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Orthogonal protein purification—Expanding the repertoire of GST fusion systems ☆

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Abstract

We have previously developed a labeling scheme that can be used to site-specifically link human glutathione transferases (hGSTs) from the alpha class to chemical entities such as fluorophores and aldehydes. The reagents are in-house synthesized derivatives of glutathione (GS-derivatives). We have focused on a lysine mutant of hGST A1:A216K. In this study, we wanted to utilize these findings and improve on protein purification schemes that are using GSTs as fusion partners. We have used random mutagenesis to scramble the hydrophobic binding site of A216K through mutations at position M208 and isolated a library of 11 A216K/M208X mutants. All mutants were easily expressed and purified and retained all or parts of the catalytic properties of the parent GST. The mutants were stable over several days at room temperature. The A216K/M208X mutants could be site-specifically labeled using our designed fluorescent reagents. Furthermore, reaction with an aldehyde-containing reagent termed GS-Al results in site-specific introduction of an orthogonal handle for subsequent conjugation with aldehyde-reactive probes. Labeling with coumarin results in a fluorescent protein-conjugate that can bind glutathione (GSH) derivatives for subsequent affinity purification. The K_d for *S*-hexyl-GSH of coumarin-labeled A216K was measured to be 2.5 μM. The candidate proteins A216K and A216K/M208F could be purified in high yield in a one-step procedure through affinity chromatography (Glutathione Sepharose™ 4B). The proteins can readily be perceived as improved GST fusion partners.

Keywords: Human GST A1-1 mutants; Site-specific covalent modification; Lysine 216; Methionine 208; Protein purification; Fusion partner

The maturing field of proteomics has inspired numerous developments in protein purification technology [1,2]. Many of the commercially available products are based on fusing the gene expressing the peptide or protein of interest with the gene of a work-horse protein that carries advantageous properties [3]. The fusion partner is often attached via a linker such that the unwanted fusion partner can be cleaved off by specific proteases following expression and purification. The fusion partner has to meet several requirements, for example; it has to be easily expressed in a high yield and easy to purify preferably through interactions with a specific ligand (affinity chromatography).

One of the commonly used commercially available fusion partners is a glutathione transferase (GST)[1] from *Schistosoma japonicum* [4]. Glutathione transferases (GSTs,

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[1] *Abbreviations used:* A216K$_{Cou}$, coumarin-labeled A216K; C_{18}, octadecyl; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione, γ-Glu-Cys-Gly; GST, glutathione transferase; hGST A1-1, GST A1-1 isoform from human; G-site, glutathione-binding site; GS-thiolester, thiolester of glutathione; GSC, γ-Glu-Cys-Cys; HPLC, high performance liquid chromatography; H-site, hydrophobic electrophile binding site; MALDI-MS, matrix assisted laser desorption ionization mass spectrometry; NA, Immobilized NeutrAvidin™; NaP_i, sodium phosphate; OD, optical density; TFA, trifluoroacetic acid; TOF, time of flight; UV, ultraviolet; wt, wild-type.

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E.C. 2.5.1.18)[2] are dimeric phase II detoxication enzymes that catalyze the nucleophilic addition of the thiol of glutathione (GSH) to a wide range of hydrophobic electrophilic molecules [5] (Figs. 1a and 2). This feature is utilized in several protein purification kits in a two-fold manner: (1) the catalytic activity of the GST can be used to monitor expression and purification and (2) the fusion protein can be purified through affinity chromatography using GSH derivatives attached to solid supports.

In our previous studies, we have worked with human glutathione transferase (hGST) A1-1 (Fig. 2), a protein that is easy to express and purify, displays a high stability, and is very tolerant to mutations [6–10]. The crystal structure has been solved both with and without ligands [11,12]. We have developed a method with which we can site-specifically link an introduced lysine (A216K) to chemical entities such as fluorophores and aldehydes [7] (Fig. 1b) both in solution and in a surface-assisted fashion [13].

In this study, we wanted to utilize these findings and improve on protein purification schemes that are using GSTs as fusion partners. We envisioned that our mutant A216K together with our labeling reaction could (a) bring in a new means of monitoring the level of expressed protein since the reaction can take place in *Escherichia coli* lysates and (b) introduce an aldehyde residue for subsequent, novel applications. We hoped to find a GST mutant that would be easily expressed and purified in a high yield, folded, thermally stable, bind GSH and GSH derivatives, could be purified by standard GST–GSH protocols, could catalyze the standard CDNB reaction (Fig. 1a) and that would react with our designed, glutathione-based labeling reagents (Fig. 1b). Since position 208 has previously been shown to be of importance in the binding of the hydrophobic substrate [14,15], we believed that by mutating M208 we could possibly enhance the interactions between the protein and the reagents used in our labeling reaction as well as the interactions with the affinity media. We thus made an A216K/M208X library and investigated the properties of eleven new double mutants.

Materials and methods

All chemicals and reagents used were of the highest purity available. The tripeptides were synthesized using solid phase Fmoc-chemistry, as previously described [13], and protected amino acids were purchased from Novabiochem (Darmstadt, Germany). Methoxycoumarin was purchased from Molecular Probes, Inc. (Eugene, Oregon, USA). EZ-Link® PEO-Iodoacetyl Biotin and Immobilized NeutrAvidin™ (NA) gel were purchased from Pierce Biotechnology, Inc. (Rockford, IL, USA). All solid-phase syntheses were carried out on a VacMaster (International Sorbent Technology). Purification of the synthesized tripeptides was carried out using a Grace Vydac EVEREST C_{18} column (4.6 × 150 mm) attached to a Varian system with a ProStar 230 Delivery System, a ProStar 330 Photodiode Array Detector, controlled by the Varian LC Software. Purification of the different mutants was performed on a 3 × 5 mL, in series, HiTrap™ SP HP cation exchange column (Amersham Biosciences) attached to an ÄKTA Purifier System (Amersham Biosciences). All mass spectra were recorded using a Voyager-DE STR™ MALDI-TOF MS (PerSeptive Biosystems, Framingham, MA) with detection in the positive mode. The UV–vis measurements were performed using a Varian Cay 100 Scan UV–visible spectrophotometer and the results analyzed with the CaryWin UV software. The fluorescence measurements were carried out on either a Safire²™ (Tecan, Switzerland), a FluoroMax-2 apparatus (Jobin Yvon, Longjumeau, France) or a Hitachi F-4500 fluorescence spectrophotometer.

Site-directed mutagenesis to obtain a A216K/M208NNN library

The vector pET-21a(+) (Novagen) containing the gene coding for the hGSTA1 mutant A216K [16] was used as a template for the replacement of M208 by site-directed mutagenesis using the Quik-Change™ Site Directed Mutagenesis Kit from Stratagene. Oligonucleotide, M208NNN forward (5′-GCCCAAGGAAGCCTCCCNNNGATGAG AAATCTTTAGAAG-3′) and its complement (M208 NNN reverse), were designed to replace codon ATG (M208) with random nucleotide bases to create codons coding for different amino acids. The mutations were checked by DNA sequencing (GATC Biotech, Germany).

Culturing, harvesting and lysis of the cells (preparative scale)

From glycerol stocks of the different mutants, 200 μL of each was plated out on agar plates containing 100 μg/mL ampicillin and incubated at 37 °C overnight. The next day the bacteria on the plates were resuspended in a few milliliter of 2YT medium (16 g/L peptone, 10 g/L yeast extract, 5 g/L NaCl) and approximately half of the bacteria were pored into 1 L of 2YT medium containing 100 μg/mL ampicillin and incubated, shaking at 37 °C until OD_{600} ~0.6–0.7. The bacteria were induced to start producing the recombinant protein by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM and incubated at 37 °C with shaking for 3.5 h. The cells were harvested by centrifugation (3000*g*, 30 min, 4 °C) and the pellets were resuspended in a protease inhibitor cocktail (Complete mini EDTA-free, Roche) containing 20 mM NaP_i, 0.02% NaN_3, pH 7 with one tablet per 10 mL of buffer. For lysing the cells, freezing at −80 °C and thawing at 37 °C were performed four times. After the final thawing the lysates were centrifuged at 11,000*g* for 30 min at 4 °C.

[2] This nomenclature is derived from that recommended by Mannervik et al., Biochem. J. 282 (1992) (Pt 1), 305–306.