Evaluation of the role of two conserved active-site residues in Beta class glutathione S-transferases

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Glutathione S-transferases (GSTs) normally use hydroxy-group-containing residues in the N-terminal domain of the enzyme for stabilizing the activated form of the co-substrate, glutathione. However, previous mutagenesis studies have shown that this is not true for Beta class GSTs and thus the origin of the stabilization remains a mystery. The recently determined crystal structure of *Proteus mirabilis* GST B1-1 (PmGST B1-1) suggested that the stabilizing role might be fulfilled in Beta class GSTs by one or more residues in the C-terminal domain of the enzyme. To test this hypothesis we mutated His^{106} and Lys^{107} of PmGST B1-1 to investigate their possible role in the enzyme's catalytic activity. His^{106} was mutated to Ala, Asn and Phe, and Lys^{107} to Ala and Arg. The effects of the replacement on the activity, thermal stability and antibiotic-binding capacity of the enzyme were examined. The results are consistent with the involvement of His^{106} and Lys^{107} in interacting with glutathione at the active site but these residues do not contribute significantly to catalysis, folding or antibiotic binding.

Key words: active-site mutation, *Proteus mirabilis*, site-directed mutagenesis.

INTRODUCTION

Glutathione S-transferases (GSTs; EC 2.5.1.18) are a large supergene family of multifunctional enzymes that metabolize a wide variety of electrophilic compounds via GSH conjugation [1–4]. This reaction is the first step in the formation of mercapturic acid, a pathway through which harmful xenobiotics and endobiotics are inactivated and eliminated [1–4]. In eukaryotes the large number of soluble GSTs so far investigated have been grouped into several classes, namely Alpha, Mu, Pi, Theta, Sigma, Kappa and Zeta, on the basis of their physical, chemical, immunological and structural properties [1–6]. Despite the low sequence identity between classes (often less than 20 %), crystallographic studies have indicated that the overall polypeptide folding of the different classes of soluble GST is very similar [3,7–10]. An additional GST family comprises membrane-bound transferases, recently called MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism) [11]. Spectroscopic and kinetic studies have demonstrated that the thiolate form of GSH is involved in the catalytic mechanism of GSTs [12,13]. A conserved tyrosine residue near the N-terminus in Alpha, Mu, Pi and Sigma class GSTs and a similarly located serine or threonine residue in Theta class GSTs have been shown to be important in catalysis, probably by stabilizing the GSH thiolate anion [3,14].

One of the best-characterized prokaryotic GSTs is one from *Proteus mirabilis*, PmGST B1-1 [15–20]. PmGST B1-1 displays biochemical and structural properties that distinguish it from the GSTs of other families and it has been identified as the prototype of a new class, Beta [18]. Unlike eukaryotic GSTs, PmGST B1-1 has a molecule of GSH covalently bound to Cys^{10} even though the enzyme has GSH-conjugating activity [18]. The mixed disulphide seems highly strained in the structure and would therefore be readily broken in the conjugation reaction. Nevertheless, mutational studies have demonstrated 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 [19].

Recent reports on the three-dimensional structure of two Beta class GSTs have indicated that His^{106} is located in close proximity to the thiol group of GSH (see Figure 1), suggesting that it might act as a catalytic residue in the conjugation reaction [18,21]. The only other positively charged group in the GSH-binding site is Lys^{107}, which interacts with the main-chain carbonyl group of the γ-glutamyl moiety of the tripeptide. It is possible that either or both of the residues could stabilize the thiolate form of GSH by direct interaction (His^{106}) or via longer-range electrostatic stabilization (His^{106} and Lys^{107}). To assess the importance of these two conserved residues in the catalytic mechanism and other properties of Beta class GSTs, site-directed mutagenesis experiments on PmGST B1-1 have been performed. The results obtained demonstrate that His^{106} and Lys^{107} are important for GSH binding but are not significant contributors to the catalytic mechanism, folding or antibiotic binding of PmGST B1-1.

MATERIALS AND METHODS

Chemicals

Isopropyl β-D-thiogalactoside (IPTG) and guanidinium chloride (GdmCl), as well as the antibiotics used in the present study, were

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

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purchased from Sigma-Aldrich (Milano, Italy). All other reagents used were of the highest grade commercially available.

Oligonucleotide-directed mutagenesis

The DNA encoding PmGST B1-1 in pBtac1 (pGPT1) [17] was used as a template in the mutagenesis procedure. The single mutations H106A, H106F, H106N, K107A and K107R were made with the following oligonucleotides: H106A, 5′-TG TAG CCT TTA GCA ACT TCA CTG GC-3′; H106F, 5′-GCT GTA GCC TTT AAA AAC TTC ACT GGC-3′; H106N, 5′-GCT GTA GCC TTT ATT AAC TTC ACT GGC-3′; K107A, 5′-GCT GTA GCC TGC ATG AAC TTC ACT G-3′; K107R, 5′-GCT GTA GCC CCT ATG AAC TTC ACT G-3′. The oligonucleotide-directed USE mutagenesis kit (Pharmacia Biotech) was used in accordance with the manufacturer's instructions. Clones with the required mutation were first identified by colony hybridization, with 5′-^{32}P-labelled mutameric oligonucleotides as probes, and confirmed by dideoxynucleotide sequencing [22].

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

To induce gene transcription, IPTG was added to a final concentration of 1 mM when *Escherichia coli* XL1-Blue strains (grown at 25 °C in Luria–Bertani medium [23] 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 sonication in the cold. The particulate material was removed by centrifugation and the resulting supernatant was subjected to isoelectric focusing on a column (LKB 8100 Ampholine Electrofocusing 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 potential of 700 V (4 °C), the content of the column was eluted and collected in 1 ml fractions. The peak of activity 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 with a DEAE column (internal diameter 1.5 cm, height 11 cm; Bio-Rad Laboratories, Milano, 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 activity were eluted in the range 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 discontinuous slab gel was performed by the method of Laemmli [24]. Protein concentration was determined by the method of Bradford [25] with γ-globulin as standard.

Enzyme assays

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was assayed at 30 °C by the method of Habig and Jakoby [26]. For the enzyme kinetic determinations either CDNB or GSH was held constant at 1 and 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 results fitted well to the equation for a rectangular hyperbola. Fitting was performed with the program ENZFITTER based on an iterative Gauss–Newton procedure [27]. The dependence of k_{cat}/K_m on pH was determined by using the following buffers (0.1 M) in the indicated pH ranges: Bis-Tris/HCl, from 5.0 to 7.0; Tris/HCl, from 7.2 to 9.0. The reaction was performed with 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 [28].

To study unfolding, all mutants (7 μM) were first incubated for 30 min at 25 °C in 0.2 M potassium phosphate buffer, pH 6.5, containing 1 mM EDTA with 0–4 M GdmCl. At the end of incubation, each sample was assayed for the remaining GST activity in a 1 ml final volume but including the same concentration of GdmCl as used in the incubation. In the refolding studies, denatured protein was rapidly diluted (1:50) in the same buffer and the activity was measured after 10 min. Thermal stability measurements of mutant enzymes (0.7 μM) were performed by incubating the samples at each temperature for 15 min. GST activity was determined at the end of the incubation.

Fluorescence measurements

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

Growth curve

A single colony of *E. coli* XL1Blue (pGPT1) was inoculated into Luria–Bertani medium [22] and grown overnight in a water-bath shaker. Luria–Bertani medium (150 ml) containing 3 ml of overnight culture and 1 mM IPTG was incubated at 25 °C in a water-bath shaker with monitoring of D_{600}. When D_{600} reached 0.250, the cells were exposed to 0.25MIC of rifamycin (MIC = 50 μg/ml). Minimum inhibitory concentration (MIC) was determined by a standard technique of broth microdilution [29].

RESULTS AND DISCUSSION

It has been suggested that the catalytic mechanism of the Alpha, Mu, Pi, Sigma and Theta class GSTs depends on their ability to lower the pK_a of the thiol group of bound GSH from pH 9.0 to 6.0, thus enhancing the rate of the enzyme's nucleophilic attack via the tripeptide towards a large number of elecrophilic substances [12–14]. This role has been assigned to a tyrosine residue in the Alpha, Mu, Pi and Sigma classes, and a serine or threonine residue in Theta class GSTs [3,14]. Previous studies from our laboratories have demonstrated that PmGST B1-1 does not use any tyrosine, serine, threonine or cysteine residue in the N-terminal domain of the protein to stabilize the thiolate form of GSH [19]. Moreover, the three-dimensional structure of PmGST B1-1 suggested that two conserved Beta class residues, His^{106} and Lys^{107}, located in the C-terminal domain of the molecule (Figure 1), might facilitate the deprotonation of the GSH thiol and stabilize the anion [18]. We performed site-directed mutagenesis to assess whether these residues are indeed involved in the catalytic mechanism.

Unlike the wild-type enzyme, the mutated enzymes (H106A, H106N, H106F, K107A and K107R) investigated here did not bind to the GSH-affinity matrix [15] and could not be purified by this standard method. The fact that the affinity for the matrix

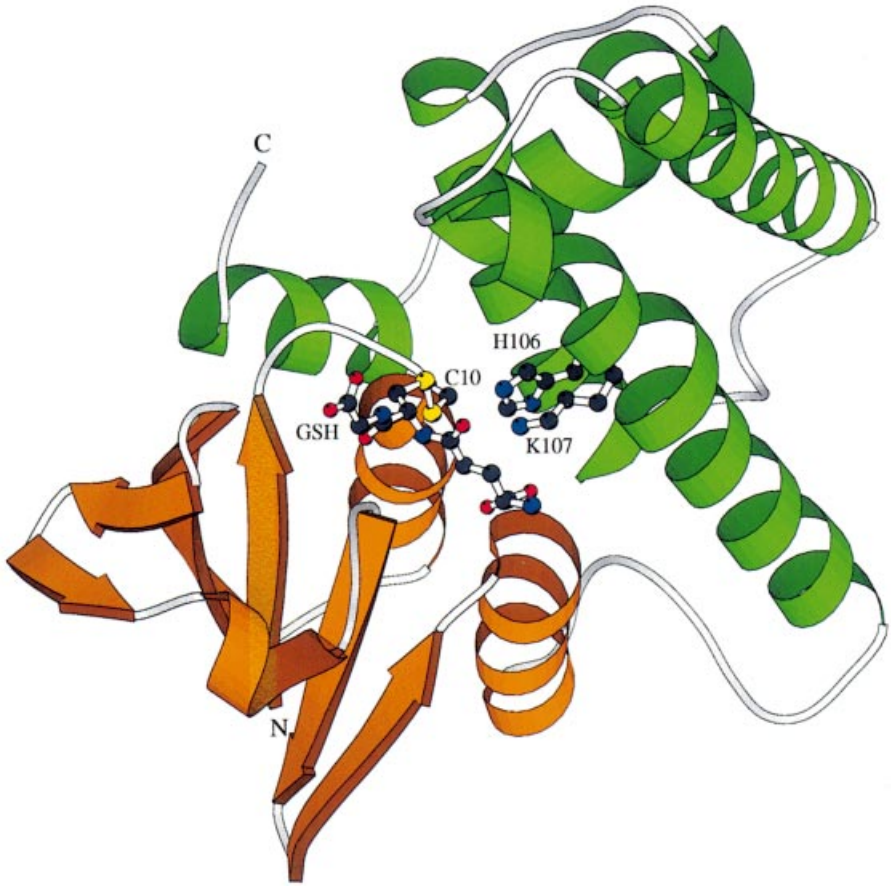

Figure 1 Schematic representation of a PmGST B1-1 monomer showing the locations of GSH bound to Cys[10], His[106] and Lys[107] in ball-and-stick representation

The Figure was generated with MOLSCRIPT [30].

Table 1 Specific activity and kinetic constants for PmGST B1-1 and the His[106] and Lys[107] mutants with CDNB as second substrate

Results are means ± S.D. for at least three independent determinations.

		GSH			CDNB			
Enzyme	Specific activity (μmol/min per mg)	K_m (μM)	k_{cat} (min^{-1})	$10^{-3}k_{cat}/K_m$ ($\mu M^{-1}\cdot min^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	$10^{-3}k_{cat}/K_m$ ($\mu M^{-1}\cdot min^{-1}$)	pK_a^{CDNB}
PmGST B1-1	1.1 ± 0.08	686 ± 91	58.1 ± 6.1	84.7	730 ± 82	69.3 ± 7	95	6.40 ± 0.27
H106A	0.048 ± 0.024	1894 ± 204	4.03 ± 0.45	2.13	2081 ± 243	9.6 ± 0.9	4.6	6.71 ± 0.30
H106F	0.06 ± 0.001	9811 ± 1079	22.5 ± 2.36	2.29	4335 ± 502	50 ± 4.9	11.5	6.54 ± 0.33
H106N	0.15 ± 0.003	12558 ± 1193	50.3 ± 5.33	3.98	1054 ± 121	34.7 ± 4.1	33	6.49 ± 0.35
K107A	0.28 ± 0.01	2070 ± 229	30.2 ± 3.14	14.6	3535 ± 428	95.4 ± 10.2	27	6.94 ± 0.24
K107R	0.44 ± 0.008	2773 ± 285	50.6 ± 5.1	18.2	3087 ± 355	145 ± 17.3	47	6.98 ± 0.28

was markedly altered by these mutations suggested that the binding for the co-substrate is largely impaired and thus His[106] and Lys[107] might contribute to the catalytic activity of the enzyme. The mutants and the wild-type enzyme were therefore purified by preparative isoelectrofocusing followed by anion-exchange chromatography, as described in the Materials and methods section. The electrophoretic mobility and apparent molecular mass, as well as the immunological properties of the mutated enzymes, were indistinguishable from those of the wild-type enzyme (results not shown).

The specific activities of the mutant enzymes compared with that of wild-type enzyme, determined with CDNB and GSH in the standard assay, are presented in Table 1. The replacement of either His[106] or Lys[107] produced a drastic decrease in activity. The replacement of His[106] with Ala, Phe and Asn resulted in an approx. 95 % decrease in activity. The substitution of Lys[107] with

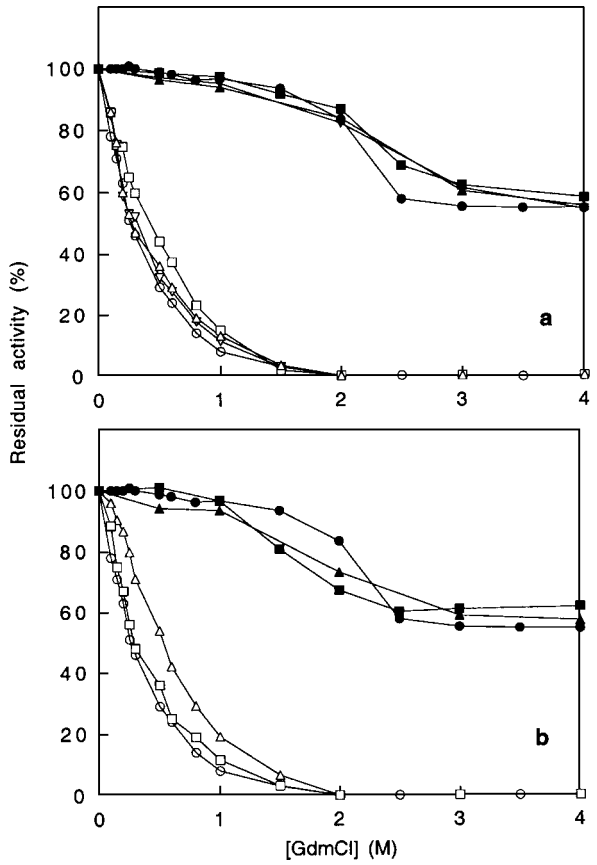

Figure 2 GdmCl-induced unfolding/refolding (open and filled symbols respectively) transition curves of wild-type and His[106] and Lys[107] mutants, monitored by changes in enzyme activity

(**a**) Wild-type (○,●), H106F (△,▲), H106N (□,■) and H106A (▽,▼); (**b**) wild-type (○,●), K107A (△,▲) and K107R (□,■).

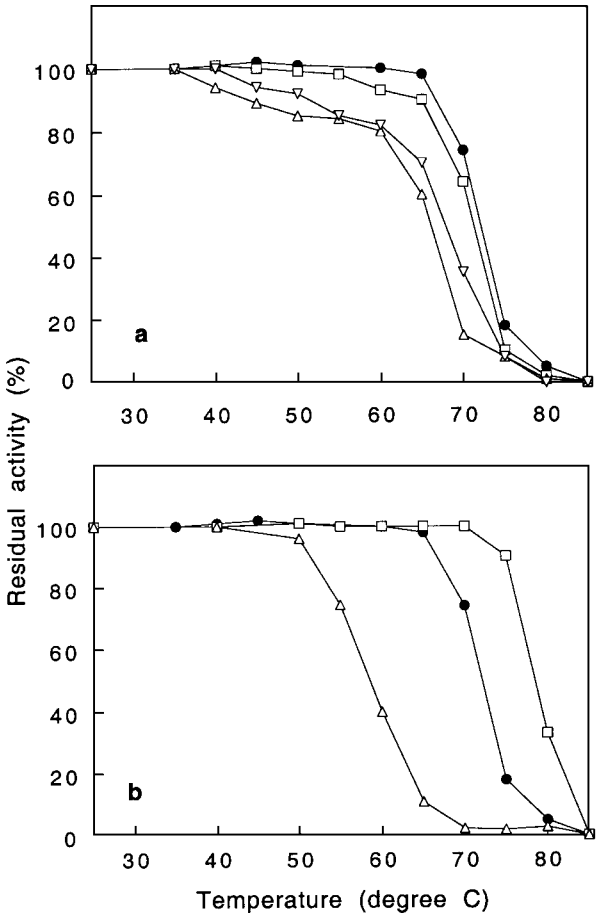

Figure 3 Effect of temperature on the stability of wild-type and His[106] and Lys[107] mutants

The enzymic activity at 25 °C was taken as 100%. (**a**) Wild-type (●), H106N (□), H106A (△) and H106F (▽); (**b**) wild-type (●), K107A (□) and K107R (△).

arginine or alanine resulted in a 70–75% decrease in activity. As a consequence the catalytic efficiencies towards GSH and CDNB were also substantially lower. Thus the degree of activity of the mutants was independent of the functional group that was introduced. With regard to the most conservative mutations and the crystal structure, the mutation of His[106] with the shorter Asn side chain would result in a loss of any possible interaction with GSH. The mutation of Lys[107] to Arg would cause the longer side chain to swing away from the active site so as to avoid steric and electrostatic clashes with GSH.

When the pH dependence of k_{cat}/K_m^{CDNB} was examined for the wild-type enzyme and the mutants, no shift in the apparent pK_a of bound GSH occurred: the estimated pK_a values were very similar to that of the wild type (Table 1). Thus all the kinetic experiments are consistent with the interpretation that His[106] and Lys[107] are important contributors to the GSH-binding site of PmGST B1-1 but they are not directly responsible for the activation and/or stabilization of the GSH thiol.

The fluorescence spectra of the wild-type and mutant enzymes, in the presence and the absence of 2–5 mM GSH, were also analysed. The overall spectra were very similar and there was no significant increase in fluorescence intensity, suggesting that the replacement of His[106] or Lys[107] with other residues did not result in a change in protein conformation (results not shown). The unfolding/refolding transition curves of the His[106] and Lys[107] mutants in comparison with that of the wild-type enzyme are shown in Figure 2. The curves of mutant enzymes are coincident with that of the wild-type enzyme, suggesting that His[106] and Lys[107] residues are not involved in the stability of the protein or in the unfolding/refolding kinetics of the enzyme. Figure 3 shows the effect of temperature on the stability of the mutant and wild-type enzymes after incubation in phosphate buffer, pH 7.0, for 15 min. It can be seen that all His[106] mutants and K107A were inactivated similarly to the wild-type enzyme, whereas K107R retained only approx. 50% of its activity at 60 °C. The reason for this last result is not known. Fluorescence analysis, unfolding/refolding curves and thermal stability studies indicate that His[106] and Lys[107] are not important for maintaining the proper conformation of the enzyme. These results are not surprising because both residues are located on the surface of the molecule.

In previous studies we have suggested a possible role for microbial GSTs in resistance to antibiotics [17]. PmGST B1-1 is able to sequester a variety of antibiotics, with a particular avidity for rifamycin [17]. His[106] and Lys[107] are both located in helix α4, which contributes to a wall of the hydrophobic binding site of PmGST B1-1. This binding site has previously been suggested as a possible location for the antibiotic-binding site [18]. We therefore examined the possibility that His[106] and Lys[107] might have a role in antibiotic binding. The growth rates of cells

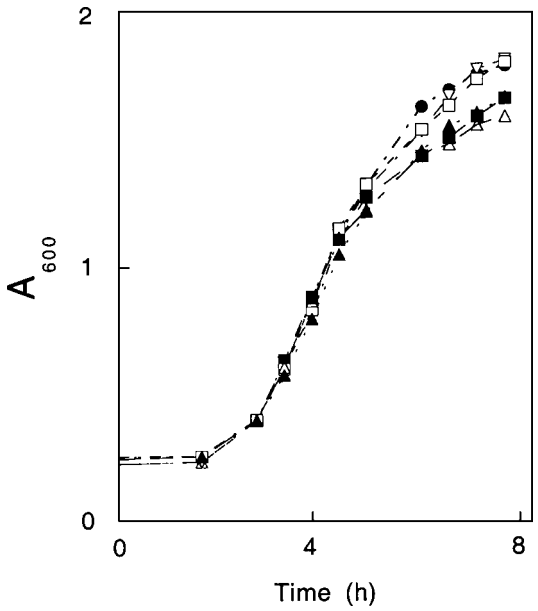

Figure 4 Effect of rifamycin (0.25 MIC) on the growth rate of cells overexpressing wild-type GST and His[106] and Lys[107] mutants

Wild-type (●), H106N (□), H106A (▽), H106F (■), K107A (▲) and K107R (△). The MIC was 50 μg/ml for rifamycin.

overexpressing mutants of these residues in the presence of rifamycin were measured and compared with that of cells overexpressing the wild-type enzyme. Similar growth curves were obtained for all bacterial strains tested, indicating that the antibiotic did not affect the growth rate of cells (Figure 4). The results presented suggest that these residues are not directly involved in antibiotic binding. Two possible binding sites were previously hypothesized on the basis of the crystal structure and biochemical data [18]. One putative site was located at the dimer interface, where the aliphatic tail of Lys[107] forms part of the wall of the site. Alternatively, antibiotics were suggested to bind to the hydrophobic binding site, where the hydrophobic electrophilic substrates normally bind. This is a much less attractive possibility because it fails to account for the non-competitive binding of antibiotics with regard to standard substrates. The results presented here rule out the possibility that antibiotic binding in either site impinges greatly on the adjacent GSH-binding site, where both His[106] and Lys[107] are located. Although Lys[107] forms part of the wall at the dimer interface binding site, it is only one of many residues at the putative binding site and might be only a small contributor to antibiotic binding.

In conclusion, our results demonstrate that His[106] and Lys[107] are involved in the interaction with GSH at the active site of PmGST B1-1 but they do not contribute significantly to the catalytic process or to the binding of antibiotics. The present results, as well as those obtained previously [19], indicate that none of the amino acid residues that have been suggested as possible candidates for GSH stabilization are essential for catalysis. The only likely candidate remaining for thiolate stabilization is the amide nitrogen of Cys[10] [18,21]. We are planning experiments to test this hypothesis.

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