Glutamic acid-65 is an essential residue for catalysis in *Proteus mirabilis* glutathione S-transferase B1-1

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The functional role of three conserved amino acid residues in *Proteus mirabilis* glutathione S-transferase B1-1 (PmGST B1-1) has been investigated by site-directed mutagenesis. Crystallographic analyses indicated that Glu^{65}, Ser^{103} and Glu^{104} are in hydrogen-bonding distance of the N-terminal amino group of the γ-glutamyl moiety of the co-substrate, GSH. Glu^{65} was mutated to either aspartic acid or leucine, and Ser^{103} and Glu^{104} were both mutated to alanine. Glu^{65} mutants ($Glu^{65} \rightarrow$ Asp and $Glu^{65} \rightarrow$ Leu) lost all enzyme activity, and a drastic decrease in catalytic efficiency was observed for $Ser^{103} \rightarrow$ Ala and $Glu^{104} \rightarrow$ Ala mutants toward both 1-chloro-2,4-dinitrobenzene and GSH. On the other hand, all mutants displayed similar intrinsic fluorescence, CD spectra and thermal stability, indicating that the mutations did not affect the structural integrity of the enzyme. Taken together, these results indicate that Ser^{103} and Glu^{104} are significantly involved in the interaction with GSH at the active site of PmGST B1-1, whereas Glu^{65} is crucial for catalysis.

Key words: circular dichroism, GST, site-directed mutagenesis.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a superfamily of dimeric multifunctional enzymes, which metabolize a wide variety of electrophilic compounds via GSH conjugation [1–4]. This reaction is the first step in mercapturic acid formation, a pathway through which harmful xenobiotics and endobiotics are inactivated and eliminated from an organism [1–4]. In eukaryotes the large number of soluble GSTs so far investigated have been grouped into several classes, i.e. Alpha, Mu, Pi, Theta, Sigma, Kappa, Zeta and Omega, on the basis of their physical, chemical, immunological and structural properties [2,4–5]. Despite their low inter-class sequence identity (often less than 20 %) crystallographic studies have indicated that the overall polypeptide fold of the different classes of soluble GSTs is very similar [3,6–9]. An additional GST family comprises membrane-bound transferases called MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism), but these bear no similarity to soluble GSTs [10].

A well-characterized prokaryotic GST is one isolated from the Gram-negative bacterium *Proteus mirabilis*, i.e. *Proteus mirabilis* glutathione S-transferase B1-1 (PmGST B1-1) [11–17]. PmGST B1-1 displays biochemical and structural properties that distinguishes it from the GSTs of other families, and it has been identified as the prototype of a new class, i.e. Beta class [14]. So far five members of the Beta class have been identified [14], all from bacteria, but many more are expected since GSTs are found in many bacterial species. A characteristic feature of PmGST B1-1 is a molecule of GSH covalently bound to Cys^{10} per subunit even though the enzyme has GSH-conjugating activity [14]. The presence of the disulphide does not appear relevant to the GSH-conjugating activity of the enzyme based on mutagenesis studies [15]. The mixed disulphide appears highly strained in the structure and hence would be readily broken in the conjugation reaction. A similar feature has recently been observed in an Omega class GST [5].

Spectroscopic and kinetic studies have emphasized that the thiolate form of GSH is involved in the catalytic mechanism of GSTs [18,19]. GSTs normally utilize hydroxy residues in the N-terminal domain of the enzyme for stabilizing the activated form of GSH: a conserved tyrosine residue in Alpha, Mu, Pi and Sigma class GSTs; and a serine or threonine residue in Theta class GSTs [3,20]. However, previous studies have shown this is not true for Beta class GSTs. In fact, we showed that none of the tyrosine, serine or cysteine residues located in the N-terminal domain of PmGST B1-1 are directly involved in its catalytic mechanism [15]. Furthermore, the crystal structure of PmGST B1-1 suggested the stabilizing role might be fulfilled in Beta class GSTs by one or more residues in the C-terminal domain of the enzyme. A recent report has demonstrated that His^{106} and Lys^{107} are important for GSH binding but are not significant contributors to the catalytic mechanism [17].

Crystallographic analyses of PmGST B1-1 have demonstrated that several residues interact to bind GSH in the active site [14]. One of these residues, Glu^{65}, is strikingly conserved between many of the GST classes. This residue is located in the generously allowed region of the Ramachandran plot and the strained stereochemistry is presumably counteracted by binding to GSH. The equivalent residue in other GST structures adopts the same conformation [5,7,8,21–23]. Moreover, mutational studies on eukaryotic enzymes suggested that the equivalent residue to Glu^{65} was involved in GSH binding [24,25].

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GST, glutathione S-transferase; IPTG, isopropyl β-D-thiogalactoside; LB, Luria–Bertani; MIC, minimum inhibitory concentration; PmGST B1-1, *Proteus mirabilis* glutathione S-transferase B1-1.

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The structural data for PmGST B1-1 indicate that Ser^{103} and Glu^{104}, together with Glu^{65}, contribute to binding of GSH through hydrogen bonding to the amino group of the γ-glutamyl moiety of the GSH molecule from the neighbouring monomer [14]. To better understand the role of these residues in the catalytic mechanism and/or binding properties of PmGST B1-1 they have been replaced by site-directed mutagenesis, and the effects of the replacements have been examined.

MATERIALS AND METHODS

Chemicals

Isopropyl β-D-thiogalactoside (IPTG) and the antibiotics used in the present study were purchased from Sigma–Aldrich (Milan, Italy). All other reagents used were of the highest grade commercially available.

Oligonucleotide-directed mutagenesis

The DNA encoding PmGST B1-1 in pBtac1 (pGPT1) [13] was used as a template in the mutagenesis procedure. The single mutations $Glu^{65} \rightarrow Asp$, $Glu^{65} \rightarrow Leu$, $Ser^{103} \rightarrow Ala$ and $Glu^{104} \rightarrow Ala$ were made with the following oligonucleotides: $Glu^{65} \rightarrow Asp$, 5′-T AGC AAC ACC GTC CGT TAA AAT ATC ACC-3′; $Glu^{65} \rightarrow Leu$, 5′-AAT AGC AAC ACC CAG CGT TAA AAT AT-3′; $Ser^{103} \rightarrow Ala$, 5′-CC TTT ATG AAC TTC AGC GGC AAG AAA G-3′; and $Glu^{104} \rightarrow Ala$, 5′-CC TTT ATG AAC TGC ACT GGC AAG AAA G-3′. The oligonucleotide-directed USE mutagenesis kit (Pharmacia Biotech) was used according to the manufacturer's instructions. Clones with the required mutation were first identified by colony hybridization, using 5′-^{32}P-labelled mutameric oligonucleotides as probes, and confirmed by dideoxynucleotide sequencing [26].

Expression and purification of wild-type and mutant PmGST B1-1 enzymes

To induce gene transcription, IPTG was added at a final concentration of 1 mM when *Escherichia coli* XL1Blue strains, grown at 25 °C in Luria–Bertani (LB) medium [27] and supplemented with tetracycline and ampicillin, reached an approximate D_{550} of 0.4, and the incubation was prolonged for a further 16 h.

The purification of enzymes was performed as follows. The bacterial cells were collected by centrifugation, washed twice and resuspended in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA (buffer A) and disrupted by cold sonication. The particulate material was removed by centrifugation, the resulting supernatant was subjected to isoelectric focusing and the concentrated enzyme obtained was further purified by anion-exchange chromatography using a DEAE resin as reported previously [17]. The supernatant was subjected to isoelectric focusing on a column (110 ml) containing 3 % (v/v) Ampholine pH 3.5–10 in a 0–40 % (w/v) sucrose density gradient. After focusing for 72 h at a final voltage of 700 V (4 °C) the content of the column was eluted and collected in 1 ml fractions. The protein peak thus separated was concentrated and dialysed against 10 mM Tris/HCl (pH 7.5) (buffer B) by ultrafiltration in an Amicon apparatus. Concentrated enzyme was further purified by anion-exchange chromatography using a DEAE column (1.5 cm × 11 cm; Bio-Rad Laboratories, Milan, Italy) equilibrated with buffer B. The enzyme was eluted with a 100 ml linear gradient of 0–0.6 M KCl in buffer B (flow rate = 0.5 ml/min; fraction volume = 1 ml). The peaks containing GST were eluted in 0.11–0.13 M KCl.

The fractions were pooled, concentrated, dialysed against buffer A by ultrafiltration and subjected to further analyses. SDS/PAGE in a discontinuous slab gel was performed using the method of Laemmli [28], and protein concentration was determined using the method of Bradford [29] with γ-globulin as a standard.

Enzyme assays

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was assayed at 30 °C according to the method of Habig and Jakoby [30]. For the enzyme kinetic determinations either CDNB or GSH was held constant at 1 or 5 mM respectively, while the concentration of the other substrate was varied (from 0.1 to 5 mM for GSH, and from 0.1 to 1.6 mM for CDNB). Each initial velocity was measured at least in triplicate. The KaleidaGraph Software package (Synergy Software, Reading, PA, U.S.A.) was used to estimate the Michaelis constant (K_m) and V_{max} values.

The dependence of k_{cat}/K_m on pH was determined by using the following buffers (0.1 M) at the indicated pH values: Bis-Tris/HCl, from 5.0 to 7.0; and Tris/HCl, from 7.2 to 9.0. The reactions were carried out using saturating GSH (5 mM) and variable CDNB concentrations. The pK_a values were obtained by computer fitting of the data to the equation:

$$\log(k_{cat}/K_m) = \log[C/(1+[H^+]/K_a)]$$

where C is the upper limit of k_{cat}/K_m at high pH [31].

Thermal stability measurements of mutant enzymes (0.7 μM) were carried out by incubating the samples at each temperature for 15 min. Following incubation, samples were divided in half, then they were utilized to determine both GST activity and intrinsic fluorescence spectra.

Fluorescence measurements

The intrinsic fluorescence spectra of the proteins were recorded on a Spex spectrofluorimeter (Fluoromax model) equipped with a thermostatically controlled sample holder at 25 °C. Emission spectra (excitation at 280 nm) were recorded in 1 nm wavelength increments and the signal was acquired for 1 s at each wavelength. Spectra were corrected by subtraction of the corresponding spectra for blank samples.

CD spectroscopy

CD measurements in the far-UV region from 200–250 nm were performed with a Jasco-600 spectropolarimeter. Samples containing native and mutant enzymes at a concentration of 22 μM were scanned at least five times at a rate of 10 nm/min and then averaged. The temperature of the sample compartment was maintained at 20.0 ± 1.0 °C with a circulating-water bath. The cuvette used had a light path of 0.1 cm. Each spectrum was corrected by subtracting the corresponding blank.

Growth curve

A single colony of *E. coli* XL1Blue (pGPT1) was inoculated into LB medium [27] and grown overnight in a water-bath shaker. LB medium (150 ml) containing 3 ml of the overnight culture and 1 mM IPTG was then incubated at 25 °C in a water-bath shaker, and the attenuance was monitored at 600 nm. When D_{600} reached a value of 0.250, the cells were exposed to 0.25× minimum inhibitory concentration (MIC) of rifamycin (MIC = 50 μg/ml). The MIC was determined by a standard broth microdilution technique [32].

RESULTS AND DISCUSSION

Although key catalytic residues in other classes of GSTs have been identified by mutagenesis studies, no key residues have been discovered in a Beta class GST despite extensive studies [15–17]. Previous crystallographic analysis of PmGST B1-1 indicated that Glu^{65}, Ser^{103} and Glu^{104} all form potential hydrogen-bonding interactions with GSH through the N-terminal amino group of the γ-glutamyl moeity of GSH [14]. To investigate the role of Glu^{65}, Ser^{103} and Glu^{104} residues in the binding of GSH and the catalytic mechanism of PmGST B1-1, site-directed mutagenesis experiments have been performed.

Glu^{65} was replaced with aspartic acid in order to extend the distance between the carboxy group and GSH. It was also replaced with leucine to see the effect of changing the carboxy group to an uncharged side chain. Both Ser^{103} and Glu^{104} residues were replaced with alanine in order to see the effect of replacing the polar or charged side chains with an uncharged group.

To test the overexpression of all mutant enzymes, total cellular extracts of induced *E. coli* XL1Blue cells were examined by immunoblot analysis using an antiserum against PmGST B1-1 [11]. All mutants co-migrated with the wild-type protein with comparable levels of expression (results not shown).

Unlike the $Ser^{103} \rightarrow Ala$ and $Glu^{104} \rightarrow Ala$ mutants, $Glu^{65} \rightarrow Asp$ and $Glu^{65} \rightarrow Leu$ did not bind to GSH-affinity matrices [15], and could not be purified by this standard method. We thus adopted a different approach to purify all mutants, as well as wild-type, to apparent homogeneity. The enzymes were purified by preparative isoelectrofocusing followed by anion-exchange chromatography as reported in the Materials and methods section. The electrophoretic mobilities and the apparent molecular masses of $Glu^{65} \rightarrow Asp$, $Ser^{103} \rightarrow Ala$ and $Glu^{104} \rightarrow Ala$ mutants were indistinguishable from those of the wild-type enzyme (Figure 1). A single band for each form of enzyme was obtained indicating the absence of contaminating proteins. Unfortunately, the $Glu^{65} \rightarrow Leu$ mutant could not be purified by this method either. As well as forming a possible hydrogen bond with GSH, Glu^{65} also forms a salt bridge with Lys^{49} and a possible interaction with Asp^{99} from the neighbouring monomer of the dimer [14]. Therefore we did not utilize this mutant for further analyses.

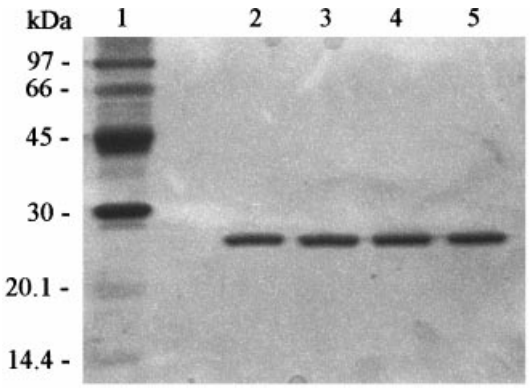

Figure 1 SDS/PAGE analysis of purified wild-type and $Glu^{65} \rightarrow Asp$, $Ser^{103} \rightarrow Ala$ and $Glu^{104} \rightarrow Ala$ mutant enzymes

Proteins (1 μg of each) were detected by silver staining. Lane 1, molecular-mass standards; lane 2, wild-type; lane 3, $Glu^{65} \rightarrow Asp$; lane 4, $Ser^{103} \rightarrow Ala$; lane 5, $Glu^{104} \rightarrow Ala$.

In Table 1 the specific activities and kinetic constants of the mutant enzymes are compared with the wild-type enzyme using GSH and CDNB as substrates. Mutation of Glu^{65} to aspartic acid led to complete loss of the ability of PmGST B1-1 to catalyse the conjugation reaction, suggesting that Glu^{65} is an essential residue for catalysis. No significant decrease in the activity was found when Ser^{103} was replaced with alanine, with 86 % of wild-type activity. In contrast, the replacement of Glu^{104} with alanine produced a decrease in activity of approx. 80 %.

The $Ser^{103} \rightarrow Ala$ and $Glu^{104} \rightarrow Ala$ mutants showed increased K_m values for GSH and CDNB compared with the wild-type enzyme. In particular, $Glu^{104} \rightarrow Ala$ exhibited 6- and 8-fold increases in K_m values for GSH and CDNB respectively. The effects of mutations on k_{cat}/K_m were also calculated. A decrease of approx. 3-fold in k_{cat}/K_m values for both GSH and CDNB were observed with the $Ser^{103} \rightarrow Ala$ mutant. The effects on the catalytic efficiency of $Glu^{104} \rightarrow Ala$ were more significant, with a decrease to approx. 7 and 25 % towards GSH and CDNB respectively. When the pH dependence of k_{cat}/K_m^{CDNB} was examined for the wild-type enzyme and the mutants, no shift of the apparent pK_a of bound GSH occurred: the estimated pK_a values were very similar to that of wild-type (Table 1). In summary, the kinetic data show that Ser^{103} and Glu^{104} are important for the binding of GSH but that they are not directly involved in the activation and/or stabilization of the GSH thiol.

The intrinsic fluorescent properties of wild-type and mutant enzymes were studied. The λ_{max} was the same, indicating a similar environment of the tryptophan residues in wild-type and mutant enzymes. Tryptophan residues are useful monitors of the folding state of the enzyme as there are two such residues in each monomer: one located in the xenobiotic binding site and the other close to the dimer interface. Furthermore, no significant increase in fluorescence intensity was observed, suggesting that the replacement of Glu^{65}, Ser^{103} and Glu^{104} with other residues did not result in a change in protein conformation (results not shown). The effect of mutations on the secondary structure of

Table 1 Specific activities and kinetic constants for wild-type and mutant enzymes, with CDNB as the second substrate

The values are presented as means ± S.D. for at least three independent determinations. n.d., no detectable activity.

		GSH			CDNB			
Enzyme	Specific activity (μmol · min^{-1} · mg^{-1})	K_m (μM)	k_{cat} (min^{-1})	$10^{-3} \times k_{cat}/K_m$ (μM^{-1} · min^{-1})	K_m(μM)	k_{cat} (min^{-1})	$10^{-3} \times k_{cat}/K_m$ (μM^{-1} · min^{-1})	pK_a^{CDNB}
Wild-type	1.100 ± 0.080	686 ± 91	58.1 ± 6.1	84.7	730 ± 82	69.3 ± 7.0	95.0	6.40 ± 0.27
$Ser^{103} \rightarrow Ala$	0.950 ± 0.024	1156 ± 204	37.1 ± 0.5	32.1	5257 ± 243	185.4 ± 0.9	35.2	6.29 ± 0.33
$Glu^{104} \rightarrow Ala$	0.220 ± 0.003	4317 ± 1193	26.1 ± 5.3	6.1	6341 ± 121	153.4 ± 4.1	24.2	6.68 ± 0.29
$Glu^{65} \rightarrow Asp$	n.d.							

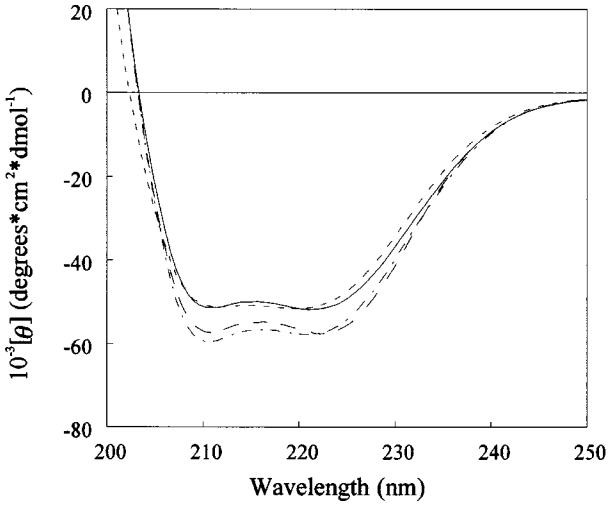

Figure 2 Far-UV CD spectra of wild-type (——) and Glu^{65} → Asp (-----) Ser^{103} → Ala (– – –) and Glu^{104} → Ala (– –) mutant enzymes

The spectra were recorded at 25 °C.

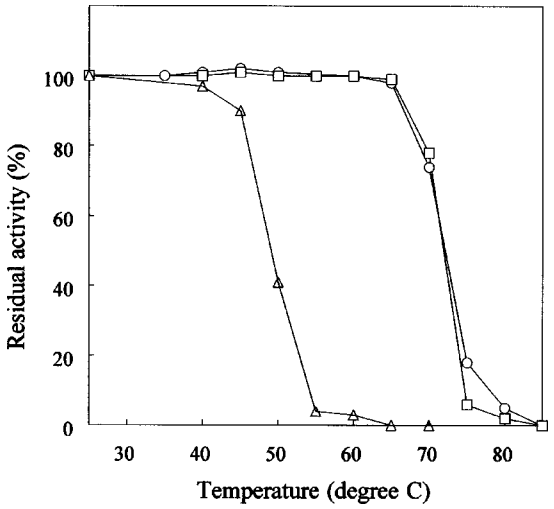

Figure 3 Effect of temperature on the stability of wild-type (○), and Ser^{103} → Ala (□) and Glu^{104} → Ala (△) mutant enzymes

The enzymic activity at 25 °C was taken to be 100%.

PmGST B1-1, monitored by CD spectroscopy in the far-UV region, was also analysed. None of the mutations had a significant effect on the secondary structure of the enzyme (Figure 2).

The effect of temperature on the stability of the mutant and wild-type enzymes is shown in Figure 3. The curve of the Ser^{103} → Ala mutant is coincident with that of the wild-type enzyme. At approx. 72 °C this mutant and the wild-type retained 50% of initial activity. In contrast, the Glu^{104} → Ala mutant is more thermolabile than the wild-type enzyme, with 50% inactivation at 49 °C. Glu^{104} is involved in two salt bridges, with Lys^{132} and Arg^{107} of the other monomer of the dimer, as well as the interaction with GSH [14]. Thus the thermal instability can be explained by the loss of all these contacts. Because the Glu^{65} mutant had no CDNB-conjugating activity, we studied the effect of temperature on this mutant form by intrinsic fluorescence. As

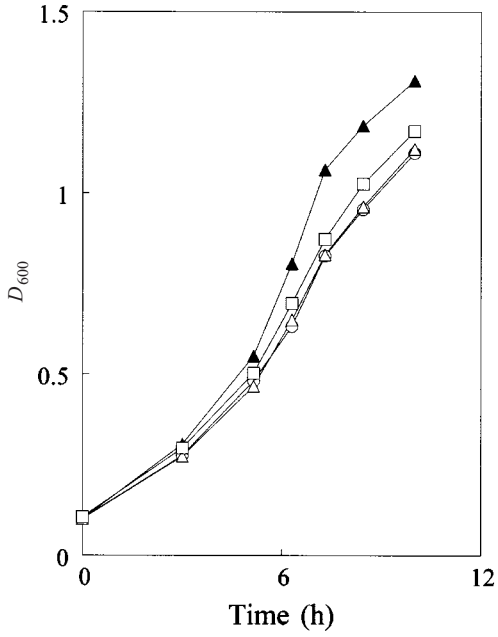

Figure 4 Effect of rifamycin (0.25 × MIC) on the growth rate of cells overexpressing wild-type (○) and Glu^{65} → Asp (▲), Ser^{103} → Ala (□) and Glu^{104} → Ala (△) mutant enzymes

The MIC value for rifamycin is 50 μg/ml.

temperature increased from 25 to 75 °C, the spectrum changed with a gradual increase of the fluorescence while maintaining the same λ_{max}. A similar pattern was observed for the other mutants and the wild-type enzyme (results not shown).

The fluorescence data, CD spectra and thermal stability experiments demonstrated that Glu^{65}, Ser^{103} and Glu^{104} are not important for maintaining the proper conformation of the protein. In particular, these results indicated that the mutation of Glu^{65}, which caused a total loss of activity, did not lead to any obvious conformational changes of the protein.

It has previously been suggested that PmGST B1-1 may play a role in antimicrobial resistance [13]. In fact, PmGST B1-1 is able to bind, *in vivo*, several classes of antimicrobial drugs, such as rifamycin. Analysis of the crystal structure of PmGST B1-1 has lead to the identification of a hydrophobic binding site as a possible location for antibiotic binding [14]. Ser^{103} and Glu^{104} are located on helix α4 which contributes to the hydrophobic binding site, and Glu^{104} forms part of the base of that site. To examine the possibility that these residues are involved in antibiotic binding, *in vivo* experiments were carried out. The growth rate of cells overexpressing Ser^{103} and Glu^{104} mutants in the presence of rifamycin was measured and compared with cells overexpressing the Glu^{65} mutant and the wild-type enzyme. As can be seen in Figure 4, similar growth curves were obtained for all bacterial strains tested, indicating that the antibiotic did not affect the growth rate of cells. These results suggest that none of the mutated residues are involved in antibiotic binding.

In conclusion, the results presented here show that Ser^{103} and Glu^{104} are involved in the interaction with GSH at the active site of PmGST B1-1 but that they do not contribute significantly to the catalytic process. Furthermore, mutation of these residues did not induce changes in protein conformation and stability of PmGST B1-1. In contrast, Glu^{65} is an essential residue for binding GSH. The mutation of this residue to the shorter

aspartic acid side-chain caused a loss of interaction with GSH and consequently a total loss of GSH activity. However, our results also indicate that this residue is not directly responsible for the activation of GSH or stabilization of the GSH thiolate ion.

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