteine free, mutant and chemically modified variants of GST M1-1) were stored at 4°C. Some of the alkylated variants (the methylated variants) appeared less stable than the unmodified variants as judged by enzyme precipitation occurring after a few days storage.

2.3. Steady-state kinetics

Steady-state kinetics were studied at 30°C in 250 mM Tris–HCl buffer, pH 7.2, at a constant and saturating concentration of GSH (5 mM), and varying concentrations of the epoxide substrates: styrene-7,8-oxide (SO) and 1-phenylpropylene oxide (PPO) 0.2–6.0 mM; trans-stilbene oxide (tSBO) 12–125 μM. The extinction coefficients used for the conjugation of the epoxides with GSH were SO: $\Delta\varepsilon_{234}$=770 M^{-1} cm^{-1}, PPO: $\Delta\varepsilon_{236}$=945 M^{-1} cm^{-1}, tSBO: $\Delta\varepsilon_{234}$=−20,300 M^{-1} cm^{-1} [5,14]. Initial rates were determined (in triplicate at six substrate concentrations) and the Michaelis–Menten equation was fitted to the data points using Prism 4.1 (GraphPad Software, San Diego, CA). Substrate–saturation curves were determined in duplicate from the same