Chemistry & Biology 13, 929–936, September 2006 DOI 10.1016/j.chembiol.2006.07.005

Replacement Surgery with Unnatural Amino Acids in the Lock-and-Key Joint of Glutathione Transferase Subunits

Usama M. Hegazy,[1] Ulf Hellman,[2] and Bengt Mannervik[1,*]

[1] Department of Biochemistry and Organic Chemistry
Biomedical Center
Uppsala University
Box 576
SE-751 23 Uppsala
Sweden

[2] Ludwig Institute for Cancer Research
Biomedical Center
Uppsala University
Box 595
SE-751 24 Uppsala
Sweden

Summary

Proteins contain amino acid residues essential to structure and function. Ribosomal protein synthesis is typically limited to the 20 amino acids of the genetic code, but posttranslational chemical modifications can greatly expand the diversity of side chain functionalities. In this investigation, a natural aromatic residue in the lock-and-key joint at the subunit interface of the dimeric glutathione transferase P1-1 was replaced by an *S*-alkylcysteine residue to give a functional enzyme. Introduction of Cys in the key position inactivates the enzyme, but subsequent alkylation of this residue enhances the catalytic efficiency up to 27,000-fold. Combinatorial modification of Cys by a mixture of reagents facilitated identification of an *n*-butyl group as the most efficient activator. Alkylation also enhanced binding affinity for active-site ligands and stabilized the enzyme against chemical denaturation and thermal inactivation.

Introduction

The soluble glutathione transferases (GSTs) are generally dimeric proteins catalyzing the conjugation of electrophilic compounds with reduced glutathione (GSH) [1, 2]. Although amino acid residues in the dimer interface are not universally conserved among different classes in the GST superfamily, the lock-and-key motif is a common feature and one of the major contributors to the subunit-subunit interactions in the alpha, mu, and pi class enzymes [3]. The conserved aromatic key residue of alpha [4, 5], mu [6], and pi [7] class GSTs has been mutated to demonstrate the significance of this key residue. In the functional dimer of the GST P1-1 protein, the Tyr^{50} residue (key) of each subunit is wedged into a hydrophobic cavity (lock) between helices α4 and α5 in the neighboring subunit (Figure 1). The key residue is part of a highly flexible structural unit, helix α2 and the following loop (residues 36–52), which forms one wall of the GSH binding site and provides hydrogen bonds to GSH from several amino acid residues (the side chains of Trp^{39}, Lys^{45}, and Gln^{52}) [8, 9]. Structural transitions of this mobile region modulate GSH affinity and control the rate of catalysis by shifting the bound GSH from a precatalysis position to the position forming the transition state. Insertion of the key residue of one subunit into the lock structure of the other subunit stabilizes the loop following the α2 helix, thereby enhancing the affinity for GSH and the rate of catalysis [10]. Truncation of the side chain of residue 50 into Ala and mutation into other amino acid residues causes a dramatic loss of catalytic activity [7], accompanied by impaired subunit interactions and decreased affinity for the thiol substrate GSH. We targeted the key residue of the lock-and-key motif at the subunit-subunit interface of GST P1-1 (Figure 1) in order to search for a synthetic residue that can replace a crucial natural aromatic amino acid. The results demonstrate that the functional properties of the enzyme can be conserved, restored, or enhanced through the proper combination of site-directed mutagenesis followed by facile chemical derivatization of the key residue. Modulation of properties of other proteins can be made by the same general approach, provided that Cys residues can be introduced in responsive sites. This notion is confirmed by previous studies involving engineered Cys side chains [11–14].

Results and Discussion

Replacement of the Aromatic Key Residue in the Subunit Interface of GST P1-1

In the present study, the key residue Tyr at position 50 was replaced by Cys. The four Cys residues of wild-type GST P1-1 were mutated into Ala (residues 48 and 102) and Ser (residues 15 and 170) in order to achieve selective chemical modification at position 50. The resulting mutant, Y50C, had a very low specific activity (0.005 μmol min^{-1} mg^{-1}), whereas the corresponding Cys-free mutant (Cys-free) with Tyr in position 50 had an activity of 15 μmol min^{-1} mg^{-1}. The primary cause of this 3000-fold weakening of catalytic function was considered to be loss of the aromatic substituent of residue 50. Thus, the Y50C protein was treated with various thiol-modifying reagents bearing an aromatic group to find out if an *S*-substituted Cys residue could mimic the functionally competent amino acids Tyr and Phe. However, none of the reagents with aromatic substituents gave a noteworthy restoration of catalytic function (Table 1). In contrast, alkylation of Y50C with some alkyl halogenides activated the enzyme by several orders of magnitude (see below). This result suggested that the gain of catalytic activity could be optimized by proper selection of a small hydrophobic alkyl side chain attached to Cys^{50} and prompted a more extensive screening of alkylating compounds as possible activating agents.

Therefore, a combinatorial approach was tested, in which the Y50C protein was treated with mixtures of reagents with similar chemical reactivities. For proof of principle, a mixture of ten electrophilic agents afforded

*Correspondence: bengt.mannervik@biokemi.uu.se

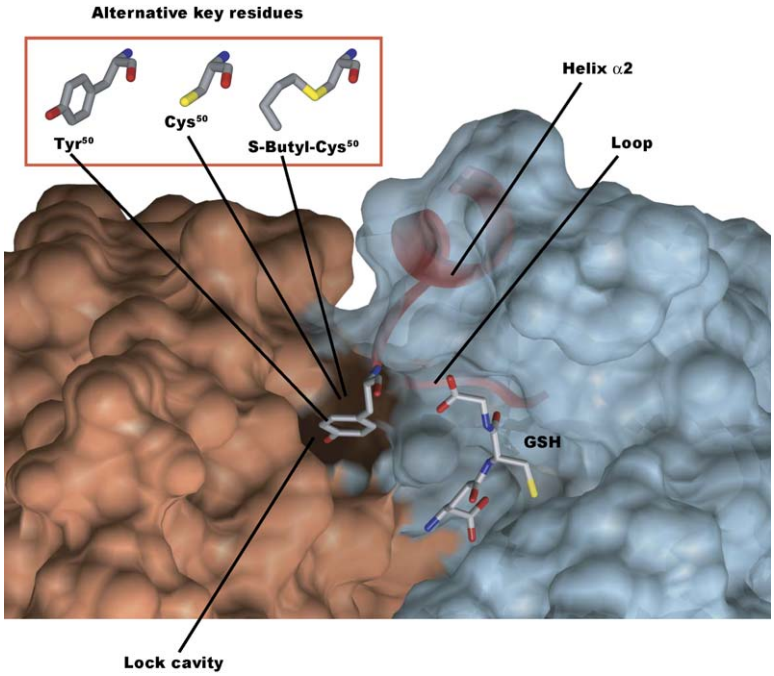

Figure 1. Structural Diagram of the GSH Binding Site of Homodimeric GST P1-1 Including the Lock-and-Key Joint and the Loop following Helix α2

The two subunits of the homodimer are represented by orange and blue surfaces, respectively. The alternative key residues of the blue subunit, Tyr^{50} in wild-type GST P1-1, Cys^{50} in Y50C, and *S*-*n*-butyl-Cys^{50} in *n*-butyl-Y50C, as well as the substrate GSH are shown in sticks. The lock cavity of the orange subunit, in which the key residue of the blue subunit fits, is rendered as a dark hole. Helix α2 and the following loop, carrying the key residue and forming one wall of the GSH binding site, are represented by a red ribbon. The figure is based on the crystal structure of the hGST P1-1-GSH complex (PDB code: 6GSS [37]).

a 60-fold increase in catalytic activity (data not shown), indicating that one or several compounds in the mixture had an activating effect of the enzyme. Deconvolution, by testing the components of the mixture individually, demonstrated a time-dependent activation with 1-iodobutane as the most efficient activator (Figure 2A). Bulky ring structures, including benzyl, pyridinyl, pyrimidinyl, or maleimide derivatives, activated Y50C $\leq$ 4-fold. On the other hand, introduction of relatively small alkyl groups activated Y50C up to 960-fold, as measured in the crude reaction mixture (Table 1). The most efficient derivatives were *n*-butyl, *n*-pentyl, and cyclobutylmethyl. Elongating the chain length to heptyl and octyl essentially abolished the activation. Also, increasing the bulkiness of the alkyl group by using the secondary haloalkanes, 1-bromo-2-ethylbutane and 1-iodo-2-methylpropane, activated Y50C modestly by 12- and 36-fold, respectively. Restricting the structural flexibility of the side chain of the key residue by using cyclobutane and cyclopropane derivatives gave less activation than the *n*-alkyl halogenides with the same number of carbon atoms, demonstrating that both shape and mass of the

Table 1. Enhanced Catalytic Activity of Y50C by Alkylation with Cysteine-Modifying Reagents

Cysteine-Modifying Reagent	Specific Activity[a] (μmol min^{-1} mg^{-1})	Activation (Fold)	Modified Cysteine[b] (%)
None	0.005	1	0
1-chloro-2,4-dinitrobenzene	0.002	0.5	77
N-ethylmaleimide	0.02	4	81
2,2′-dithiodipyridine	0.02	4	ND
4-vinylpyridine	0.015	3	86
Benzyl bromide	0.01	2	65
2-bromo-pyrimidine	0.01	2	73
2-fluoro-5-methylpyridine	0.01	2	ND
4-fluoro-benzylbromide	0.01	2	ND
1-iodomethane	0.03	6	100
1-iodoethane	0.2	40	ND
1-iodopropane	0.9	180	100
1-iodobutane	4.8	960	90
1-iodopentane	2.3	460	89
1-iodohexane	0.4	80	ND
1-iodoheptane	0.002	0.5	67
1-iodooctane	0.002	0.5	ND
1-bromo-2-ethylbutane	0.06	12	19
1-iodo-2-methylpropane	0.18	36	20
Bromomethyl cyclopropane	0.15	30	65
Bromomethyl cyclobutane	1.4	280	67

[a] The specific activity was determined in the standard assay system after alkylation for 20 hrs at 22°C followed by removing the excess of alkylating reagent by gel filtration but without further purification of the modified protein.
[b] The modified cysteine was calculated by subtracting the remaining cysteine residue of the chemically modified protein from that of unmodified protein. The remaining cysteine residue was determined by Ellman's reagent.

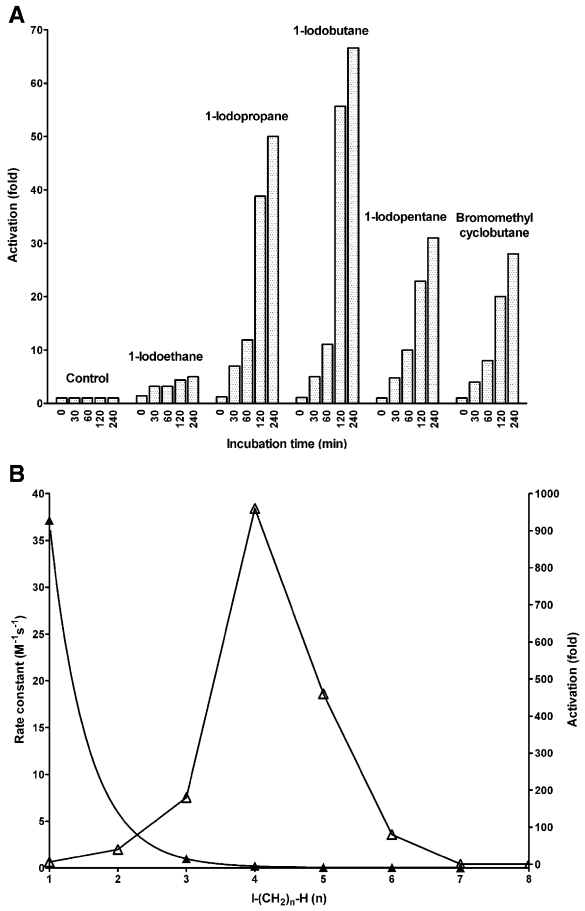

Figure 2. Chemical Activation of Y50C with Different Haloalkanes

(A) Time dependence of the chemical activation of Y50C. Y50C was incubated at 22°C with 100 mM 1-iodoethane, 1-iodopropane, 1-iodobutane, 1-iodopentane, and bromomethyl cyclobutane for different periods of time, 0, 30, 60, 120, and 240 min; the control, like the samples, contained 10% (v/v) methanol (solvent of the reagents). The activity was measured on aliquots from the reaction mixture.

(B) Influence of chain length of 1-iodoalkanes on chemical reactivity and activation of Y50C. The second-order rate constants of the nonenzymatic reaction of different 1-iodoalkanes with GSH was determined at 22°C (▲) in 50 mM glycine-NaOH buffer (pH 10) containing 1 mM EDTA. The degree of activation of Y50C afforded by the 1-iodoalkanes Y50C is shown for comparison (Δ). The specific activity reached after 20 hr incubation of Y50C with a 1-iodoalkane was determined in the standard assay system after removing the excess of reagent by gel filtration.

key-residue side chain influence the interaction with the lock structure.

Reactivity of Iodoalkanes versus Gain of Catalytic Activity

The iodoalkanes used differ in their intrinsic chemical reactivities, and the degree of activation of Y50C is therefore influenced by possible differences in the extent of Cys alkylation at a given time point. The second-order rate constants for the S-alkylation of GSH were therefore determined with a homologous series of 1-iodoalkanes and compared with the activating effects of the series on the Y50C protein. The rate constant of 1-iodomethane is markedly higher than those of all other 1-iodoalkanes tested (Figure 2B). The reactivity of the reagents decreases monotonously with the chain length. In contrast, the activation has a distinct optimum at a carbon-chain length of four (Figure 2B). In particular, it can be noted that 1-iodomethane is 177-fold more chemically reactive than 1-iodobutane, whereas the latter gives 160-fold higher activation of Y50C than does 1-iodomethane (Figure 2B). Further, the activation effect of 1-iodoheptane on Y50C is 1920-fold lower than that of 1-iodobutane, whereas the reactivities of 1-iodoheptane and 1-iodobutane differ by 7-fold only.

Direct determination of unmodified cysteine residues was also made by use of Ellman's reagent (Table 1). With the exception of the secondary iodoalkanes, all the investigated reagents modified 65%–100% of the Cys in Y50C. Similar results were obtained by measurement of S-alkylcysteines in hydrolysates of Y50C alkylated by 1-iodobutane and 1-iodopentane; the elution time and the ninhydrin reaction in the amino acid analyzer were standardized by the corresponding S-alkylglutathiones (data not shown). These results concordantly show that the differential activation effects of 1-iodoalkanes on Y50C are due to differences in the structure of the alkyl chain rather than to the degree of alkylation of Y50C. In quantitative terms, the activation observed with 1-bromo-2-ethylbutane and 1-iodo-2-methylpropane may underestimate their potency, since only 19%–20% of the Cys was modified (Table 1). The action of these secondary iodoalkanes may also be influenced by the fact that they are both chiral molecules that were tested as racemates (the only form available to us).

Selectivity of the Chemical Modification

In order to test the specificity of the chemical modification, different 1-iodoalkanes were incubated with the following GST P1-1 variants: C48 (the mutant containing a sulfhydryl group in position 48 only), Cys-free, and Y50C for different periods of time (Figure 3). The catalytic activity of Cys-free was not affected by incubation with $\leq$100 mM of different 1-iodoalkanes for 5 hr at 22°C. On the other hand, the activity of C48 decreased by 50% after 97 and 57 min incubation at 22°C with 1-iodopropane and 1-iodobutane, respectively, at the lower concentration of 10 mM. In striking contrast, Y50C was activated by incubation for up to 5 hr at 22°C with 100 mM of different 1-iodoalkanes. The crucial structural difference between Cys-free, C48, and Y50C is the absence or the presence of Cys residues in different positions. Consequently, the differential effect of alkylation on these three mutants demonstrates that the activation of Y50C by 1-iodoalkanes is indeed due to the chemical modification of Cys^{50}.

Mass Spectrometric Analyses

For further conformation of the site-specific alkylation, a mass-spectrometric analysis of protein digests was made. After in-gel tryptic digestion of the appropriate GST P1-1 variants, peptide mass fingerprinting of Y50C and *n*-butyl-Y50C demonstrated the presence of the expected native and modified peptides: ASALCGQLPK (MH^+ 987.53) and ASAL(butyl-C)GQLPK (MH^+ 987.53 + 56.06 = 1043.59), respectively (Figures 4A and 4B). This was further confirmed by sequencing the modified

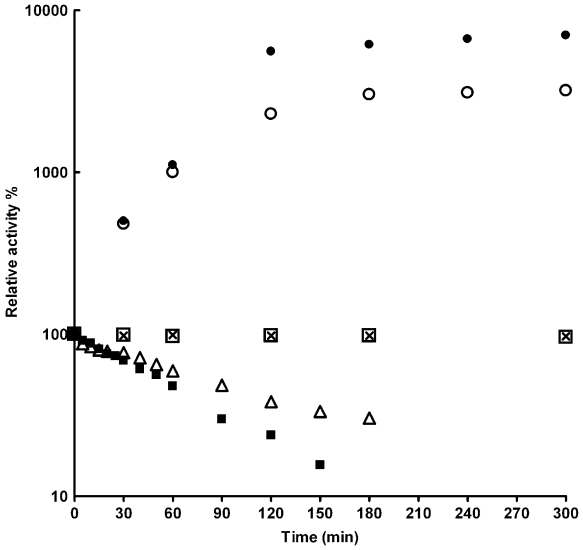

Figure 3. Differential Effects of 1-Iodoalkanes on Mutants C48, Cys-Free, and Y50C

The enzyme variants were incubated with alkylating agents at 22°C for different periods of time; Y50C with 100 mM of 1-iodobutane (●), 1-iodopentane (○); Cys-free with 100 mM of 1-iodobutane (□), 1-iodopentane (X); and C48 with 10 mM with 1-iodopropane (Δ), 1-iodobutane (■). The activity (percent of unmodified protein), plotted on a logarithmic scale, was measured on aliquots from the reaction mixture.

peptide after lysine blockage, sulfonation, and post-source decay analysis by MALDI-TOF/TOF (Figure 4C). In the position for the modified Cys^{50} residue, the sequence analysis revealed a mass difference of 159.17, which results from Cys (103.01) + C_4H_8 (56.06). No indication of alkylation of positions other than residue 50 of *n*-butyl-Y50C was obtained by mass spectrometry.

Steady-State Kinetic Characterization of Purified Alkylated Enzyme

Steady-state kinetic analysis of *n*-butyl-Y50C, first activated by 1-iodobutane and subsequently purified from nonalkylated Y50C by affinity chromatography, demonstrated that the K_M value for the electrophilic standard substrate 1-chloro-2,4-dinitrobenzene (CDNB) had not been markedly changed (Table 2). In contrast, the K_M value for GSH had decreased by >500-fold and 37-fold in comparison with the values for Y50C and Cys-free, respectively. These results support the notion that the key residue in position 50 secures the tight binding of the thiol substrate [7, 10]. The k_{cat} value was also elevated by two orders of magnitude by the alkylation, and the combined effects of the decreased K_M value for GSH and the elevated k_{cat} value thus contributed to the 28,000-fold enhanced catalytic efficiency (k_{cat}/K_M) of *n*-butyl-Y50C (Table 2). The absolute value, $1.38 \bullet 10^5$ $M^{-1}s^{-1}$, is 10-fold higher than that of the "parental" Cys-free GST P1-1.

Effect of Alkylation on Affinity for Active-Site Ligands

In order to further probe the nature of the activation, the effect of alkylation on binding of the competitive inhibitors *S*-methyl glutathione and *S*-hexyl glutathione was investigated. Chemical modification of Cys^{50} in Y50C by 1-iodobutane increased the affinity for *S*-hexyl glutathione as shown by a decrease of the K_i value by more than 2100- and 56-fold in comparison with the K_i values of Y50C and Cys-free, respectively (Table 2). Also, the K_i value of *n*-butyl-Y50C for *S*-methyl glutathione was decreased 53-fold in comparison with that of Cys-free for the same inhibitor. These results show that *n*-butyl-Y50C has acquired markedly enhanced binding affinity

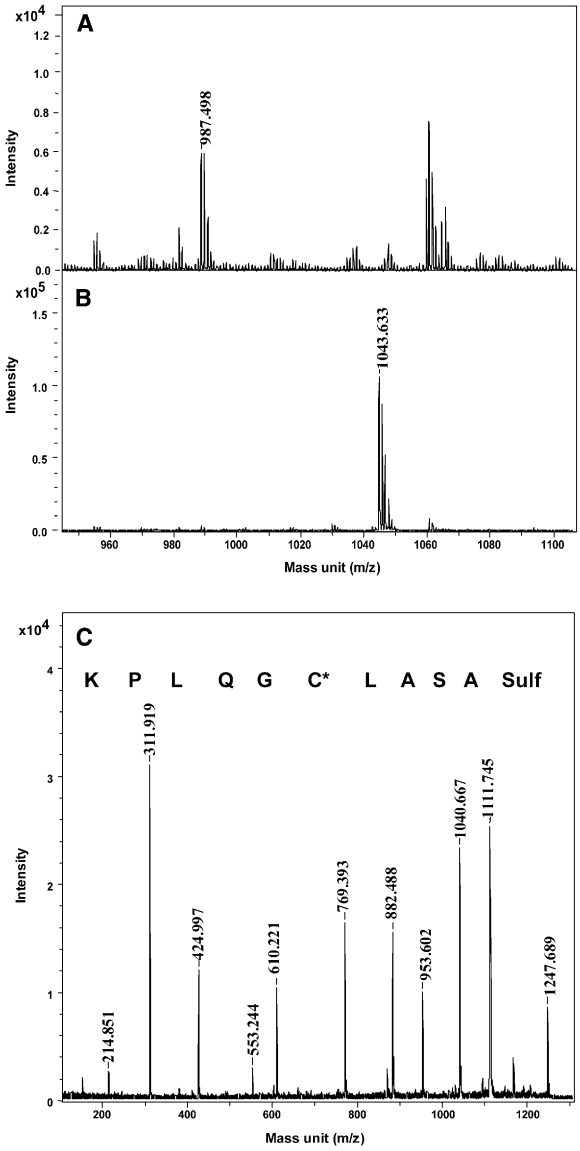

Figure 4. Mass Spectrometric Analyses of Y50C and Y50C Alkylated with 1-Iodobutane

A detail of peptide mass fingerprints of Y50C (A) and *n*-butyl-Y50C (B) showing the presence of the unmodified peptide ASALCGQLPK (MH^+ 987.498) and the modified peptide ASAL(butyl-C)GQLPK (MH^+ 1043.633), respectively. Sequence analysis demonstrating the position and mass of the modified C^{50} (C). Block letters in between the peaks represent the amino acid residues (one-letter code, N terminus to the right). C* has the mass of 159.17 Da (103.01 + 56.06). "Sulf" represents the 136 Da that the sulfonation reaction adds to the N terminus. Intensities are given in arbitrary units [33].

Table 2. Steady-State Kinetic Parameters of GST P1-1 Variants

GST Variant (Residue 50)	Kinetic Parameters[a]				Inhibition Constants (K_i)	
	k_{cat} (s^{-1})	K_M^{GSH} (mM)	K_M^{CDNB} (mM)	k_{cat}/K_M^{GSH} ($mM^{-1}s^{-1}$)	*S*-Methyl glutathione (μM)	*S*-Hexyl glutathione (μM)
Cys-free[b] (Tyr)	331 ± 27	11.5 ± 1	2.6 ± 0.33	15 ± 0.97	10800	410
Y50C[b] (Cys)	0.8 ± 0.2	160 ± 46	0.4 ± 0.08	0.005 ± 0.0006	ND	16000
n-Butyl-Y50C[c] (*n*-Butyl-Cys)	62.4 ± 2.4	0.32 ± 0.039	0.8 ± 0.046	138 ± 15	204	7.3

ND, not determined due to the low affinity of *S*-methyl glutathione for the mutant.
[a] The kinetic parameters were determined with purified proteins. Parameter values ± SE were estimated by nonlinear regression analysis of experimental data.
[b] Steady-state kinetics were carried out by varying GSH concentration from 0.1 to 50 mM, at a constant CDNB concentration of 2 mM, and by varying CDNB concentration from 0.1 to 2 mM, at a constant GSH concentration of 100 mM.
[c] Steady-state kinetics were carried out by varying GSH concentration from 0.1 to 16 mM, at a constant CDNB concentration of 2 mM, and by varying CDNB concentration from 0.1 to 2 mM, at a constant GSH concentration of 5 mM.

in comparison with the unmodified enzyme. In combination with the effect on the K_M value for GSH, this finding implies that alkylation of the Cys residue of Y50C enhances binding affinity for the GSH moiety as such. However, the elevated k_{cat} value shows that the enhanced catalytic efficiency is to a large proportion due also to the 80-fold increased substrate turnover as mentioned above (Table 2).

Effect of Alkylation on Stability of the Protein

Furthermore, alkylation affected the physical properties of Y50C. Heat inactivation experiments were carried out at 50°C to elucidate the effect of the alkylation on protein stability. The chemical modification of Y50C with 1-iodobutane increased the thermal stability from a half-life of 1.5 min up to 200 min. In comparison with the half-life of "parental" Cys-free, this corresponds to a 2-fold increase (Table 3). Enhanced protein stability following alkylation was also demonstrated by the effect of the chemical denaturant guanidine hydrochloride (GuHCl) on the enzymatic activity. The midpoint of denaturation for Y50C increased from 0.02 to 0.26 M GuHCl by the *S*-butyl modification; the midpoint of Cys-free was 0.06 M (Table 3).

Selective Alkylation Optimizes the Catalytic Properties

The striking regain of catalytic efficiency and ligand binding affinity of Y50C by select alkylating reagents demonstrates that unnatural amino acids can substitute for ordinary residues in a crucial position of a GST. A pertinent question is whether the catalytic properties could be further enhanced since the k_{cat}/K_M value 138 ± 15 $mM^{-1}s^{-1}$ (Table 2) is already somewhat higher than the value 98.2 ± 7.5 $mM^{-1}s^{-1}$ reported for the most active allelic variant of the wild-type GST P1-1/Ile105 [15]. Judicious choice of the chemical modifier could perhaps further enhance the activity, but it appears more likely that any further improvements would be obtained by mutations at sites other than the key residue of the lock-and-key joint. The level of improvement of catalytic efficiency is ultimately limited by the diffusion-controlled association (or dissociation) of the reactants and the enzyme [16]. GSTs generally have k_{cat}/K_M values $\leq 10^3$ $mM^{-1}s^{-1}$, even with their most active substrates, and in the present case, we have consequently reached an activity not far from the highest level that can be expected. A similar limiting k_{cat}/K_M value applies generally to all enzymes catalyzing two-substrate reactions.

Table 3. Alkylation with 1-Iodobutane Stabilizes Y50C against Heat and Chemical Denaturation

GST Variant	Half-Life (min)[a]	Midpoint Concentration (M)[b]
Cys-free	99	0.06
Y50C	1.5	0.02
n-Butyl-Y50C	200	0.26

[a] Time for 50% decreased enzymatic activity upon incubation at 50°C in the presence of 10 mM GSH.
[b] GuHCl concentration at which the enzymatic activity has decreased by 50%.

Comparison with Other Methods for Mutagenesis with Unnatural Amino Acids

Alternative methods used for site-specific incorporation of unnatural amino acids into proteins include engineering of the ribosomal translation system by various schemes [17, 18] and chemical peptide synthesis [19]. However, the targeted chemical modification has the advantage over these alternative methods that Cys in the selected position can be modified by numerous electrophilic chemical reagents, which can be introduced in a combinatorial manner. The crucial issue in the design is identification of a suitable site in the protein into which a unique Cys residue (or an alternative amino acid with high reactivity) can be site-specifically introduced. If susceptible sites are unknown, it is possible to make a mutational Ala [20] or Cys [21–23] screening linked with combinatorial chemical alkylation.

Significance

Biotechnical applications of proteins may be limited by the chemical and physical properties of crucial amino acid residues. For example, a Tyr, such as the key residue in GST P1-1, may be susceptible to oxidation or nitration under conditions of oxidative or nitrosative stress [24] or have an undesired phenolic hydroxyl group. For exchange of such unwanted residues, recombinant DNA techniques may in some cases be sufficient, but the restrictions of the genetic code limit the number of possible substitutions. The combination of site-specific introduction of a Cys residue complemented with chemical modification

widens the scope of feasible changes [25, 26]. Replacement of Tyr[50] in GST P1-1 by *S-n*-butyl-Cys is an example of successful molecular replacement surgery that apparently conserves all essential characteristics of the GST protein. The present approach to introducing unnatural amino acid residues has been applied to other proteins for gain of novel functional and structural properties [25, 26]. The applications of this method include (but are not limited to) modification of catalytic efficiency, substrate selectivity, affinity for other molecules, and allosteric or cooperative behavior. Other examples of enhanced properties include altered stability in relation to physical, chemical, or biological factors and processes, such as thermal denaturation, organic solvents, and biodegradation. For biotechnical purposes, the chemically modified protein can be produced in significant quantities at reasonable cost, if the target protein is available in large amounts from microbial cultures or other expression systems.

Experimental Procedures

Construction of the GST P1-1 Mutants Named C48, Cys-Free, and Y50C

Human GST P1-1 naturally contains four Cys residues per monomer. Site-directed mutagenesis was used to change residues Cys[15] and Cys[170] into Ser and Cys[102] into Ala, thereby constructing C48. Standard recombinant DNA techniques were applied as described [27]. The mutant was generated in a stepwise manner by three rounds of inverted polymerase chain reaction (IPCR). The pKK-D plasmid [28] containing the cDNA encoding human GST P1-1 (allelic variant Ile105 [29]) was used as template for PCR. The plasmid containing the cDNA encoding C48 was subsequently used as template for constructing Cys-free by mutating Cys[48] residue into Ala. The following 5′ phosphorylated primer pairs were used (altered nucleotides are underlined): Cys15Ser For, 5′-TGG TCG TTC TGC TGC ACT G-3′; Cys15Ser Rev, 5′-CGA ACT GGG AAG TAA ACA ACT G-3′; Cys48Ala For, 5′-AAA GCC TCC GCT CTA TAC GGG CAG-3′; Cys48Ala Rev, 5′-GAG TGA GCC CTC CTG CCA CGT CTC-3′; Cys102Ala For, 5′-CCT CCG CGC TAA ATA CAT CTC CCT CAT C-3′; Cys102Ala Rev, 5′-TCC TCC ACG CCG TCA TTC ACC ATG-3′; Cys170Ala For, 5′-CCC TGG CTC TCT GGA TGC GTT-3′; Cys170Ala Rev, 5′-GCT AGG ACC TCA TGG ATC AGC AG-3′.

Y50C was constructed by mutating the Tyr[50] residue of Cys-free into Cys[50] followed by adding six histidine residues to the N terminus of Y50C by IPCR. The histidine tag has previously been shown not to affect the functional properties of GSTs [7]. The plasmid containing the cDNA encoding Cys-free was used as a template for PCR. The following 5′ phosphorylated primer pairs were used (altered or inserted nucleotides are underlined): Tyr50Cys For, 5′-TCC GCT CTA TGC GGG CAG CTC-3′; Tyr50Cys Rev, 5′-GGC TTT GAG TGA GCC CTC CTG -3′; Histag For, 5′-CAC CAC CAC ATG CCT CCA TAC ACA GTT GTT TAC-3′; Histag Rev, 5′-GTG GTG GTG CAT TTT GTC ACC TTT GAA TTC TGT TTC-3′.

The reaction mixtures contained 0.2 mM dNTPs, 0.5 μM of each primer, various amounts of the template, and five units Pfu DNA polymerase (Stratagene, La Jolla, CA) in the buffer provided with the enzyme. PCR started with denaturation at 95°C for 5 min and was followed by 25 cycles of 95°C for 1 min, 52–66°C for 1 min, and 72°C for 9 min. The program was completed with a further extension period at 72°C for 30 min. The DNA was isolated from 1% (w/v) agarose gel after electrophoresis, purified with the QIAquick kit (Bio 101, Inc., Vista, CA), and ligated with T4 DNA ligase (Roche Diagnostics, Mannheim, Germany). The circularized DNA was used to transform *E. coli* XL-1 Blue by electroporation. The entire cDNA was sequenced by using Big Dye terminator kit v1.1 (Applied Biosystems, Foster City, CA) and ABI Model 310 DNA sequencer (Applied Biosystems) to verify that no unwanted mutation had been introduced.

Expression and Purification of C48, Cys-free, and Y50C

C48, Cys-free and Y50A were expressed in *E. coli* strain XL-1 Blue in presence of 0.2 mM IPTG as described by Kolm et al. [29] at 37°C (except for Y50C, which was grown at 25°C due to its thermal instability). The cells were harvested by centrifugation and disrupted by ultrasonication. C48 and Cys-free were purified by affinity chromatography with *S*-hexylglutatione-Sepharose 6B, while Y50C was purified by using Ni-IMAC as described by Hegazy et al. [10] owing to its low GSH affinity. The purity of enzymes was checked by SDS/polyacrylamide gel electrophoresis and isoelectric focusing with precast gels (Pharmacia LKB PhastSystem).

Cysteine-Specific Chemical Modification

Y50C, Cys-free were chemically modified by incubation with 100 mM alkyl halogenide, and C48 with 10 mM halogenide. All reactions were performed in 50 mM glycine-NaOH buffer (pH 10) containing 0.1 mM dithiothreitol (DTT) and 2.5 mM EDTA at 22°C. Y50C was also chemically modified by incubating the enzyme with 0.01–20 mM N-ethylmaleimide or 2 mM 1-chloro-2,4-dinitrobenzene (CDNB) in 10 mM Tris-HCl buffer (pH 7.8) containing 0.1 mM DTT and 2.5 mM EDTA or with 0.1 mM 2,2′-dithiodipyridine in 0.1 M phosphate buffer (pH 6.5) at 22°C for different periods of time. The specific activity of chemically modified enzyme was measured in the standard assay system. All Cys modifying reagents were dissolved in methanol.

Quantification of the Modified Cysteine

The amount of modified cysteine was determined with the Ellman's reagent [30] by subtracting the measured amount of the remaining cysteine after chemical modification of Y50C from the measured amount of the cysteine of the control (unmodified Y50C). The protein sulfhydryl titer was determined by incubating of protein sample with 0.2 mM 5,5-dithio-bis(2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 8.0) containing 1 mM EDTA in the absence and the presence of 6 M GuHCl for 10 min at room temperature and reading the absorbance at 412 nm.

Determination of Reaction Rate Constant of 1-Iodoalkanes with GSH

The different concentrations of 1-iodoalkane were incubated with constant GSH concentration of 1 mM in 50 mM glycine-NaOH buffer (pH 10) containing 2.5 mM EDTA at 22°C. The reaction was followed by measuring the decrease in GSH concentration with Ellman's reagent for quantitation of free thiols. Aliquots of the reaction mixture at different periods of time were drawn and mixed with Ellman's reagent in 0.1 M phosphate buffer (pH 8.0) containing 1 mM EDTA. The absorbance was monitored at 412 nm with a millimolar absorptivity of 13.6. A second-order plot of the extent of reaction at selected times gives the rate of the reaction. The second-order rate constant is the slope of the linear relationship of the measured reaction rates with the reagent concentrations.

Preparation and Purification of S-*n*-Butyl-Y50C

The S-*n*-butyl-Y50C derivative was prepared by incubating 18 μM Y50C with 100 mM of 1-iodobutane in 50 mM glycine-NaOH buffer (pH 10) containing 0.1 mM DTT and 2.5 mM EDTA at 22°C for 20 hr. After chemical alkylation of Cys[50] residue of Y50C, the excess of 1-iodobutane was removed by gel filtration in 10 mM Tris-HCl buffer (pH 7.8). The S-*n*-butyl-Y50C was purified from nonalkylated Y50C by affinity chromatography with *S*-hexylglutatione-Sepharose 6B [10], taking advantage of the difference in the binding affinity of alkylated and nonalkylated Y50C for the matrix.

Mass Spectrometric Analyses

The homogeneous Coomassie brilliant blue-stained protein bands of GST P1-1 variants were excised from SDS/PAGE gels and digested in situ with porcine trypsin as described [31]. After digestion and extraction, a small aliquot of the peptide mixture was analyzed by MALDI-TOF on a Bruker UltraflexTOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The instrument settings were those recommended by the manufacturer for proteolytic peptide analysis; the matrix used was α-cyano-4-hydroxycinnamic acid or dihydrobenzoic acid. Sequence analysis by post-source decay (PSD) was done after Lys blockage with 2-methoxy-4,5-dihydro-1H-imidazole [32] and N-terminal derivatization with 3-sulfopropionic NHS ester (CAF-MALDI

Sequencing kit from GE Healthcare, Uppsala, Sweden) [33]. The blocking reaction added 68 Da per Lys residue, and the sulfonation added 136 Da at the N-terminal amino group of the peptide. Hence, by PSD, the y1 ion for Lys measures 147 + 68 = 215 Da.

Specific Activity Measurement

The enzymatic activity of the GST P1-1 variants was determined at 30°C by a spectrophotometric standard assay involving 1 mM GSH and 1 mM CDNB in 0.1 M phosphate (pH 6.5) [34]. The reaction was monitored at 340 nm. Protein concentrations were determined by using Bio-Rad protein assay reagent with bovine serum albumin as a standard [35].

Determination of Steady-State Kinetic Parameters

Steady-state kinetics of Cys-free, Y50C, and *n*-butyl-Y50C were investigated by varying the GSH concentration in the range of 0.05 to 50 mM, at a constant CDNB concentration of 2 mM, and by varying the CDNB concentration from 0.1 to 2 mM, at a constant GSH concentration (see Table 2). All measurements were performed in triplicate in 0.1 M phosphate (pH 6.5) at 30°C. Kinetic parameters were determined by fitting the Michaelis-Menten equation to the experimental data with the SIMFIT package [36]; k_{cat} values were calculated on a dimer basis (M_r = 46000 Dalton).

Inhibition Studies

The K_i values for *S*-methyl and *S*-hexyl glutathione for the enzyme variants were determined by measuring the catalytic activities in the presence of 1.0, 1.6, and 2.0 mM GSH, 1 mM CDNB, and different concentrations of inhibitor. The inhibition constants were determined from Dixon plots.

Thermal Stability

The thermal stability of Cys-free, Y50C and *n*-butyl-Y50C was examined by incubating 2 μM enzyme at 50°C for different time periods in 10 mM Tris-HCl (pH 7.8) containing 10 mM GSH and 5 mM DTT. The remaining enzymatic activity was measured in the standard assay system.

Chemical Denaturation

The loss of enzymatic activity of 1.6 μM Cys-free, Y50C, and *n*-butyl-Y50C was measured in the standard assay system after incubation for 30 min at 22°C in the presence of increasing concentration of GuHCl (0–1 M) in 0.1 M phosphate buffer (pH 8).

Acknowledgments

This work was supported by grants from the Swedish Research Council. U.M.H. was supported by a scholarship from the Egyptian Government, Department of Culture and Education.

Received: March 9, 2006
Revised: June 26, 2006
Accepted: July 7, 2006
Published: September 22, 2006

References

1. Mannervik, B., and Danielson, U.H. (1988). Glutathione transferases-structure and catalytic activity. CRC Crit. Rev. Biochem. *23*, 283–337.
2. Armstrong, R.N. (1997). Structure, catalytic mechanism, and evolution of the glutathione transferases. Chem. Res. Toxicol. *10*, 2–18.
3. Sinning, I., Kleywegt, G.J., Cowan, S.W., Reinemer, P., Dirr, H.W., Huber, R., Gilliland, G.L., Armstrong, R.N., Ji, X., Board, P.G., et al. (1993). Structure determination and refinement of human alpha class glutathione transferase A1-1, and a comparison with the Mu and Pi class enzymes. J. Mol. Biol. *232*, 192–212.
4. Sayed, Y., Wallace, L.A., and Dirr, H.W. (2000). The hydrophobic lock-and-key intersubunit motif of glutathione transferase A1-1: implications for catalysis, ligandin function and stability. FEBS Lett. *465*, 169–172.
5. Vargo, M.A., Nguyen, L., and Colman, R.F. (2004). Subunit interface residues of glutathione S-transferase A1-1 that are important in the monomer-dimer equilibrium. Biochemistry *43*, 3327–3335.
6. Hornby, J.A., Codreanu, S.G., Armstrong, R.N., and Dirr, H.W. (2002). Molecular recognition at the dimer interface of a class mu glutathione transferase: role of a hydrophobic interaction motif in dimer stability and protein function. Biochemistry *41*, 14238–14247.
7. Stenberg, G., Abdalla, A.M., and Mannervik, B. (2000). Tyrosine 50 at the subunit interface of dimeric human glutathione transferase P1-1 is a structural key residue for modulating protein stability and catalytic function. Biochem. Biophys. Res. Commun. *271*, 59–63.
8. Reinemer, P., Dirr, H.W., Ladenstein, R., Huber, R., Lo Bello, M., Federici, G., and Parker, M.W. (1992). Three-dimensional structure of class pi glutathione S-transferase from human placenta in complex with S-hexylglutathione at 2.8 Å resolution. J. Mol. Biol. *227*, 214–226.
9. Garcia-Saez, I., Parraga, A., Phillips, M.F., Mantle, T.J., and Coll, M. (1994). Molecular structure at 1.8 Å of mouse liver class pi glutathione S-transferase complexed with S-(p-nitrobenzyl)glutathione and other inhibitors. J. Mol. Biol. *237*, 298–314.
10. Hegazy, U.M., Mannervik, B., and Stenberg, G. (2004). Functional role of the lock and key motif at the subunit interface of glutathione transferase P1-1. J. Biol. Chem. *279*, 9586–9596.
11. Hendrickson, T.L., de Crécy-Lagard, V., and Schimmel, P. (2004). Incorporation of nonnatural amino acids into proteins. Annu. Rev. Biochem. *73*, 147–176.
12. Plettner, E., DeSantis, G., Stabile, M.R., and Jones, J.B. (1999). Modulation of esterase and amidase activity of subtilisin *Bacillus lentus* by chemical modification of cysteine mutants. J. Am. Chem. Soc. *121*, 4977–4981.
13. Corey, D.R., Pei, D., and Schultz, P.G. (1989). Generation of a catalytic sequence-specific hybrid Dnase. Biochemistry *28*, 8277–8286.
14. Sutton, C.L., Mazumder, A., Chen, C.H., and Sigman, D.S. (1993). Transforming the *Escherichia coli* Trp repressor into a site-specific nuclease. Biochemistry *32*, 4225–4230.
15. Johansson, A.-S., Stenberg, G., Widersten, W., and Mannervik, B. (1998). Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. J. Mol. Biol. *278*, 687–698.
16. Fersht, A. (1999). Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding (New York, NY: Freeman).
17. Wang, L., Xie, J., Deniz, A.A., and Schultz, P.G. (2003). Unnatural amino acid mutagenesis of green fluorescent protein. J. Org. Chem. *68*, 174–176.
18. Josephson, K., Hartman, M.C., and Szostak, J.W. (2005). Ribosomal synthesis of unnatural peptides. J. Am. Chem. Soc. *127*, 11727–11735.
19. Nilsson, B.L., Soellner, M.B., and Raines, R.T. (2005). Chemical synthesis of proteins. Annu. Rev. Biophys. Biomol. Struct. *34*, 91–118.
20. Cunningham, B.C., and Wells, J.A. (1989). High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis. Science *244*, 1081–1085.
21. Sahin-Toth, M., and Kaback, H.R. (1993). Cysteine scanning mutagenesis of putative transmembrane helices IX and X in the lactose permease of *Escherichia coli*. Protein Sci. *2*, 1024–1033.
22. Pinto, L.H., Dieckmann, G.R., Gandhi, C.S., Papworth, C.G., Braman, J., Shaughnessy, M.A., Lear, J.D., Lamb, R.A., and DeGrado, W.F. (1997). A functionally defined model for the M2 proton channel of influenza A virus suggests a mechanism for its ion selectivity. Proc. Natl. Acad. Sci. USA *94*, 11301–11306.
23. Kubo, Y., Yoshimichi, M., and Heinemann, S.H. (1998). Probing pore topology and conformational changes of Kir2.1 potassium channels by cysteine scanning mutagenesis. FEBS Lett. *435*, 69–73.
24. Dalle-Donne, I., Scaloni, A., Giustarini, D., Cavarra, E., Tell, G., Lungarella, G., Colombo, R., Rossi, R., and Milzani, A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. Mass Spectrom. Rev. *24*, 55–99.

25. Tann, C.M., Qi, D., and Distefano, M.D. (2001). Enzyme design by chemical modification of protein scaffolds. Curr. Opin. Chem. Biol. *5*, 696–704.
26. Qi, D., Tann, C.M., Haring, D., and Distefano, M.D. (2001). Generation of new enzymes via covalent modification of existing proteins. Chem. Rev. *101*, 3081–3111.
27. Sambrook, J., and Russell, D.W. (1989). Molecular Cloning: A Laboratory Manual, Third Edition (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
28. Björnestedt, R., Widersten, M., Board, P.G., and Mannervik, B. (1992). Design of two chimaeric human-rat class alpha glutathione transferases for probing the contribution of C-terminal segments of protein structure to the catalytic properties. Biochem. J. *282*, 505–510.
29. Kolm, R.H., Stenberg, G., Widersten, M., and Mannervik, B. (1995). High-level bacterial expression of human glutathione transferase P1-1 encoded by semisynthetic DNA. Protein Expr. Purif. *6*, 265–271.
30. Ellman, G.L. (1959). Tissue sulfhydryl groups. Arch. Biochem. Biophys. *82*, 70–77.
31. Hellman, U. (2000). Sample preparation by SDS/PAGE and in-gel digestion. EXS *88*, 43–54.
32. Peters, E., Horn, D., Tully, D., and Brock, A. (2001). A novel multifunctional labeling reagent for enhanced protein characterization with mass spectrometry. Rapid Commun. Mass Spectrom. *15*, 2387–2392.
33. Hellman, U., and Bhikhabhai, R. (2002). Easy amino acid sequencing of sulfonated peptides using post-source decay on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer equipped with a variable voltage reflector. Rapid Commun. Mass Spectrom. *16*, 1851–1859.
34. Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974). Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. *249*, 7130–7139.
35. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248–254.
36. Bardsley, W.G. (2006). Simfit (http://www.simfit.man.ac.uk/).
37. Oakley, A.J., Lo Bello, M., Battistoni, A., Ricci, G., Rossjohn, J., Villar, H.O., and Parker, M.W. (1997). The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. J. Mol. Biol. *274*, 84–100.