Contribution of the two conserved tryptophan residues to the catalytic and structural properties of *Proteus mirabilis* glutathione S-transferase B1-1

Nerino ALLOCATI^{*1}, Michele MASULLI^{*}, Marilena PIETRACUPA^{*}, Bartolo FAVALORO^{*†}, Luca FEDERICI[‡] and Carmine DI ILIO^{*†}

^{*}Dipartimento di Scienze Biomediche, Università 'G. d'Annunzio', Via dei Vestini 31, I-66013 Chieti, Italy, [†]Centro Studi per l'Invecchiamento, Università 'G. d'Annunzio', Via dei Vestini 31, I-66013 Chieti, Italy, and [‡]Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, U.K.

PmGSTB1-1 (*Proteus mirabilis* glutathione S-transferase B1-1) has two tryptophan residues at positions 97 and 164 in each monomer. Structural data for this bacterial enzyme indicated that Trp⁹⁷ is positioned in the helix α 4, whereas Trp¹⁶⁴ is located at the bottom of the helix α 6 in the xenobiotic-binding site. To elucidate the role of the two tryptophan residues they were replaced by site-directed mutagenesis. Trp⁹⁷ and Trp¹⁶⁴ were mutated to either phenylalanine or alanine. A double mutant was also constructed. The effects of the replacement on the activity, structural properties and antibiotic-binding capacity of the enzymes were examined. On the basis of the results obtained, Trp⁹⁷ does not seem to be involved in the enzyme active site and structural stabilization. In contrast, different results were achieved for Trp¹⁶⁴ mutants. Conservative substitution of the Trp¹⁶⁴ with phenylalanine enhanced enzyme activity 10-fold, whereas replacement with alanine enhanced enzyme activity 17-fold. Moreover, the catalytic efficiency for both GSH and 1-chloro-2,4-dinitrobenzene

substrates improved. In particular, the catalytic efficiency for 1-chloro-2,4-dinitrobenzene improved for both W164F (Trp¹⁶⁴ → Phe) and W164A by factors of 7- and 22-fold respectively. These results are supported by molecular graphic analysis. In fact, W164A presented a more extensive substrate-binding pocket that could allow the substrates to be better accommodated. Furthermore, both Trp¹⁶⁴ mutants were significantly more thermolabile than wild-type, suggesting that the substitution of this residue affects the overall stability of the enzyme. Taken together, these results indicate that Trp¹⁶⁴ is an important residue of PmGSTB1-1 in the catalytic process as well as for protein stability.

Key words: bacterial glutathione S-transferase (GST), CD spectroscopy, *Proteus mirabilis* glutathione S-transferase B1-1 (PmGSTB1-1), 2-(*p*-toluidino)naphthalene-6-sulphonic acid (TNS), tryptophan mutant.

INTRODUCTION

GSTs (glutathione S-transferases; EC 2.5.1.18) are dimeric multifunctional enzymes that play a key role in cellular detoxification [1–3]. GSTs metabolize a wide variety of electrophiles through GSH conjugation [1–4]. GSTs are also involved in other cell functions, such as peroxidase and isomerase activities, and are capable of binding a large number of endogenous and exogenous compounds non-catalytically [1,2]. The cytosolic GSTs have been grouped into at least ten gene-independent classes on the basis of their physical, chemical, immunological and structural properties [5]. Despite their low-sequence similarity, they share a similar three-dimensional organization with each subunit divided in two domains. The active site of GSTs consists of two binding sites: the G-site where GSH binds and the H-site where hydrophobic electrophiles bind [3,5]. The Beta class is one of the cytosolic GSTs, and the bacterial GST from *Proteus mirabilis* (PmGSTB1-1, *Proteus mirabilis* GSTB1-1) is a member of this class [6–13]. PmGSTB1-1 is characterized by the presence of a GSH molecule covalently bound to Cys¹⁰ per subunit although the enzyme has GSH-conjugating activity [10,14]. Furthermore, PmGSTB1-1 is also capable of redox activity with comparable efficiency [15]. Recent results suggested that this bacterial enzyme has an active role in the protection against oxidative stress generated by H₂O₂ [16]. A possible role for this enzyme in the detoxification of the antimicrobial agents has been suggested previously [8,17]. In fact, PmGSTB1-1 is capable of binding several antibiotics *in vitro* and *in vivo* [8,16,17].

PmGSTB1-1 contains two tryptophan residues at positions 97 and 164 [7,10]. Alignment of the beta class GST sequences present in the databases (www.ncbi.nlm.nih.gov/Entrez/index) has revealed that Trp¹⁶⁴ is conserved, whereas other hydrophobic residues may replace Trp⁹⁷. The crystallographic data for PmGSTB1-1 indicated that Trp⁹⁷ is positioned in the helix α 4, whereas Trp¹⁶⁴ is located at the bottom of the helix α 6 in the hydrophobic-binding site [10] (see Figure 1A). Furthermore, on the basis of intrinsic fluorescence measurements, we suggested the location of at least one tryptophan residue close to the antibiotic-binding site [10,17]. To understand better the role of these conserved tryptophan residues in the catalytic mechanism and/or binding properties of PmGSTB1-1, they have been replaced by site-directed mutagenesis, and the effects of the replacements have been examined.

MATERIALS AND METHODS

Site-directed mutagenesis, expression and purification of wild-type and mutant PmGSTB1-1 enzymes

The DNA encoding PmGSTB1-1 in pBtac1 (pGPT1) [8] was used as a template in the mutagenesis procedure. The single mutations W97F (Trp⁹⁷ → Phe), W97A, W164F and W164A were made with the following oligonucleotides: For W97F: 5'-CATCA-AATTGAGTTTCTAACTTTCTTGC-3'; Rev W97F: 5'-GC-AAGAAAGTTTGTAACTCAATTTGATG-3'; For W97A, 5'-CATCAAATTGAGGCGCTAACTTTCTTGC-3'; Rev W97A,

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; GdmCl, guanidinium chloride; GST, glutathione S-transferase; PmGSTB1-1, *Proteus mirabilis* glutathione S-transferase B1-1; TNS, 2-(*p*-toluidino)naphthalene-6-sulphonic acid; for brevity, the single-letter system for amino acids has been used, e.g. W164F means Trp¹⁶⁴ → Phe.

¹ To whom correspondence should be addressed (email n.allocati@dsb.unich.it).

Figure 1 Structures and electrostatic potential surfaces of wild-type and W164A

(A) Structure of a PmGSTB1-1 monomer showing the locations of Trp⁹⁷, Trp¹⁶⁴ and GSH bound to Cys¹⁰. The secondary-structure representation was produced using PyMOL [27]. (B) Trace of the superimposed G-site for wild-type crystal structure (green trace) and W164A homology model (blue trace); GSH (red trace). Electrostatic potential surface representation of wild-type crystal structure (C) and the W164A homology model (D). Negative and positive charges are represented in red and blue respectively. The Figures were generated using the program GRASP [30]. Co-ordinates of PmGSTB1-1 are deposited in the Protein Data Bank with accession code 1PMT.

5'-GCAAGAAAGTTTAGCGCCTCAATTTGATG-3'; For W164F, 5'-GTTTACGTAAAGTCAATTTGCACCTCATGTGGC-3'; Rev-W164F, 5'-GCCACATGAGGTGCAAAATTGACTTAACGTAAAC-3'; For W164A, 5'-GTTTACGTAAAGTCAAGCGGCACCTCATGTGGC-3'; Rev W164A, 5'-GCCACATGAGGTGCCGCTTGACTTAACGTAAAC-3'. The Quik Change Site-Directed Mutagenesis kit (Stratagene) was used according to the manufacturer's instructions. Clones with the required mutation were confirmed by DNA sequencing [18].

To induce gene transcription, isopropyl β -D-thiogalactoside (Sigma-Aldrich, Milano, Italy) was added to a final concentration of 1 mM when *Escherichia coli* XL1-Blue strains, grown at 25 °C in Luria-Bertani medium [19] supplemented with tetracycline (Sigma-Aldrich) and ampicillin (Sigma-Aldrich), reached a D_{550} of approx. 0.4 and the incubation was prolonged for a further 16 h.

The purification of enzymes was performed as described previously [12]. 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, the resulting supernatant was subjected to isoelectric focusing on column and the concentrated enzyme

obtained was further purified by anion-exchange chromatography using a DEAE resin (Bio-Rad Laboratories, Milano, Italy). 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 [20]. Protein concentration was determined by the method of Bradford [21] with γ -globulin as standard.

Enzyme assays

GST activity with CDNB (1-chloro-2,4-dinitrobenzene) was assayed at 30 °C by the methods of Habig and Jakoby [22]. For the enzyme kinetic determinations, either CDNB or GSH was held constant at 1 and 5 mM respectively, whereas the concentration of the other substrate was varied (0.1–5 mM for GSH and 0.1–1.6 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 Michaelis constant (K_m) and V_{max} values.

The selenium-independent glutathione peroxidase activity of GST was measured with cumene hydroperoxide (Cu-OOH) as described previously [23].

To study unfolding, all mutants (7 μ M) were first incubated for 30 min at 25 °C in 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM EDTA with 0–4 M GdmCl (guanidinium chloride). At the end of the incubation period, 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 (1:40) in the same buffer and the activity was measured after 10 min. Thermal stability measurements of mutant enzymes (0.7 μ M) were performed by incubating the samples at each temperature for 15 min. GST activity was determined at the end of the incubation.

The effects of antibiotics as inhibitors were measured by using 1 mM CDNB and 1 mM GSH in 0.1 M potassium phosphate buffer (pH 6.5) plus 1 mM EDTA. Stock solutions of antibiotics were freshly prepared immediately before use by the method of Anhalt and Washington [24]. The enzyme was preincubated with antibiotics for at least 1 min before the addition of substrates. The inhibition of PmGSTB1-1 was determined by using at least ten different concentrations of each antibiotic. The IC_{50} value represents the concentration of drug causing 50 % inhibition of activity.

Spectroscopic studies

Fluorescence measurements were performed on a Spex spectrofluorimeter (model Fluoromax) equipped with a thermostatically controlled sample holder at 25 °C. The enzyme samples were in buffer A. The intrinsic fluorescence spectra of the proteins (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. The surface hydrophobicity of the proteins was studied by using the specific hydrophobic probe, TNS [2-(*p*-toluidino)naphthalene-6-sulphonic acid; Sigma]. A 2 mM aqueous solution of TNS (15 μ l) was added to 3 μ M protein in 2 ml of buffer A. Fluorescence emission spectra were recorded between 350 and 600 nm with the excitation wavelength set at 323 nm. Binding studies were also performed by using the TNS probe, according to the procedures described previously [25]. Wild-type and mutant enzymes (3 μ M) were titrated with increasing concentrations of TNS up to 100 μ M. Fluorescence emission spectra were recorded between 350 and 600 nm with the excitation wavelength set at 323 nm. The spectra were corrected for dilution.

CD measurements in the far-UV region from 190 to 250 nm were performed with a Jasco-810 spectropolarimeter (Jasco Europe, Cremella, Lecco, Italy). Samples containing wild-type and mutant enzymes at a concentration of 17 μ M were scanned at least five times at a rate of 20 nm/min and then averaged. The temperature of the sample compartment was maintained at 20 °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. Secondary-structure estimation from CD spectra of wild-type and mutant enzymes was obtained by the method of Chang et al. [26].

Molecular modelling

The secondary-structure representation was produced using PyMOL [27]. Models of mutant PmGSTB1-1 were prepared and energy minimized using program MODELLER (version 6.2) [28]. For each mutant, 20 models were prepared and the best models were chosen on the basis of minimum energy and minimal geometrical clashes. The models were further validated for correct geometry using the server VERIFY3D (http://hannon.mbi.ucla.edu/DOE/Services/Verify_3D/) and the program PROCHECK

[29]. Structural superimpositions were performed and displayed using program QUANTA (Molecular Structure, The Woodlands, TX, U.S.A.). Electrostatic potential surface calculations were performed and displayed using program GRASP [30].

RESULTS AND DISCUSSION

In the present study, we report the different contributions of the tryptophan residues of PmGSTB1-1 and the effect of the mutations on the properties of the protein. PmGSTB1-1 contains two conserved tryptophan residues, Trp⁹⁷ and Trp¹⁶⁴. One is positioned in the helix α 4 of the enzyme, and the other is buried at the bottom of the helix α 6 in the H-site. Whereas the residues forming the G-site are well conserved in all GSTs, the residues forming the H-site are not conserved in all structures because of the broad substrate specificities of the GSTs. The conserved Trp¹⁶⁴ residue is one of the main residues making up this hydrophobic site [10]. Furthermore, binding of antibiotics to PmGSTB1-1 has been monitored by tryptophan fluorescence [17], suggesting the location of at least one tryptophan residue close to the antibiotic-binding site [10]. Thus to evaluate the role of the conserved Trp⁹⁷ and Trp¹⁶⁴ residues in the catalytic mechanism and/or binding properties of PmGSTB1-1, site-directed mutagenesis was used.

Design, expression and purification of mutant enzymes

Conservative substitutions of the tryptophan residues were produced with phenylalanine. They were also replaced with the aliphatic residue alanine to eliminate all the side interactions of the residue. To test the overexpression of all tryptophan mutants, total cellular extracts of induced *E. coli* XL1-Blue cells were examined by immunoblot analysis using an antiserum against PmGSTB1-1 [6]. All single mutants co-migrated with the wild-type protein with comparable levels of expression (results not shown). However, the double mutant W97A/W164A did not express a detectable amount of protein probably due to the crucial role played by tryptophan residues in protein folding/stability.

Unlike the W97A, W97F and W164F mutants, W164A did not bind to GSH-affinity Sepharose, and could not be purified by this standard method suggesting that the binding of GSH is impaired. Trp¹⁶⁴ is located at the interface between the G-site and the H-site (Figure 1A). Even if this residue does not directly contact the GSH, its substitution with alanine might account for local structural changes that would not allow the enzyme to bind the GSH rigidly attached to the matrix. Indeed the homology model of the W164A superimposed with the native structure (Figure 1B) shows a number of side-chain movements in both the G- and H-sites. Therefore the Trp¹⁶⁴ residue might also contribute to the catalytic activity of PmGSTB1-1. Consequently, the mutant enzymes, as well as wild-type, were purified by preparative iso-electrofocusing followed by anion-exchange chromatography as described previously [12]. All enzymes were purified to homogeneity and a single band for each form was obtained indicating the absence of contaminating proteins (results not shown).

Enzymic studies

The specific activities and kinetic constants of the mutant enzymes compared with the wild-type enzyme, determined with GSH and CDNB as substrates, are summarized in Table 1. The specific activity of W97A and W97F was quite similar to that of the wild-type. The substitution of Trp⁹⁷ with Ala does not affect the kinetic parameters for GSH when compared with the wild-type enzyme. Furthermore, W97F exhibited a decreased K_m^{GSH} value of 3-fold coupled with an increased catalytic efficiency of

Table 1 Specific activity and kinetic constants for wild-type and tryptophan mutant enzymes with CDNB as second substrate

The results are means for at least three independent determinations; S.D. values never exceed 10 % of the mean.

Enzyme	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)	GSH			CDNB		
		K_m (μM)	k_{cat} (min^{-1})	$10^{-3} \times k_{\text{cat}}/K_m$ ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)	K_m (μM)	k_{cat} (min^{-1})	$10^{-3} \times k_{\text{cat}}/K_m$ ($\mu\text{M}^{-1} \cdot \text{min}^{-1}$)
Wild-type	1.1	683	56	81.9	730	71	97.2
W97A	1.2	530	52	98.1	5658	297	52.5
W97F	0.7	223	40	179.3	5393	190	35.2
W164A	19.1	1888	955	506.1	546	1177	2155.6
W164F	11.5	671	333	496.2	2886	2114	732.5

approx. 2-fold relative to the wild-type enzyme. Instead, Trp⁹⁷ mutants showed limited but significant kinetic differences for CDNB when compared with wild-type. In particular, K_m values exhibited an increase of approx. 7-fold for both Trp⁹⁷ mutants. Thus W97A and W97F displayed a catalytic efficiency 2- and 3-fold lower respectively. Although Trp⁹⁷ could not play a direct role in the catalysis, such effects might be due to local structural perturbations of the residues involved in the active site as a consequence of the mutation. The replacement of Trp¹⁶⁴ with Phe or Ala produced a significant increase in the specific activity of the enzyme when compared with the wild-type. In fact, W164F and W164A mutants had specific activities of approx. 11- and 17-fold higher respectively. Furthermore, in comparison with wild-type enzyme, higher k_{cat}/K_m values for both substrates were observed. W164F and W164A mutants displayed an enhancement of approx. 6-fold in catalytic efficiency with GSH when compared with wild-type enzyme. The effect of mutations on the catalytic efficiency for CDNB was more significant, with an increase of approx. 7- and 22-fold for W164F and W164A respectively. This improvement of catalytic efficiency for Trp¹⁶⁴ mutants may be explained by an increased accessibility of the substrate-binding pocket.

PmGSTB1-1 is also capable of reducing cumene hydroperoxide [6]. Consequently, mutant enzymes were also assayed with this substrate. The specific activities were quite similar to that obtained with wild-type (results not shown), indicating that both tryptophan residues are not involved in peroxidase activity.

Structural studies

The intrinsic fluorescence properties of wild-type and tryptophan mutant enzymes were analysed (Figure 2). Emission spectra were obtained after excitation at 280 nm. Wild-type, W164F and W164A mutants had emission maxima at 339 nm. Furthermore, compared with wild-type, W164F and W164A showed a decrease in the fluorescence intensity of approx. 15 and 30 % respectively. In contrast, W97F showed a decrease of approx. 40 % in the fluorescence intensity. The emission maximum of this mutant was at 331 nm, blue-shifted by approx. 8 nm. Finally, W97A had a very low fluorescence with a decrease of approx. 85 %, and displayed an emission maximum at 310 nm probably due to the presence of the tyrosine residues in PmGSTB1-1 [7]. These results indicate that the contribution of Trp¹⁶⁴ to the overall protein fluorescence is quite low. This is in accordance with the crystal structure, which showed Trp¹⁶⁴ located in a more buried region [10]. On the other hand, these results show that Trp⁹⁷ is positioned on the exterior of the protein.

The structure of tryptophanyl mutants in comparison with wild-type enzyme was monitored by far-UV CD spectroscopy. As shown in Figure 3, the CD profiles revealed no significant differences between wild-type and mutant enzymes. The percentages

Figure 2 Fluorescence emission spectra of the wild-type and tryptophan mutant enzymes

Wild-type (○), W164A (◆), W164F (□), W97A (△) and W97F (▽). Protein concentration was 3 μM in 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Excitation was at 280 nm.

of secondary structures estimated by CD spectroscopy were also obtained. Structure estimation confirmed that the secondary structure of wild-type and mutant enzymes was essentially identical. All forms are composed of α -helix (48–50 %), β -sheet (14–16 %), β -turn (9–11 %) and random (25–28 %) structures. These results disclosed that none of the mutations has a significant effect on the secondary structure of the enzyme.

The unfolding/refolding transition curves of tryptophan mutants in comparison with that of the wild-type enzyme are shown in Figure 4. The curves of Trp⁹⁷ and Trp¹⁶⁴ are coincident with that of the wild-type suggesting that these residues are not involved in the unfolding/refolding kinetics of the enzyme. The thermal stability of the mutant and wild-type enzymes is shown in Figure 5. The curves of the W97A and W97F mutants are coincident with that of the wild-type enzyme, retaining 50 % of initial activity at approx. 70 °C. In contrast, the W164F and W164A mutants are more thermolabile than the wild-type enzyme. Figure 5 shows that after incubation at 35 °C, W164F was already inactivated by approx. 20 %. The W164A mutant is more unstable retaining approx. 50 % of initial activity at approx. 55 °C. It was completely inactivated at 70 °C, suggesting that the hydrophobic interactions

Figure 3 Far-UV CD spectra for wild-type and tryptophan mutant enzymes

Protein concentration was 17 μ M in 10 mM phosphate buffer (pH 7.0). The spectra were recorded at 25 °C. Wild-type (—), W97F (---), W97A (-.-.-), W164F (.....) and W164A (-----).

Figure 4 GdmCl-induced unfolding/refolding (open and filled symbols respectively) transition curves of wild-type and tryptophan mutants, monitored by changes in enzyme activity

(a) Wild-type (○/●), W164A (△/▲) and W164F (□/■). (b) Wild-type (○/●), W97A (△/▲) and W97F (▽/▼).

Figure 5 Effect of temperature on the stability of wild-type and tryptophan mutants

The enzyme activity at 25 °C was taken as 100 %. Wild-type (○), W164A (◆), W164F (□), W97A (△) and W97F (▽).

Figure 6 Fluorescence emission spectra of TNS binding with wild-type and tryptophan mutants

Protein concentration was 3 μ M in 10 mM phosphate buffer (pH 7.0) containing 1 mM EDTA. Excitation was at 323 nm. The spectra were corrected for the contribution of free unbound TNS. The inset shows the complete emission spectrum of the W164A mutant.

of the Trp¹⁶⁴ residue are essential for enzyme stability. The effect of mutations on the structure of PmGSTB1-1 was also examined by using the TNS extrinsic fluorescence technique. TNS is a hydrophobic compound that becomes highly fluorescent on binding to protein hydrophobic sites and it is usually employed to explore structural changes in the proteins. When TNS was added to the solution containing the wild-type or the mutant enzymes, an increase in the fluorescence intensity and a concomitant large blue-shift in the emission maximum for all forms were observed (Figure 6). The fluorescence spectra

of W97F (emission maximum at 450 nm) and W97A (449 nm) were quite similar to wild-type (456 nm). Different effects were observed for Trp¹⁶⁴ mutants when compared with wild-type. W164F showed higher fluorescence intensity although it had similar wavelength maximum (448 nm). Addition of TNS to W164A resulted in a 22-fold enhancement in fluorescence intensity and this was accompanied by a large blue-shift in the emission maximum at 432 nm, implying that the probe occupies a more hydrophobic environment (Figure 6, inset). The TNS dissociation constants (K_{dTNS}) for tryptophan mutants as well as wild-type enzyme were calculated. The results obtained indicate that TNS is capable of binding to all mutants with the same affinity as wild-type. TNS binds wild-type enzyme with a K_{d} value of 6.1 μM . The K_{dTNS} values for W97F (5.8 μM) and W97A (4.6 μM) were found to be essentially similar to wild-type. Moreover, W164F (12.4 μM) showed a K_{dTNS} value 2-fold higher than that obtained for wild-type. Finally, the K_{dTNS} value for W164A was 1.4 μM , 4-fold lower than that found for wild-type, indicating that for Trp¹⁶⁴ the mutation to Ala is followed by an increase in the affinity of the enzyme for the probe.

Molecular graphics analyses

In Figures 1(C) and 1(D), the electrostatic potential surfaces of the wild-type crystal structure and the homology model of the W164A mutant are shown. As expected, the mutation does not significantly alter the electrostatic potential in the H-site. On the other hand, the substitution of the bulky tryptophan residue into alanine is responsible for a significant increase in the accessible surface area in the H-site. This larger and more accessible H-site probably allows the substrates to be readily accommodated in a productive orientation.

Interaction with antibiotics

Previously, we have suggested a possible role for bacterial GSTs in resistance to antibiotics [8,16,17]. The binding capacity of several antibiotics to PmGSTB1-1 was previously determined by the quenching of intrinsic tryptophan fluorescence, suggesting the location of at least one tryptophan residue close to the antibiotic-binding site [17]. Furthermore, the three-dimensional structure of PmGSTB1-1 highlighted the possible involvement of Trp¹⁶⁴ in the antibiotic-binding site [10]. Consequently, to examine which of the two tryptophan residues is involved in the interaction of the enzyme with antibiotics, tryptophan mutant enzymes were tested for sensitivity to the action of two representative molecules [8], tetracycline and rifamycin, by inhibition studies. In fact, tetracycline and rifamycin were found to be strong inhibitors for PmGSTB1-1, having IC_{50} values of 49 and 85 μM respectively [8]. W164F, W164A, W97F and W97A showed similar IC_{50} values to wild-type for both the antibiotics (results not shown). The results obtained suggest that the antibiotic-binding site is not significantly altered by any of these mutations, indicating that these residues do not seem to be implicated in the binding of antimicrobial agents.

Conclusions

Mutations of the conserved Trp¹⁶⁴ demonstrated that this residue contributes significantly to the catalytic process of PmGSTB1-1. In fact, direct effects due to the substitution of the tryptophan residue on catalysis were observed. Trp¹⁶⁴ mutants showed a significant enhancement of catalytic efficiency for both substrates. Moreover, for W164A an increase in the affinity of the enzyme

for TNS was observed, indicating easier access of the probe in the hydrophobic area. These results are in agreement with molecular modelling analysis obtained for W164A mutant. W164A displayed a wider substrate-binding pocket and it may be slightly more flexible, resulting in a positive modulation on the catalytic efficiency of the enzyme. The results presented in the present study show that Trp¹⁶⁴ is also important for stabilizing the normal conformation of the enzyme, since the structure of the mutant is more readily damaged at high temperatures.

These results are consistent with previous observations on other GSTs that there seems to be a trade-off during evolution between catalytic efficiency and structural stability [31,32]. It has been suggested that some GST classes may be evolved towards product retention at the expense of catalytic efficiency [31,32]. Considering that Trp¹⁶⁴ is conserved in all beta class GSTs, it is possible that this residue is maintained to increase product retention and lower catalytic efficiency.

Finally, on the basis of the results obtained, Trp⁹⁷ does not seem to be involved in catalysis and structural stabilization. Moreover, Trp⁹⁷ and Trp¹⁶⁴ residues do not appear to be important determinants of antibiotic binding.

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