Programmed Delivery of Novel Functional Groups to the Alpha Class Glutathione Transferases†

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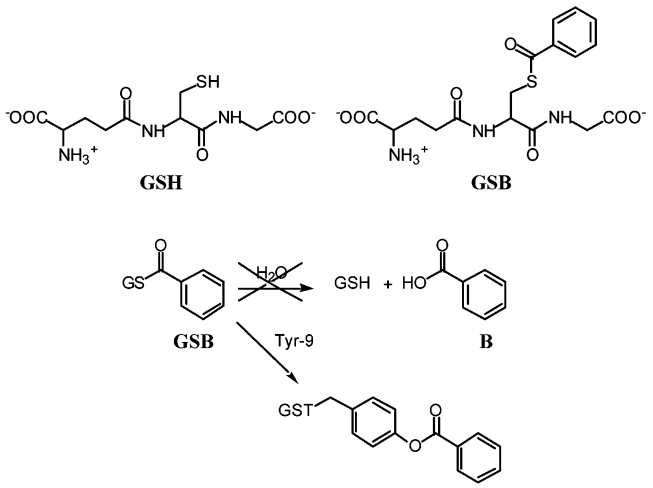
ABSTRACT: Here we describe a new route to site- and class-specific protein modification that will allow us to create novel functional proteins with artificial chemical groups. Glutathione transferases from the alpha but not the mu, pi, omega, or theta classes can be rapidly and site-specifically acylated with thioesters of glutathione (GS-thioesters) that are similar to compounds that have been demonstrated to occur in vivo. The human isoforms A1-1, A2-2, A3-3, and A4-4 from the alpha class all react with the reagent at a conserved tyrosine residue (Y9) that is crucial in catalysis of detoxication reactions. The yield of modified protein is virtually quantitative in less than 30 min under optimized conditions. The acylated product is stable for more than 24 h at pH 7 and 25 °C. The modification is reversible in the presence of excess glutathione, but the labeled protein can be protected by adding *S*-methylglutathione. The stability of the ester with respect to added glutathione depends on the acyl moiety. The reaction can also take place in *Escherichia coli* lysates doped with alpha class glutathione transferases. A control substance that lacks the peptidyl backbone required for binding to the glutathione transferases acylates surface-exposed lysines. There is some acyl group specificity since one out of the three different GS-thioesters that we tried was not able to acylate Y9.

The glutathione transferases (GSTs, EC 2.5.1.18)[1,2] constitute a large family of phase II detoxication enzymes that catalyze the nucleophilic addition of glutathione (GSH, Scheme 1) to a broad range of electrophilic molecules (*1*−*4*). The catalytic function is highly dependent on a conserved tyrosine, serine, or cysteine residue in the glutathione-binding site (G-site) that aids in the ionization of GSH to form the more reactive thiolate ion (*1*). The proteins are found as homo- or heterodimers (*3*, *5*), and the mode of binding of GSH in the N-terminal domain is essentially conserved throughout the classes (*1*, *6*). The differences between the isoenzymes are mostly located in the hydrophobic electrophile binding sites (H-sites) to provide the broad substrate specificity that is the hallmark of GST catalysis (*7*).

Scheme 1



The modular features of these proteins, in combination with their stability, ease of purification (*8*), and the wealth of accumulated knowledge of structure−activity relationships (*9*), are all factors that point to the GSTs as ideal candidates in protein engineering experiments (*10*−*17*) with the goal of obtaining novel function. Creating new function such as enzymatic activity in a protein scaffold is, however, a challenging task, even though progress has been made lately through rational design (*18*−*20*) and combinatorial approaches (*21*, *22*). A less explored route to novel enzymes is that of chemical modification of a protein scaffold to introduce an artificial functional group (*23*−*25*).

With the purpose of obtaining information that would guide us in the construction of a novel thioester hydrolase, we designed and synthesized a thioester of glutathione (GS-

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[1] Abbreviations: ACN, acetonitrile; ANT-NHS, succinimidyl *N*-methylanthranilate; C18, octadecyl; DCM, dichloromethane; DIPCDI, *N*,*N*′-diisopropylcarbodiimide; DIPEA, diisopropylethylamine; DMF, dimethylformamide; Fmoc, 9-fluorenylmethyloxycarbonyl; GSH, glutathione (γ-Glu-Cys-Gly); GST, glutathione transferase; hGST A1-1, GST A1-1 isoform from human; rGST A1-1, GST A1-1 isoform from rat; G-site, glutathione-binding site; GS-ANT, thioester of glutathione and *N*-methylanthranilate; GSB, thioester of glutathione and benzoic acid; GSB_{d5}, thioester of glutathione and benzoic acid-d_5; GS-thioester, thioester of glutathione; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; H-site, hydrophobic electrophile binding site; NMR, nuclear magnetic resonance; MALDI-MS, matrix-assisted laser desorption mass spectrometry; MeOH, methanol; Mmt, 4-methoxytrityl; NaP_i, sodium phosphate; TFA, trifluoroacetic acid; UV, ultraviolet.

[2] This nomenclature is derived from that recommended by Mannervik et al. (*53*).

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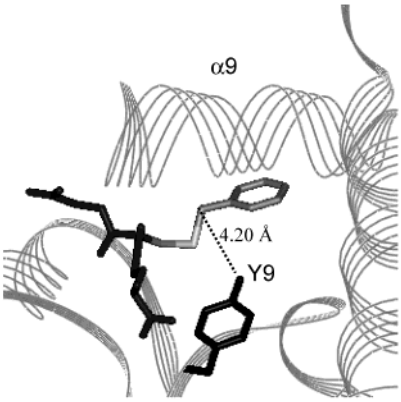


FIGURE 1: Close-up of the crystal structure (1GUH) of hGST A1-1 in a complex with *S*-benzylglutathione (*6*). The distance between the hydroxyl oxygen of Y9 and the ligand benzyl CH_2 is 4.2 Å.

thioester) termed GSB (Scheme 1) modeled from the crystal structure of hGST A1-1 with *S*-benzylglutathione (*6*) (Figure 1). We then studied the outcome of adding GSB, a molecule that is similar to compounds reported to be found in vivo, to a panel of recombinant GSTs from five different classes (alpha, mu, omega, pi, and theta). The GSTs exist as dimers, each dimer contains two active sites, and we report all protein concentrations in terms of active sites, i.e., the "monomeric" concentration. We used hGST A1-1 as a model protein for the bulk of the experiments.

MATERIALS AND METHODS

Benzoyl-d_5 chloride was purchased from QMX Laboratories Ltd., Essex, U.K. *S*-Lactoylglutathione was purchased from Sigma-Aldrich, St. Louis, MO. The HPLC experiments were carried out with a Varian system using a ProStar 230 delivery system and a ProStar 330 photodiode array detector together with the Varian LC software. UV measurements were performed with a Varian Cary 100 scan UV−visible spectrophotometer. Microcuvettes (submicrocells) were purchased from Varian. The glutathione transferases were kind gifts from the group of Professor Bengt Mannervik at the Department of Biochemistry, Uppsala University, Sweden. All chemicals and reagents used were of the highest purity available.

Synthesis of GS-Thioesters. The tripeptide γ-Glu-Cys-Gly (0.7 mmol scale) was synthesized using standard Fmoc protocols starting from Fmoc-Gly-Wang resin. The thiol was orthogonally protected with the Mmt group to enable selective deprotection with 1% TFA in DCM. After deprotection of Cys, the polymer was neutralized with 20% piperidine and washed with DMF, and the subsequent coupling was performed in DMF using benzoic or *N*-methylanthranilic acid−HOBt−DIPCDI−DIPEA (2:3:3:6). In the case of GSB_{d5} we used benzoyl-d_5 chloride and DIPEA (2:5) in the coupling reaction. Global deprotection and simultaneous cleavage from the solid support were achieved with TFA−H_2O (97.5:2.5). Following precipitation with ice-cold diethyl ether, the peptides were purified by reversed-phase HPLC using a C8 column (Kromasil) and a shallow H_2O−ACN (both 0.1% TFA) gradient. The purities of the products were checked with analytical HPLC and the identities confirmed with MALDI MS (Voyager System 4212, Applied Biosystems) using a α-cyano-4-hydroxycinnamic acid matrix with detection in the positive mode. The measured masses of GSB, GSB_{d5}, and GS-ANT were 411.97, 416.99, and 441.76 g/mol, respectively, in good agreement with the calculated masses of $GSBH^+$ (412.39 g/mol), $GSB_{d5}H^+$ (416.39 g/mol), and GS-$ANTH^+$ (441.44 g/mol). The thioester functionality of GSB displayed an absorbance at 266 nm, and we determined the extinction coefficient to be 7889 M^{-1} cm^{-1} in buffered solutions at neutral pH.

Glutathione Transferases. The activities of the GSTs were checked with a standard assay (*26*) containing 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH (pH 6.5 in 0.1 M NaP_i at 25 or 30 °C). Protein concentrations were estimated using previously published extinction coefficients for hGSTs A1-1 (*27*), A2-2 (*28*), A3-3 (*29*), A4-4 (*14*), M2-2 (*30*), M4-4 (*31*), O1-1 (*32*), P1-1 (*33*), mGST M5-5 (*34*), and rGST T2-2 (*35*). The GSTs were stored in NaP_i-buffered solutions containing NaCl (between 1 and 150 mM) and 10% glycerol at −80 or −20 °C until used in the experiments.

Modification Reaction. A typical modification reaction was performed in the following way: 1 μL of protein (100 μM stock solution) was diluted with 18 μL of buffer (0.1 M NaP_i, pH 7), and the reaction was initiated by adding 1 μL of reagent (2 mM stock solution). The reaction was allowed to proceed at 25 °C from 30 min to 24 h. When the reaction was followed by UV spectroscopy, the volume was scaled up to 110 μL to match the volume of the cuvette.

Digestion Experiments. The digestions were performed in the following way: 10 μL of the modification mixture was diluted with 10 μL of buffer (0.2 M $NaHCO_3$, pH 8), and the reaction was started by addition of 1 μL of trypsin (0.2 μg/μL stock solution in 0.1 mM HCl). The mixture was then incubated at 25 °C from 1 to 3 h. In the case of *Staphylococcus aureus* protease V8 (Pierce Biotechnology, Inc., Rockford, IL), we added 5 μL of the modification mixture to 15 μL of buffer (50 mM NH_4OAc, pH 4), and the digestion was initiated with 1 μL of protease V8 (1 μg/μL stock solution, pH 4). The reactions were then incubated at 25 °C from 1 to 3 h.

MALDI-MS Experiments. Most of the samples were desalted and concentrated with ZipTips (Millipore, C-18) prior to MALDI-MS analysis. An equal amount of 0.1% TFA (e.g., 20 μL to 20 μL reaction mixture) was added to the digestion mixtures to quench and acidify the sample, and we then proceeded as directed in the ZipTip manual. In the MALDI-MS experiments we used an α-cyano-4-hydroxycinnamic acid matrix with detection in the positive mode.

Pulse and Chase Experiment. hGST A1-1 (5 μM) was incubated with 50 μM GSB (0.1 M NaP_i, pH 7) in a microcuvette in a UV spectrophotometer. After 3 h there was no longer any decrease in the absorbance at 266 nm, i.e., amount of GSB, and we judged that the modification reaction was completed. Thereafter, an equal amount of GSB_{d5} was added so that the total concentration of GSB and GSB_{d5} was 100 μM and continued the incubation overnight at 25 °C. The reaction mixture was then digested with *S. aureus* protease V8, desalted, and analyzed with MALDI-MS.

Addition of GSH and S-Methylglutathione to the Reaction Mixture. Samples of benzoic acid-modified hGST A1-1 (5 μM) were incubated with GSH (5, 50, 200, or 1000 μM) at pH 7 (0.1 M NaP_i, 25 °C) for 10 min. The reaction mixtures were then digested with *S. aureus* protease V8, desalted, and subjected to MALDI-MS analysis. Mixtures with premodi-

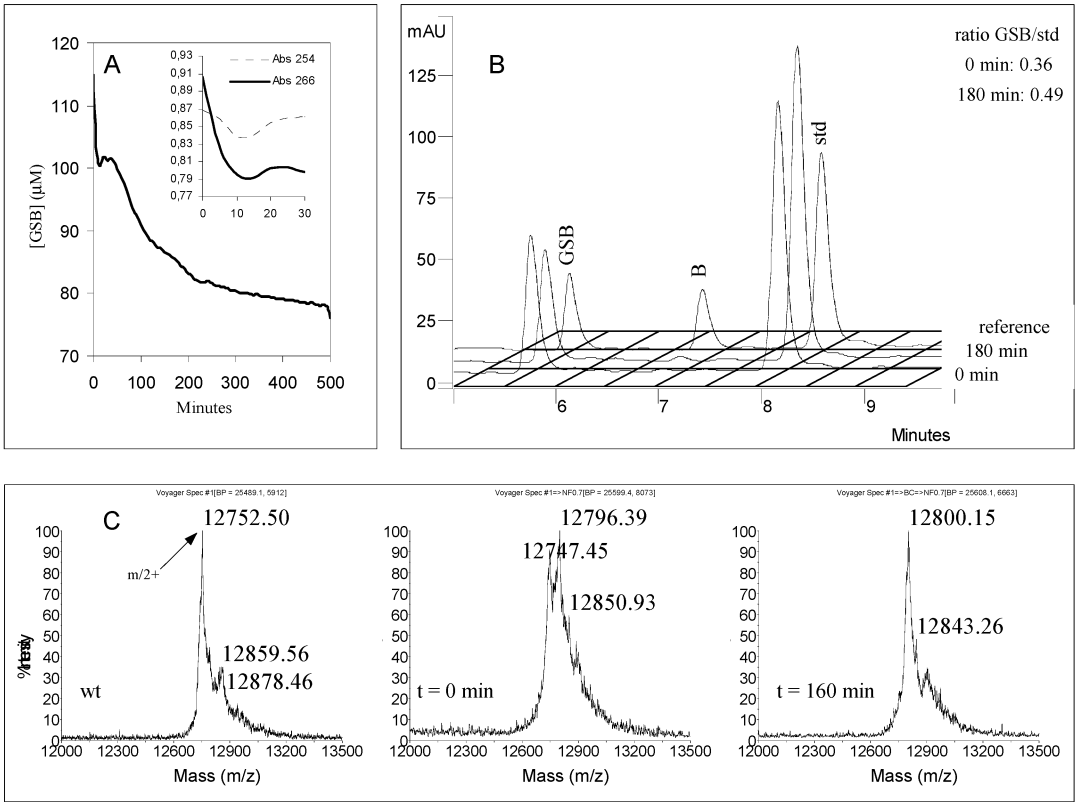


FIGURE 2: Reaction between GSB (110 μM) and hGST A1-1 (25 μM) at pH 7 monitored by (A) UV spectroscopy, (B) HPLC, and (C) MALDI-MS. The UV trace is shown at the maximum absorbance of the thioester functionality (266 nm); the first 30 min are magnified and shown for both 266 and 254 nm. The increase in absorbance at 254 nm is probably due to formation of the Y9 ester. The HPLC traces are shown at 229 nm. The calculated mass of the *m*/+2 peak is 12750 Da. The protein is covalently modified with one benzoic acid moiety in a stoichiometric fashion.

Table 1: Peptide Fragments Containing Y9 in the Alpha Class GSTs[a]

	trypsin			*S. aureus* protease V8		
hGST	fragment	MW	+B/+ANT	fragment	MW	+B/+ANT
A1-1	LHY*FNAR	920.47	1024/1053	AEKPKLHY*FNARGRME[b]	1947.00	2051/2080
A2-2	LHY*SNIR	902.48	1006/1035	AEKPKLHY*SNIRGRME[b]	1929.01	2033/2062
A3-3	LHY*FNGR	906.46	1010/1039	MAGKPKLHY*FNGRGRME	1991.00	2094/2123
A4-4	LHY*PNGR	856.44	960/989	MAARPKLHY*PNGRGRME	1984.01	2088/2117
A4-4 Y9F	LHFPNGR	840.44	944/973	MAARPKLHFPNGRGRME	1968.01	2072/2101

[a] +B or +ANT corresponds to the MW of the fragment after proteolysis if the acyl group is covalently attached. Tyrosine 9 is marked with an asterisk. [b] There is one missed cleavage when *S. aureus* protease V8 is used.

fied protein and *S*-methylglutathione were allowed to preequilibrate for 5 min prior to the addition of GSH.

Cell Lysate Experiments. Escherichia coli BL21(DE3) cells (50 mL) were grown for 18 h before being harvested by centrifugation. The bacterial cell pellet was then resuspended in 500 μL 0.1 M NaP_i buffer, pH 7. Freezing at −80 °C and thawing at 25 °C were performed four times to lyse the cells. The material was then centrifuged for 5 min at 15000*g*, and the resulting supernatant was stored at −80 °C until used in the experiments. The reaction conditions (buffer, pH, temperature) were comparable to what has been described (see above), but the final GS-thioester concentration was slightly higher (140 μM) as was the hGST A1-1 concentration (70 μM). In experiments containing the Complete Mini EDTA-free protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), one tablet was dissolved in 1.5 mL of MQ water. The maximum amount of added inhibitor solution was 10 μL to a reaction volume of 30 μL. Mixtures with the inhibitor solution were allowed to preequilibrate for 5 min prior to the addition of lysate. The *S. aureus* protease V8 (an aspartic acid protease) was used to digest the reaction mixtures since the inhibitor cocktail targets serine proteases such as trypsin (see Roche instruction manual).

RESULTS

Addition of GSB to hGST A1-1. An excess of GSB (110 μM) was added to a solution of hGST A1-1 (pH 7, 25 μM GST, 25 °C), and the disappearance of the thioester functionality was monitored by UV spectroscopy (266 nm). There was an initial fast decrease in the absorbance at 266 nm and roughly 1 equiv of GSB (25 μM) was consumed, but after that the reaction halted (Figure 2A). Concomitant with the decrease in absorbance at 266 nm there was also an increase at 254 nm that is not due to the formation of benzoic acid (Scheme 1 and Supporting Information). The increase at 254 nm precluded detailed analysis of the rate of disappearance of GSB. When the reaction mixture was

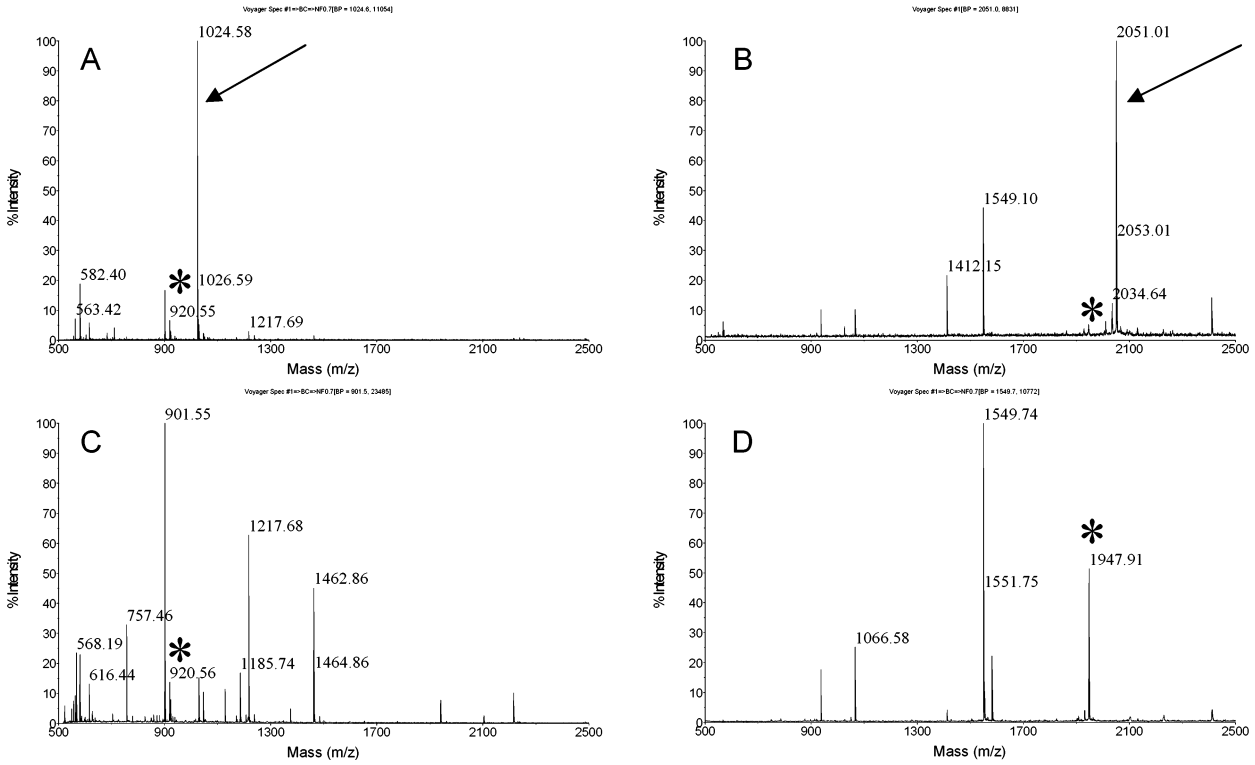


FIGURE 3: MALDI-MS spectra showing proteolytic peptide fragments from hGST A1-1 following overnight incubations with 100 μM GSB using (A) trypsin and (B) *S. aureus* V8 protease. The corresponding MALDI-MS spectra of unmodified hGST A1-1 resulting from proteolysis with (C) trypsin and (D) *S. aureus* V8 are also shown. Parent fragments are denoted with asterisks, and modified fragments (+B) are indicated with arrows.

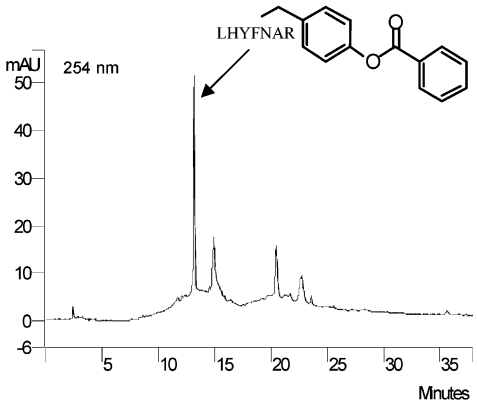


FIGURE 4: Reversed-phase HPLC chromatogram (λ = 254 nm) of proteolytic (*S. aureus* V8) peptide fragments from hGST A1-1 (5 μM) following overnight incubation with GSB (100 μM). Fractions were collected with 1 min intervals and identified with MALDI-MS. The peak containing the benzoic acid-modified fragment LHY*FNAR is indicated with an arrow.

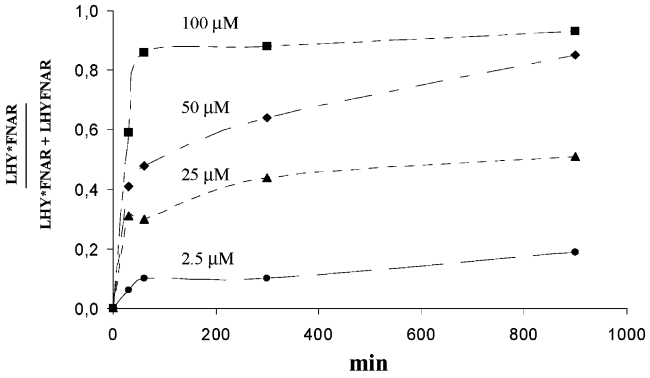


FIGURE 5: Semiquantitative analysis of the reaction between GSB (2.5, 25, 50, and 100 μM) and hGST A1-1 (5 μM) obtained from MALDI-MS experiments. The ratio of the modified fragment (LHY*FNAR) divided by the total amount of the fragment (LHY*FNAR + LHYFNAR) is plotted vs time.

analyzed by reversed-phase HPLC, we could not detect any formation of benzoic acid (Figure 2B and Scheme 1). The extinction coefficient of benzoic acid at 229 nm is much higher than that of GSB (Supporting Information), and the small peak of benzoic acid observed in the reaction mixture amounts to a maximum of 0.7 μM GSB, which is no more than in the starting material within the error limits (Supporting Information). A MALDI-MS analysis of the reaction mixture showed that the protein became covalently modified with one benzoic acid moiety in a stoichiometric fashion (Figure 2C).

Detailed Analysis of the Modified Protein. Modified hGST A1-1 was digested with both trypsin (Figure 3A) and *S. aureus* protease V8 (Figure 3B), and the peptide fragments were identified with MALDI-MS and compared with those obtained from unmodified protein (Figure 3 and Table 1). Both experiments identified Y9 as the site of modification. In addition, a larger batch of modified hGST A1-1 (5 μM) was digested and analyzed with reversed-phase HPLC following an overnight incubation with GSB (100 μM) at pH 7 (Figure 4). Fractions were collected at 1 min intervals and analyzed by MALDI-MS. Only one peak (retention time 13.3 min) showed the characteristic absorption spectrum of the benzoic acid moiety, and it corresponded to modified Y9.

Kinetics of the Modification Reaction. By adding increasing concentrations of GSB (2.5, 10, 25, 50, 100, and 500 μM) to hGST A1-1 (5 μM), we found, through MALDI-

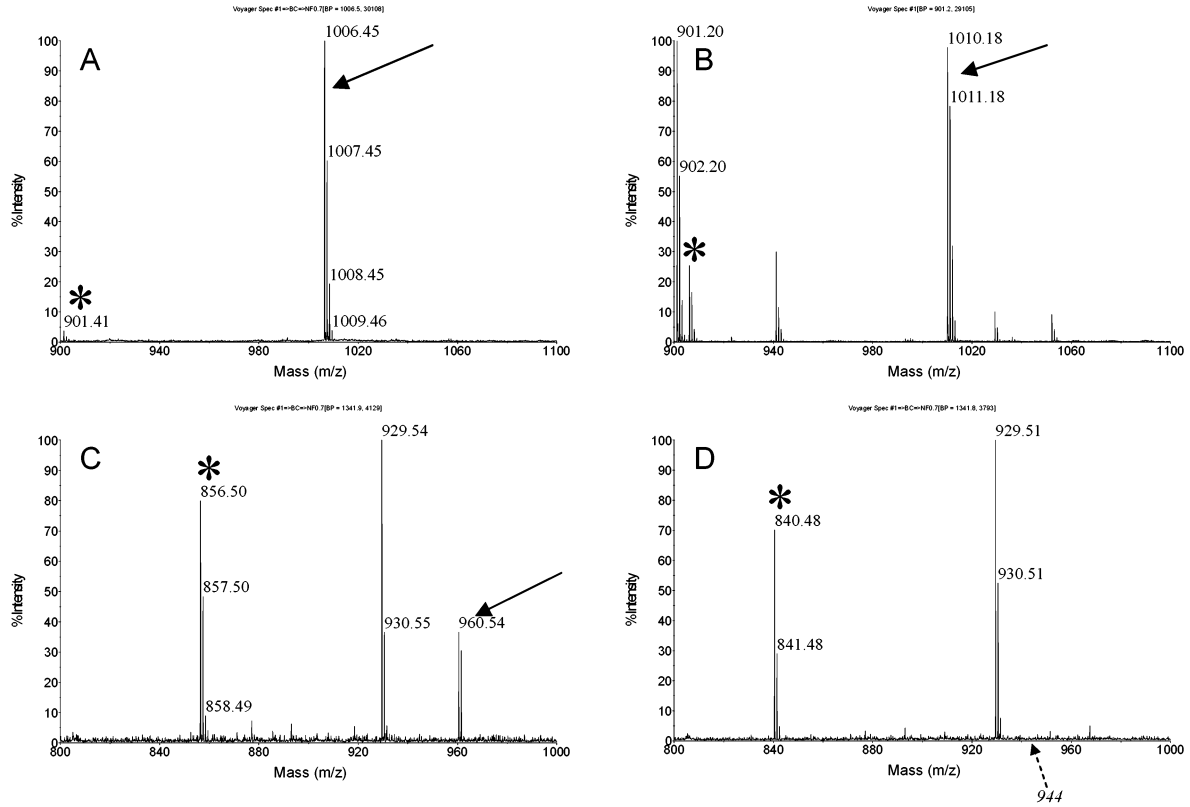


FIGURE 6: MALDI-MS spectra of proteolytic (trypsin) peptide fragments from hGSTs (A) A2-2, (B) A3-3, (C) A4-4, and (D) A4-4 Y9F following overnight incubations with 100 μM GSB. Parent fragments (if any) are indicated with an asterisk, and modified fragments (+B) are highlighted with arrows. The theoretical masses can be found in Table 1.

MS experiments, that the yield of modified protein increased from roughly 10% with 2.5 μM GSB (here, the maximum yield is 50%) to >90% with 100 μM GSB (Figure 5). There was also an increase in the rate of formation of modified protein. We did not attempt to extract the rate constant from the acylation step by UV spectroscopy because of the entangled signal from the concomitant decrease at 266 nm (the thioester; Supporting Information) and the increase at 254 nm (Supporting Information) that presumably is due to the formation of the Y9 ester with benzoic acid. In a separate experiment, we found that the time to reach completion was reduced when a higher protein concentration was used; for example, with 25 μM protein and 75 μM GSB the reaction was almost completed within 30 min (data not shown).

Addition of GSB to a Panel of Five Classes of GSTs. We used proteolytic digestion and MALDI-MS to investigate the outcome of adding GSB to ten isoenzymes from five classes (hGSTs A1-1, A2-2, A3-3, A4-4, M2-2, M4-4, O1-1, P1-1, mGST M5-5, and rGST T2-2). The reactions were performed at both pH 7 and pH 8 with the same outcome; only in the case of GSTs from the alpha class could we detect modified protein (Figure 6). The mutant hGST A4-4 (Y9F) did not show any sign of modification after 24 h incubation with GSB (Figure 6D).

pH Dependence of the Reaction between GSB and hGST A1-1. The yield of the reaction between hGST A1-1 (5 μM) and GSB (100 μM) after 30 min incubation at 25 °C was investigated with respect to pH (0.1 M NaPi, pH 7.0, 7.5, 8.0, and 8.5). We used proteolytic digestion and MALDI-MS to analyze the reactions and determined the ratio of modified fragment (Figure 7). There appears to be a slight optimum around pH 7.5.

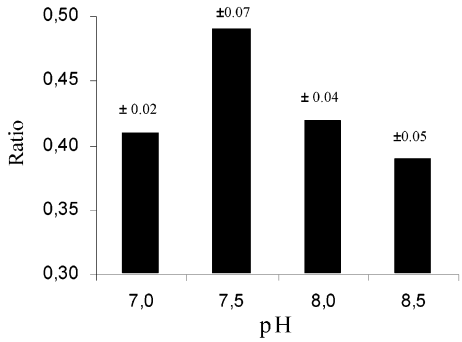


FIGURE 7: Semiquantitative analysis of the pH dependence of the reaction between GSB (100 μM) and hGST A1-1 (5 μM) obtained from MALDI-MS after proteolytic digestion (*S. aureus* V8). The figure shows the ratio, i.e., the modified Y9 fragment divided by the total amount of the Y9-containing fragment, after 30 min incubation at 25 °C.

GSB Analogues. We synthesized a thioester with an *N*-methylanthranilic acid moiety (GS-ANT; Scheme 2) to investigate if the modification reaction was unique for benzoic acid. We found that this reagent could also modify Y9 of the four isoenzymes from the alpha class (Figure 8). On the other hand, the naturally occurring *S*-lactoylglutathione (*36*) (Scheme 2) could not modify hGST A1-1 under the same reaction conditions.

Pulse and Chase Experiment Using GSB and GSB$_{d5}$. To study whether free GSB was in equilibrium with the protein-linked benzoic acid moiety, we synthesized the deuterated analogue of GSB (GSB$_{d5}$). Control experiments showed that GSB and GSB$_{d5}$ react with hGST A1-1 and are ionized in the MALDI-MS experiment in identical ways (Figure 9). We

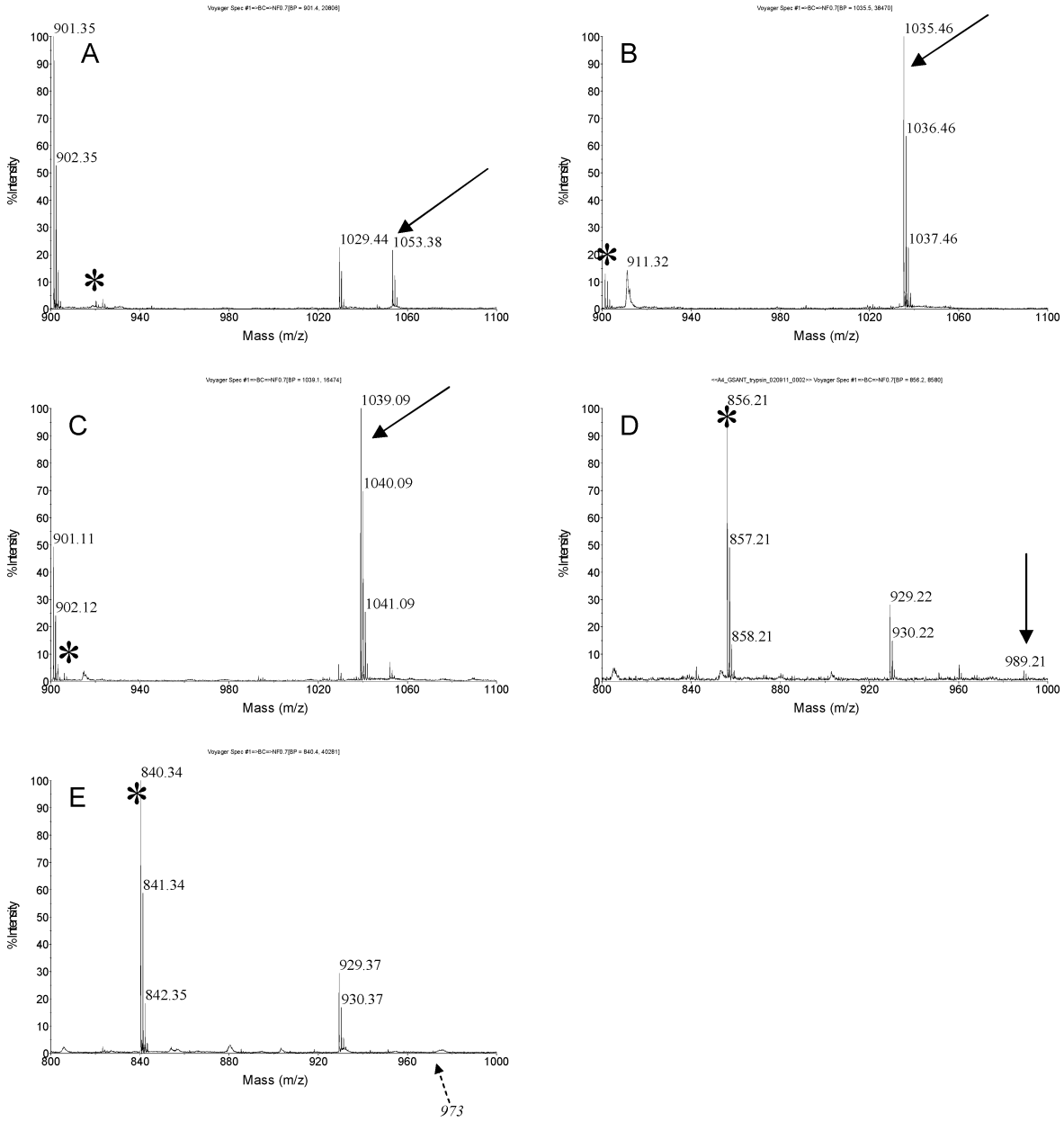
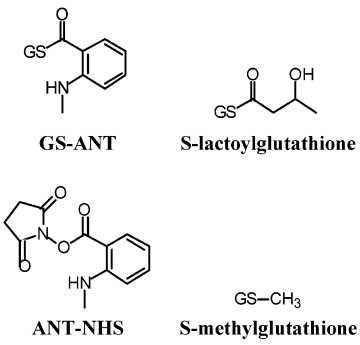


FIGURE 8: MALDI-MS spectra of proteolytic (trypsin) peptide fragments from hGSTs (A) A1-1, (B) A2-2, (C) A3-3, (D) A4-4, and (E) the mutant A4-4 Y9F following overnight incubations with 100 μM GS-ANT. Parent fragments (if any) are indicated with an asterisk, and modified fragments (+ANT) are highlighted with arrows. The theoretical masses can be found in Table 1.

Scheme 2



also determined that there is no exchange of bound benzoic acid during the proteolytic step. The only difference between GSB_{d5} and GSB is the mass of the benzoic acid moiety (109 Da instead of 104 Da), thus enabling a pulse and chase experiment. An excess of GSB (50 μM) was added to hGST A1-1 (5 μM), and after completion of the modification reaction (as judged by UV), we added an equal amount of GSB_{d5} (50 μM) and continued the incubation overnight. After proteolytic digestion and MS analysis we found that none of the added GSB_{d5} had displaced its protium analogue (Figure 9).

Reagent Requirements. To investigate the impact of the glutathionyl part on the reaction, we used ANT-NHS (succinimidyl *N*-methylanthranilate; Scheme 2), a substance that contains an active ester but lacks the peptidyl backbone required for binding to the GSTs. When ANT-NHS was added to hGST A1-1, we found that, in addition to a small amount of Y9, several of the surface-exposed lysine residues were modified (Figure 10).

We also tried to break the ester by adding up to 1 M MeOH to the modified protein but were not able to displace the tyrosine hydroxyl (data not shown). We tried to measure

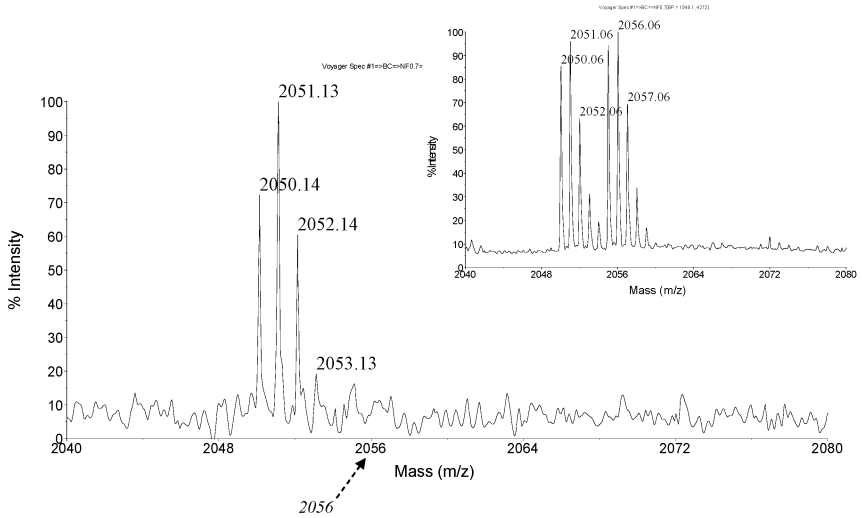


FIGURE 9: MALDI-MS spectrum recorded after a pulse and chase experiment where GSB_{h5} (50 μM) was added to hGST A1-1 (5 μM) followed by a chase of GSB_{d5} (50 μM) after 5.5 h incubation. The reaction was then allowed to continue overnight. Control experiments showed that GSB_{h5} and GSB_{d5} react with hGST A1-1 and are ionized in the MALDI-MS experiment in identical ways and that there is no exchange of bound benzoic acid during the proteolytic step (inset).

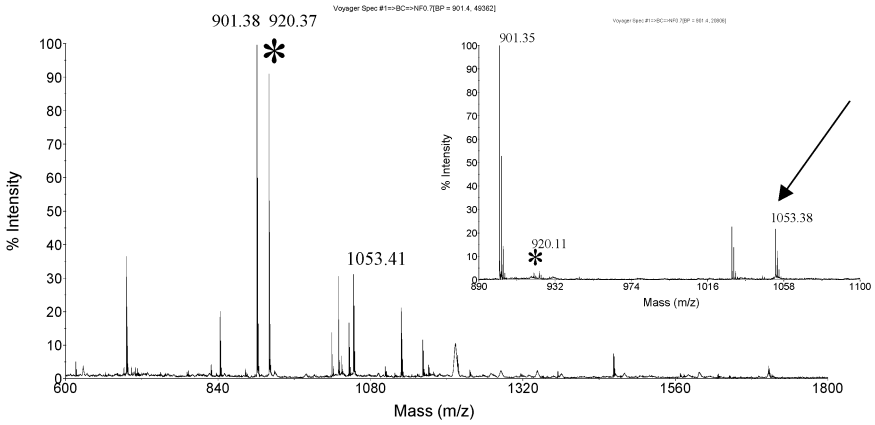


FIGURE 10: MALDI-MS spectrum showing tryptic fragments from hGST A1-1 after 1 h incubation with 100 μM ANT-NHS. The spectrum resulting from incubation with GS-ANT is shown as an inset where the modified Y9 fragment (+ANT) is indicated with an arrow.

the enzymatic activity of the benzoic acid-modified hGST A1-1 in the conjugation reaction between CDNB and GSH but found through MALDI-MS analyses that the high concentrations of GSH used (1 mM) completely removed the ester during the experiment. The protein is therefore no longer modified, and the activity was measured to be the normal hGST A1-1 activity. In fact, even lower concentrations of GSH liberated the tyrosine. For example, incubation of 5 μM benzoic acid-modified hGST A1-1 with 50 μM GSH for 10 min removed the ester completely (data not shown).

The ester (5 μM modified protein) could, however, be protected by addition of 200 μM *S*-methylglutathione (Scheme 2) to a mixture with 100 μM GSH. The tyrosine ester with anthranilic acid was more resilient toward displacement; even in the presence of 50 μM GSH, there was still a large amount of modified protein left after 10 min incubation (data not shown).

Modification Reactions in E. coli Lysate. To test whether the modification reaction might occur in a more in vivo-like setting, we added GSB or GS-ANT to *E. coli* lysates that were doped with hGST A1-1. The samples were incubated (25 °C), and aliquots were withdrawn, digested with *S. aureus* protease V8, and analyzed with MALDI-MS to obtain semiquantitative data (Table 2). We also added benzoic acid-modified protein to study the stability of the Y9 ester separately. We found that in order for the premodified protein to survive in the lysate we had to add a protease inhibitor cocktail to the reaction mixture. However, GS-ANT was able to react with hGST A1-1 to some extent even in the absence of inhibitors. The experiment was repeated for hGST A2-2 with similar result.

Table 2: Amount of Modified Y9 after 30 min (25 °C) Incubation in *E. coli* Lysate[a]

	benzoic acid-modified hGST A1-1	GSB + hGST A1-1	GS-ANT + hGST A1-1
no inhibitor	nd[b]	nd	+
with inhibitor	+[b]	+	+

[a] In relation to the amount of modified Y9 fragment without added lysate. [b] nd = not detectable; + = approximately the same amount modified as without lysate.

DISCUSSION

We have found that it is possible to acylate human GSTs from the alpha class in a site-specific fashion using GS-thioesters as modification reagents. The covalent modification takes place at Y9, and the reaction can be completed within

30 min under optimal conditions. All GSTs have a tyrosine (alpha, mu, and pi), serine (theta), or cysteine (omega) in the G-site in the equivalent position, and this residue is crucial in the normal detoxication reaction (*1*, *3*). Despite this, we could not detect modified protein when GSB was added to GSTs from four other classes. The reason the alpha class and not the others react with GSB is still not clear. The differences could potentially be due to binding preferences, but similar compounds have been shown to bind to the alpha, mu, and pi class with K_D values that are comparable (*37*). Also, within the alpha class, the enzymes display different substrate preferences (*29*, *38*, *39*), and the crystal structures (*6*, *40*) also reveal differences; for example, the H-site of hGST A4-4 appears to be more narrow and elongated than that of hGST A1-1. The depressed pK_a values of the Y9 in the alpha class ranging from 6.7 (hGST A4-4) (*41*) to 9.2 (hGST A2-2) (*12*) probably contribute to their reactivity, but the value is reported to be in this range for the pi enzyme also (*42*, *43*). Also, rat GST A1-1, an enzyme with a tyrosine pK_a of 8.3 (*44*), has previously been shown not to react covalently with the glutathione thioester of ethacrynic acid (*45*). The equivalent tyrosine in rGST M1-1 has a pK_a of 10, which is close to the value observed for a free tyrosine in solution. The theta class employs a serine (*46*), and the omega class has a cysteine residue (*32*) that could potentially engage in a trans-thioesterification reaction with GSB.

The observation that alpha class GSTs can become covalently modified on a vital catalytic residue by synthetic GS-thioesters that are similar to compounds that occur naturally raises several questions. Do related things happen in vivo? Can this observation be exploited in the construction of novel class-specific GST inhibitors? Can this finding be utilized in vitro to modify GST scaffolds with new functional groups in order to create novel catalysts or labeled proteins?

A Slight Optimum at pH 7.5 in the Reaction between GSB and hGST A1-1. The yield of modified protein (hGST A1-1) peaks around pH 7.5, which is lower than the pK_a of Y9 in hGST A1-1, which is 8.1 (*47*). The bell-shaped pH profile is likely due to several events since even though the concentration of reactive tyrosinate ion increases with increasing pH, there is also an increased breakdown of the thioester as well as a decreased stability of the Y9 ester at higher pH values.

Not All GS-Thioesters Are Able To Modify the Alpha Class GSTs. It is important to know whether this is a unique reaction for GSB, and we therefore tested two additional compounds, GS-ANT and *S*-lactoylglutathione. Out of the three reagents we tried, two (GSB and GS-ANT) were able to acylate Y9, but the naturally occurring *S*-lactoylglutathione did not become covalently attached under the reaction conditions. In light of these results and the thiol esterase activity reported for rGST A1-1 with the GS-thioester of ethacrynic acid (*45*), it is tempting to draw the conclusion that there is a need for aryl thioesters for the covalent modification reaction to work. However, we feel that the library of compounds tested (three in our hands) is far too small to state anything conclusive. The relative reactivities of the GS-thioesters correlate with the leaving group, which is GSH in all cases, and also with the pK_a value of the acyl group. The pK_a values of the acids in this set are reported (*48*) or calculated (*49*) to be 4.2 for benzoic acid, 4.0 for *N*-methylanthranilic acid, 3.9 for lactic acid, and 2.9 for ethacrynic acid. We do not believe that the difference in pK_a values between *N*-methylanthranilic acid and lactic acid alone is responsible for the different behaviors of the reagents. As of now, we can conclude that not all GS-thioesters are able to modify the alpha class GSTs, but we do not yet know the requirements for a successful modification reagent.

The Glutathione Backbone Is Required. In contrast to the more solvent-exposed pi class (*50*), the alpha class isoenzymes have fairly hydrophobic H-sites (*6*, *40*), and we speculated that unspecific hydrophobic interactions might contribute to the reactivity of the alpha class. We therefore tested a compound that is similar to GS-ANT but that lacks the peptidyl backbone required to bind to the GSTs (ANT-NHS; Scheme 2). However, ANT-NHS modified surface-exposed lysine residues except Y9 to only a low extent. In addition, the tyrosine ester also withstood 1 M MeOH. The combination of a suitably activated thioester and a binding module is thus ideal in achieving a high level of specific tagging with minimal side reactions.

No Exchange between Free GSB and Bound Benzoic Acid. The pulse and chase experiment showed that the covalently linked benzoic acid moiety does not equilibrate with GSB in solution. That means that the amount of GSH that is produced from 1 equiv of reacted GSB is not enough to pull off the bound acyl group. By addition of more than 1 equiv of GSH, the modification reaction could be reversed, but the modified protein could be protected by including non-nucleophilic *S*-methylglutathione to out-compete GSH in the reaction mixture. These experiments also show that the modified protein retains the affinity for glutathione-like substances even when the key residue Y9 is modified. Interestingly, the *N*-methylanthranilic acid-modified hGST A1-1 was significantly more resilient toward GSH; even 200 μM could not liberate all Y9 in a 5 μM protein sample. The known concentration of GSH in mammalian cells ranges from 1 to 10 mM, and it is perhaps not likely that any modification can survive in vivo for any length of time. However, since even the compounds within this limited set display a variety of behaviors, we cannot exclude that some GS-thioesters might actually modify the enzymes in vivo.

Modification in an in Vivo-like Setting. If we turn to biotechnological purposes such as selective tagging of an overexpressed recombinant alpha class GST, it is important that the modification reaction can occur in a cell lysate prior to purification of the protein. Again, the two acylating reagents behave differently; GS-ANT in itself seems to be more resistant to hydrolysis, and the Y9 ester with *N*-methylanthranilic acid is also more resilient. However, by adding a protease inhibitor cocktail, we could modify the GSTs and increase the lifetime of the Y9 esters in biologically relevant settings irrespective of the acyl group, and this adds to the versatility of the modification reaction. The concentration of hGST A1-1 (70 μM) in the doped cell lysates was lower than what is achieved during normal expression and purification since the yield frequently is >100 mg of pure protein from 1 L of cell culture concentrated in 10 mL of buffer or less.

CONCLUSION

We have identified a novel route to site- and class-specific GST modification. This finding presents us with the op-

portunity to, for example, specifically tag recombinant GSTs in a cell lysate with fluorescent probes in order to measure the protein concentration prior to purification. Because of the reversibility of the reaction, the tag would be easily removed upon addition of GSH. Another possibility is to create novel functional proteins through the design and synthesis of GS-thioesters with appropriate chemical groups that would redirect the protein scaffold toward non-GST activity, for example, a phenanthroline derivative that through complexed Cu^{2+} ions can aid in hydrolysis reactions of amides (*51*, *52*).

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SUPPORTING INFORMATION AVAILABLE

Two figures showing (i) the reaction between GSB and hGST A1-1 monitored by UV spectroscopy (266 and 254 nm) and UV spectra of GSB and benzoic acid and (ii) the HPLC chromatogram of GSB (229 nm). This material is available free of charge via the Internet at http://pubs.acs.org.

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