Functional analysis of the evolutionarily conserved proline 53 residue in *Proteus mirabilis* glutathione transferase B1-1

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Abstract The role of the evolutionarily conserved residue Pro-53 in *Proteus mirabilis* glutathione transferase B1-1 has been examined by replacing it with a serine residue using site-directed mutagenesis. The effect of the replacement on the activity, thermal stability and antibiotic binding capacity of the enzyme was examined. The results presented support the view that Pro-53 participates in the maintenance of the proper conformation of the enzyme fold rather than playing a direct role in the catalytic reaction. Furthermore, this residue appears to be an important determinant of the antibiotic binding to the enzyme. Experiments with wild type and mutated enzymes provide evidence that glutathione transferases may play an important role in antibiotic resistance exhibited by bacteria.
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Key words: Proteus mirabilis; Glutathione transferase; Site-directed mutagenesis

1. Introduction

Cytosolic glutathione transferases (GSTs; EC 2.5.1.18) are a family of dimeric multi-functional proteins which catalyse the conjugation of glutathione (GSH) to a large variety of endogenous and exogenous electrophilic molecules [1–3]. GSTs are widely distributed in nature: they are found in all eukaryotes and many prokaryotes [1–9]. Cytosolic GSTs exist as dimers with a subunit molecular mass of 23–25 kDa and have been grouped into at least seven distinct classes, namely alpha, mu, pi, theta, sigma, kappa and zeta on the basis of immunological properties and primary structure [1–3,10,11]. GST isoenzymes belonging to the same class show greater than 60% sequence identity in their primary structure, whereas GSTs belonging to different classes have less than 30% sequence identity [2]. However, despite their low inter-class sequence identity, crystallographic studies have indicated that the overall polypeptide fold of the different GSTs are very similar [3,12–15].

In our laboratories, a GST isoenzyme, originally named Pm-GST-6.0, was purified and characterised from the Gram-negative bacterium *Proteus mirabilis* [4]. Its sequence was first resolved at the protein level [16], but the corresponding gene sequence is now available [17]. We found that *P. mirabilis* GST was predominantly located in the periplasmic space, supporting its suggested role in cellular detoxification [18]. Recently, its three-dimensional crystal structure has also been resolved [19]. This enzyme exhibits several kinetic, structural and immunological characteristics that distinguish it from GSTs of other classes. For example, it does not appear to utilise either a Tyr, Ser or Cys residue to activate glutathione and it was subsequently found to have a molecule of GSH covalently bound to a cysteine residue at position 10 of the enzyme [19,20]. Because of its peculiar structural and immunological properties, *P. mirabilis* GST has been identified as the prototype of a new GST class i.e. 'beta class' and following the guidelines adopted for mammalian GSTs, it is now referred to as PmGST B1-1 [19].

Comparing the amino acid sequence of PmGST B1-1 with the sequences of the GSTs from other classes present in the Swiss-Prot protein sequence data bank, it appears that only two residues, namely Pro-53 and Asp-155, are strictly conserved. These residues thus become obvious candidates for playing crucial structural and/or catalytic roles in this class of enzymes. A recent study has shown that the aspartic acid residue belongs to a local conserved motif 'Ser/Thr-Xaa-Xaa-Asp' which may have a role in the folding of the protein [21]. Pro-53 adopts a *cis* configuration in all known GST structures [12,15] and is also present in homologous structures from the thioredoxin superfamily from which GSTs are thought to have evolved [19]. Pro-53 is located in a β-turn that lines the base of the GSH binding site (G-site) and is thought important for the proper folding and maintenance of the G-site. Most importantly, the *cis*-proline ensures that the main-chain atoms of the proceeding residue in the sequence are able to form an anti-parallel β-sheet interaction with the main-chain atoms of the cysteinyl moiety of the substrate [12,15]. Given the importance of this residue it is surprising that there are no experimental data confirming the role of this residue in any GST to date. In order to evaluate the role of Pro-53 in PmGST B1-1, this residue was replaced with Ser by site-directed mutagenesis and the effect of the replacement on the activity, thermal stability and antibiotic binding capacity of the enzyme was examined.

2. Materials and methods

2.1. Chemicals
Isopropyl-β-D-thiogalactopyranoside (IPTG), guanidinium chloride (GdmCl), as well as antibiotics used in the present work were purchased from Sigma-Aldrich (Milan, Italy). All other reagents used were of the highest grade commercially available.

2.2. Multiple sequence alignment
GST sequences were taken from the Swiss-Prot protein sequence data bank. The multiple sequence alignment was obtained by ClustalW [22] at Network Protein Sequence Analysis, IBCP, France.

Table 1
Specific activity and kinetic constants for PmGST B1-1 and P53S mutant with CDNB as second substrate

Enzyme	Specific activity (μmol min^{-1} mg^{-1})	GSH			CDNB		
		K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})
PmGST B1-1	1.1	686	58.1	84	730	69.3	95
P53S	0.062	1337	2.5	1.87	1346	5.64	4.19

2.3. Oligonucleotide-directed mutagenesis

The DNA encoding PmGST B1-1 in pBtac1 (pGPT1) [17] was used as a template in the mutagenesis procedure. The single mutation P53S was made with the following oligonucleotide: 5′-C TAA TTG AAG AAC CGA AAC TTG CCC-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 hybridisation, using 5′-^{32}P-labelled mutameric oligonucleotides as probes, and confirmed by dideoxynucleotide sequencing [23].

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

To induce the mutant and wild type gene transcription, IPTG was added to a final concentration of 1 mM when Escherichia coli XL1-Blue strains, grown at 37°C in a Luria-Bertani (LB) medium [24] supplemented with tetracycline and ampicillin, reached an approximate A_{550} of 0.4, and the incubation was prolonged for a further 16 h.

The purification of mutant and wild type enzymes was performed as reported by Di Ilio et al. [4]. Briefly, 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 and the resulting supernatants were applied to a GSH-Sepharose affinity column [25]. The column was exhaustively washed with buffer A, supplemented with 50 mM KCl. The enzyme was eluted with Tris-HCl buffer pH 9.6 containing 10 mM GSH. The fractions containing GST activity were pooled, concentrated by ultrafiltration, dialysed against buffer A and subjected to further analyses. SDS-PAGE in discontinuous slab gel was performed by the method of Laemmli [26]. Protein concentration was determined by the method of Bradford [27] with γ-globulin as standard.

2.5. Enzyme assays

GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB) was assayed at 30°C according to the method of Habig and Jakoby [28]. For the enzyme kinetic determinations either CDNB or GSH was held constant at 1 mM and 5 mM respectively, whilst the concentration of the other substrate was varied, (0.1–5 mM for GSH, and 0.1–1.6 mM for CDNB). The selenium-independent glutathione peroxidase activity of GST was measured with cumene hydroperoxide (Cu-OOH) as previously described [29]. For the enzyme kinetic determinations either Cu-OOH or GSH was held constant at 0.8 mM and 1.3 mM respectively, whilst the concentration of the other substrate was varied (0.1–3 mM for GSH, and 0.05–1.5 mM for Cu-OOH). For both types of assay, the initial velocity was measured at least in triplicate. Fitting was carried out using the computer program ENZFITTER based on an iterative Gauss-Newton procedure [30]. The data fitted well to the equation for a rectangular hyperbola.

To study unfolding, PmGST B1-1 and the P53S mutant (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 remaining GST activity in 1 ml of 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 measured after 10 min. Thermal stability measurements of native and mutant enzymes (0.7 μM) were carried out by incubating the samples at each temperature for 15 min. GST activity was determined at the end of incubation.

2.6. 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, and the signal was acquired for 1 s at each wavelength. Spectra were corrected by subtraction of the corresponding spectra for blank samples.

2.7. Growth curve

A single colony of E. coli XL1Blue (pGPT1) was inoculated with LB medium [24] and growth overnight in a water bath shaker. 150 ml of LB medium containing 3 ml of overnight culture and 1 mM IPTG was incubated at 37°C in a water bath shaker, monitoring the absorbance at 600 nm. When a 0.250 value in absorbance was reached, the cells were exposed to 1/8 and 1/2×MIC of rifamycin (MIC=50 μg/ml). A sample without IPTG was used as control. MIC was determined by a standard broth microdilution technique [31].

3. Results and discussion

Purification of the recombinant enzyme was based on affinity chromatography on GSH-Sepharose and was carried out as previously described [4]. A final yield of about 9 mg was obtained from 1 l of culture. Judging from the results of the purification it appears that the binding of enzyme to the GSH-affinity matrix was not altered noticeably by the mutation, suggesting that the binding of the ligand was largely unaffected. The electrophoretic mobility and the immunological reactivity of the mutated enzyme were indistinguishable from those of the wild type enzyme (data not shown). The purified recombinant enzyme was not very stable in 10 mM potassium phosphate buffer pH 7.0 containing 1 mM EDTA at 25°C. The addition of glycerol to a final concentration of 10% helped to stabilise the enzyme at −20°C. In this condition the enzyme retains 100% activity for at least 3 months.

The specific activities of the mutant enzyme compared to the wild type enzyme, measured with both CDNB and Cu-OOH as substrates, are presented in Tables 1 and 2. The results indicate that the specific activities of the mutant enzyme toward CDNB and Cu-OOH were about 6% and 10% of that of the wild type, respectively. Tables 1 and 2 also show

Table 2
Specific activity and kinetic constants for PmGST B1-1 and P53S mutant with cumene hydroperoxide (Cu-OOH) as second substrate

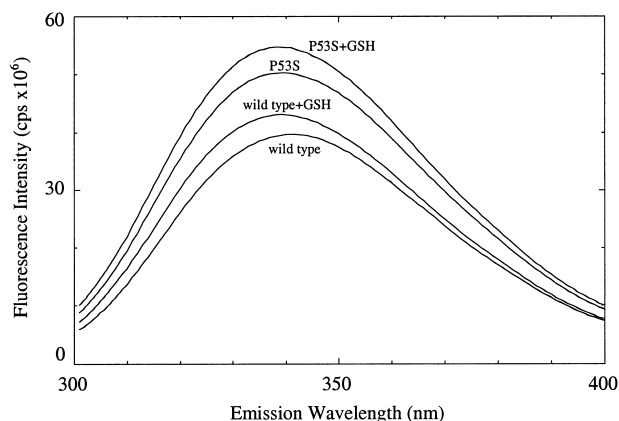| Enzyme	Specific activity (μmol min^{-1} mg^{-1})	GSH			Cu-OOH		
		K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})	K_m (μM)	K_{cat} (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})
PmGST B1-1	0.23	691	15.2	22	3359	34.8	10.4
P53S	0.024	674	2.3	3.4	9284	12.7	1.4

Fig. 1. Fluorescent emission spectra of wild type and the Pro-53-Ser mutant, in the present and absence of 2 mM GSH.

the kinetic parameters obtained by using CDNB and Cu-OOH as co-substrate with GSH. The catalytic efficiency of the mutant enzyme toward GST substrates was found to be significantly lower than that of the wild type. This reduction is caused mainly by a much lower K_{cat} and a slightly higher K_m value than that of the wild type enzyme. Thus, the affinity for GSH of the enzyme was not significantly altered by the replacement of Pro-53 with Ser. This is not surprising since GSH is bound to the enzyme through multiple interactions including three salt bridges, eight polar interactions and nine van der Waals interactions [19]. The loss of up to two polar interactions from Val-52, the residue proceeding the *cis*-proline, would be predicted to have a marginal effect on the affinity of the enzyme for GSH.

The fluorescence spectra of the wild type and mutant enzyme, in the presence and absence of 2 mM GSH, are shown in Fig. 1. The overall spectra are very similar, i.e. the same excitation wavelength at 280 nm and emission maxima at 339 nm. However, there was a significant increase in fluorescence intensity with the mutant enzyme indicating that the replacement of Pro-53 with Ser results in a change in protein conformation. Fig. 2 shows the unfolding/refolding transition curves of both wild type and mutant enzymes induced by

Fig. 3. Effect of temperature on the stability of wild type (○,●) and Pro-53-Ser mutant (△,▲) in the presence (closed symbols) and absence (open symbols) of 2 mM GSH. The enzymatic activity at 25°C was taken as 100%.

GdmCl. It can be seen that the curves of wild type and mutant are coincident with each other (within experimental error) suggesting that the substitution of Pro-53 with Ser changes neither the overall stability of the protein nor the unfolding/refolding kinetics of the protein. Fig. 3 shows the enzyme activity remaining for both wild type and mutant after their incubation in 10 mM potassium phosphate buffer pH 7.0 at various temperatures for 15 min, in the presence and absence
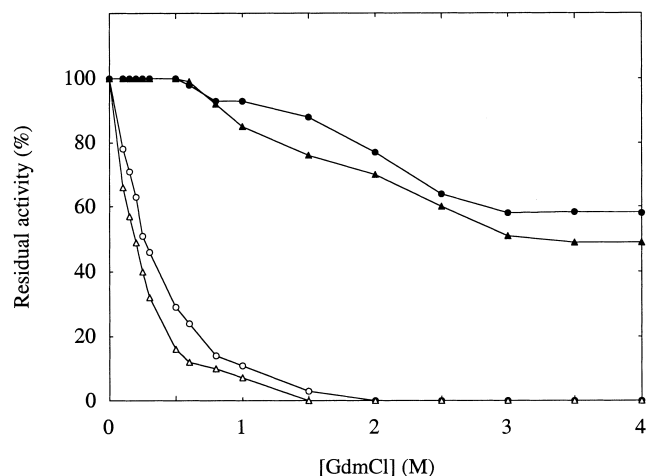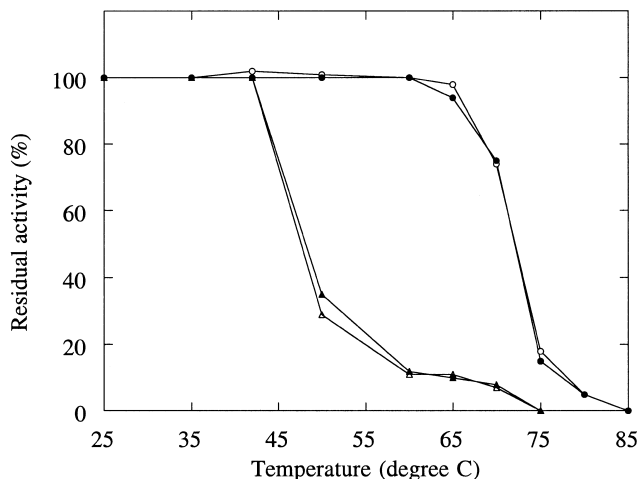

Fig. 2. Unfolding/refolding transition curves of wild type (○/●) and Pro-53-Ser (△/▲) mutant induced by GdmCl, monitored by changes in enzyme activity.
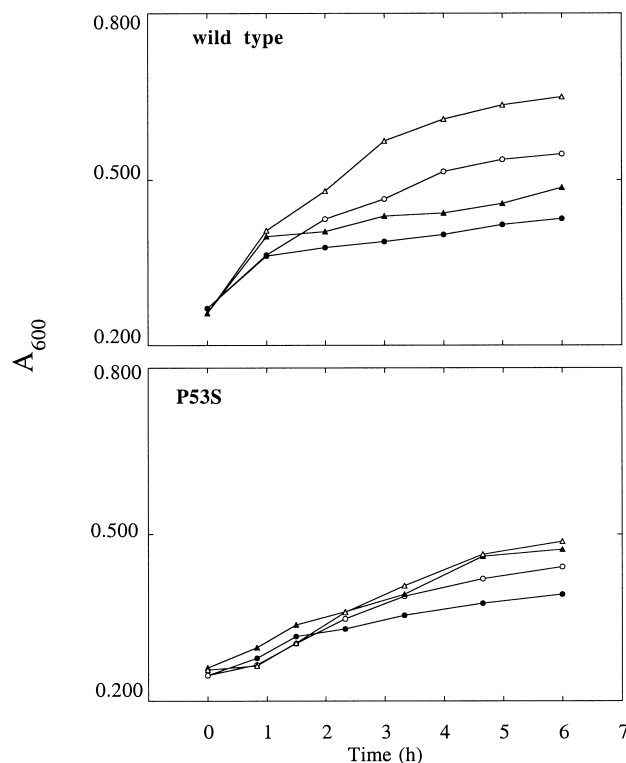
Fig. 4. Effect of rifamycin on the growth rate of cells over-expressing wild type and the Pro-53-Ser mutant. ○, 1/2×MIC value plus IPTG; ●, 1/2×MIC value without IPTG; △, 1/8×MIC value plus IPTG; ▲, 1/8×MIC value without IPTG. MIC value is 50 μg/ml for rifamycin.

of 2 mM GSH. The data presented demonstrate that the mutant enzyme is more easily inactivated by heat treatment than wild type. In fact, the wild-type enzyme retains more than 80% of its activity after 15 min incubation at 70°C, whereas the mutant enzyme after an identical treatment retains only 5% of its activity. No protective effect on heat inactivation was obtained in the presence of GSH. These results strongly suggest that Pro-53 might be critical in maintaining the proper conformation of the enzyme.

We have previously demonstrated that PmGST B1-1 is able to bind avidly, in vivo, to different classes of antibiotics, including rifamycin, suggesting a possible role for the bacterial enzyme in antibiotic resistance [17]. To date, no crystals of PmGST B1-1 complexed with rifamycin have been successfully grown and as a consequence the identity of the amino acid residues contacting the drugs are unknown. On the basis of the wild type structure it has suggested that a hydrophobic cavity located at the dimer interface may be involved in binding drugs [19].

To examine the possible involvement of Pro-53 in antibiotic binding, the growth rate of Pro-53-Ser mutant-overexpressed cells in the presence of different concentrations of rifamycin was measured and compared with cells overexpressed for wild type. For comparison, the same experiment with cells not overexpressing for mutant and wild type enzymes has been carried out.

As can be seen in Fig. 4, rifamycin differentially affected the growth rate of cells overexpressed for Pro-53-Ser mutant and wild type. In fact, in the presence of 6.25 and 25 μg/ml rifamycin, the cells overexpressing wild type protein continued to grow, whereas the growth of cells overexpressing Pro-53-Ser mutant was substantially inhibited.

These results suggest that Pro-53 may play a role in the direct binding of antibiotics. Pro-53 is located at the base of the G-site, positioned adjacent to both the H-site (the binding site for hydrophobic electrophiles) and the dimer interface. Previous studies of PmGST B1-1 by fluorescence spectroscopy have suggested that the antibiotic binding site is close to a tryptophan residue [5]. The two most likely sites, based on this and the crystallography data [19], are either in the H-site or in the dimer interface. However, antibiotics bind to the enzyme in a non-competitive manner with respect to GSH or CDNB, ruling out the G- and H-sites as possible locations of the antibiotic binding site. Thus we hypothesise that the replacement of Pro-53 with Ser causes conformational changes at the dimer interface which in turn affect antibiotic binding. Such changes are in accord with the fluorescence results reported here which suggest localised conformational changes (Fig. 1).

In conclusion, the results of our experiments suggest that Pro-53, even though located in a β-turn that lines the G-site, is not a major contributor to GSH binding nor is it directly involved in catalytic reaction. The mutation of Pro-53 does not cause any large scale conformational changes to the enzyme as judged by the electrophoresis, antibody, fluorescence and unfolding data. However, small, localised changes are evidenced by the fluorescence and antibiotic binding data. The heat lability data indicate that Pro-53 is important for stabilising the normal conformation of the enzyme's active site since the active site structure of the mutant is more readily damaged at high temperatures. Finally, Pro-53 appears to be an important determinant of antibiotic binding.

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