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Delineation of the structural and functional role of Arg111 in GSTU4-4 from *Glycine* max by chemical modification and site-directed mutagenesis

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ABSTRACT

The structural and functional role of Arg111 in GSTU4-4 from *Glycine* max (*Gm*GSTU4-4) was studied by chemical modification followed by site-directed mutagenesis. The arginine-specific reagent 2,3-butanedione (BTD) inactivates the enzyme in borate buffer at pH 8.0, with pseudo-first-order saturation kinetics. The rate of inactivation exhibited a non-linear dependence on the concentration of BTD which can be described by reversible binding of reagent to the enzyme (K_D 81.2 ± 9.2 mM) prior to the irreversible reaction, with maximum rate constants of 0.18 ± 0.01 min^{-1}. Protection from inactivation was afforded by substrate analogues demonstrating the specificity of the reaction. Structural analysis suggested that the modified residue is Arg111, which was confirmed by protein chemistry experiments. Site-directed mutagenesis was used in dissecting the role of Arg111 in substrate binding, specificity and catalytic mechanism. The mutant Arg111Ala enzyme exhibited unchanged K_m value for GSH but showed reduced affinity for the xenobiotic substrates, higher k_{cat} and specific activities towards aromatic substrates and lower specific activities towards aliphatic substrates. The biological significance of the specific modification of Arg111 by dicarbonyl compounds and the role of Arg111 as a target for engineering xenobiotic substrate specificity were discussed.

1. Introduction

Glutathione transferases (GSTs, EC 2.5.1.18) comprise a large family of ubiquitous detoxifying enzymes that catalyze a wide variety of conjugations of glutathione (GSH) to hydrophobic electrophile compounds [1–4]. GSTs are widespread in nature, being found in mammals, bacteria, yeast, plants, insects and other sources [1–4]. GSTs form a complex enzyme superfamily that has been subdivided into a number of classes according to amino acid/nucleotide sequence [5,6]. GSTs from tau (GSTU) and phi (GSTF) classes are the most abundant isoenzymes in plants [1,3,4].

GSTs exhibit wide substrate specificity and catalyze the conjugation of GSH with a broad range of electrophilic substrates including xenobiotics and endogenous electrophilic compounds such as pesticides, chemical carcinogens, environmental pollutants, leading to their detoxification [2,3,12–15]. Therefore, there, has been a particular interest in plant GSTs with regard to herbicide selectivity and environmental safety. Different classes of herbicides such as triazines, thiocarbamates, chloroacetanilides, diphenylethers, aryloxyphenoxypropionates, etc, can be detoxified by GSTs [7–11]. It is widely assumed that the catalytic promiscuity of GSTs and wide substrate specificity are the results of their structural plasticity and flexibility [1–3].

During the last decade, delineation of important structural characteristics have laid the groundwork for experiments involving both rational and random redesign of these enzymes, to create new forms of GSTs with engineered specificities [8,12–18]. GSTs can, be considered as an adaptable platform for engineering new catalytic activities, based on the use of natural and catalytic diversity, particularly with respect to the detoxification of synthetic compounds [8,12–17].

Chemical modification is a useful tool for the identification and probing of specific, catalytic and regulatory sites in purified enzymes and proteins [19]. Chemical modification experiments complement the results from crystallography and provide structural information of proteins and enzymes in free solution. This approach has been widely used to characterize several GST isoenzymes [20–21].

Abbreviations: BTD, 2,3-butanedione; CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; GSH, glutathione; GST, glutathione transferase; GSTUs, tau class GSTs; Nb-GSH, S-nitrobenzyl-glutathione.

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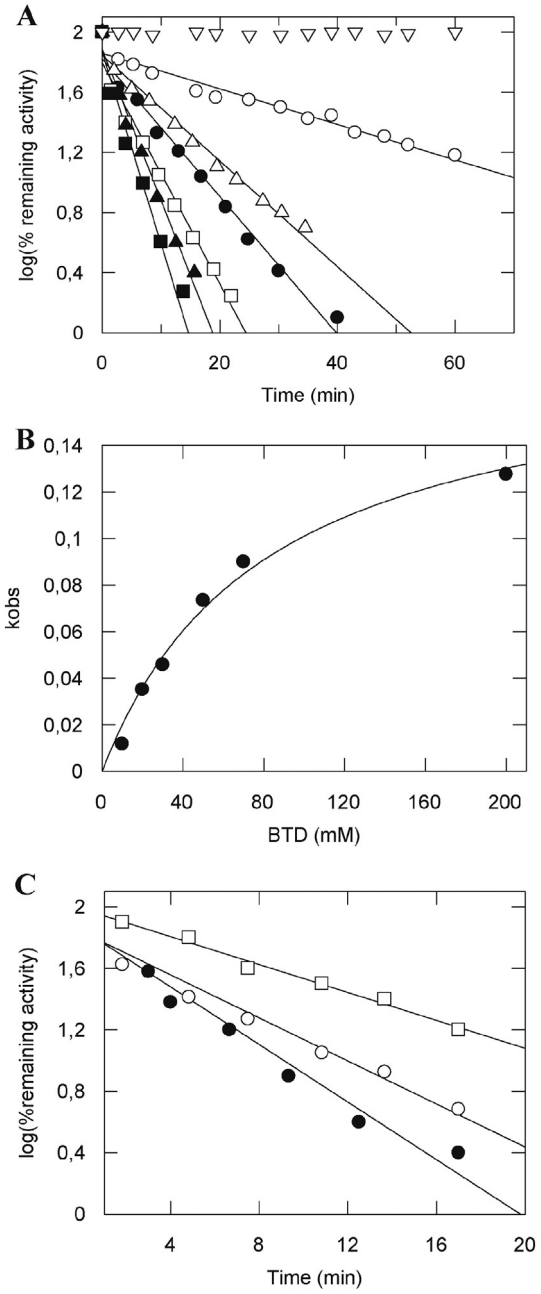


Fig. 1. A. Inactivation of *Gm*GSTU4-4 by BTD pH 8.0 and 25 °C. Enzyme (0.1 units) was incubated in the absence (▽) of BTD or in its presence at concentrations of 10 mM (○); 20 mM (△); 30 mM (●); 50 mM (□); 70 mM (▲); 200 mM (■). At the times indicated, aliquots were withdrawn and assayed for enzymatic activity. B. The effect of BTD concentration on the observed rate of inactivation (k_{obs}) of *Gm*GSTU4-4 by BTD. The concentration of BTD was in the range 10–200 mM. C. Inactivation of *Gm*GSTU4-4 by BTD (70 mM, ●) in the presence of 1 mM S-methyl-GSH (○) and 1 mM (□) S-nitrobenzyl-GSH.

In *Glycine max*, 42 GST isoenzymes have been identified so far [22]. Several of them have been characterized in detail [12,13,17,18]. The isoenzyme *Gm*GSTU4-4 has been the major focus of interest as a model for herbicide detoxification [9,10,11,17,18]. *Gm*GSTU4-4 known to be the most abundant *Glycine max* GST and is a homodimer protein of 214 amino acids [17,18]. *Gm*GSTU4-4 has been explored for the development of transgenic plants with increased resistance to abiotic stress [9,10,11]. Therefore, detailed characterization of this enzyme is of great importance.

In the present work, the structural and functional role of Arg111 in *Gm*GSTU4-4 was studied by chemical modification and site-directed mutagenesis. The results showed that the arginine-specific reagent BTD [23] reacts with high rate and specificity with Arg111. The mutant Arg111Ala enzyme exhibited unchanged K_m values for GSH but showed reduced affinity for the xenobiotic substrate and altered specificity towards a range of electrophile substrates. The results may provide the basis for the rational design of specially engineered forms of *Gm*GSTU4-4 with potential application in biotechnology.

2. Materials

GSH, 1-chloro-2,4-dinitrobenzene (CDNB), 2,3-butanedione (BTD), and all other reagents and analytical grade chemicals were obtained from Sigma-Aldrich Co (USA). Molecular biology reagents, enzymes and kits were obtained from Invitrogen (USA).

3. Methods

3.1. Cloning, expression and purification of *Gm*GSTU4-4

Cloning, expression and purification of *Gm*GSTU4-4 were carried out as described by Axarli et al. (2009) [18].

3.2. Assay of enzyme activity and protein and kinetic analysis

GST assays were performed by monitoring the formation of the conjugate between CDNB and GSH at 340 nm ($\varepsilon = 9.6\ mM^{-1}\cdot cm^{-1}$) according to a published method [24]. Observed reaction velocities were corrected for spontaneous reaction rates when necessary. Enzyme-dependent catalysis was determined by subtracting observed rates for conjugate formation in the absence of *Gm*GSTU4-4 from observed rates in the presence of *Gm*GSTU4-4. Kinetic constants were calculated from the initial velocities of enzyme-dependent conjugate formation. Michaelis-Menten equation was fitted to the experimental data using the computer program GraFit (Erithacus Software Ltd., Horley, U.K.). All initial velocities were determined in triplicate in buffers equilibrated at constant temperature. One unit of enzyme is defined as the amount of enzyme that produces 1.0 μmol of product per min.

3.3. Site-directed mutagenesis

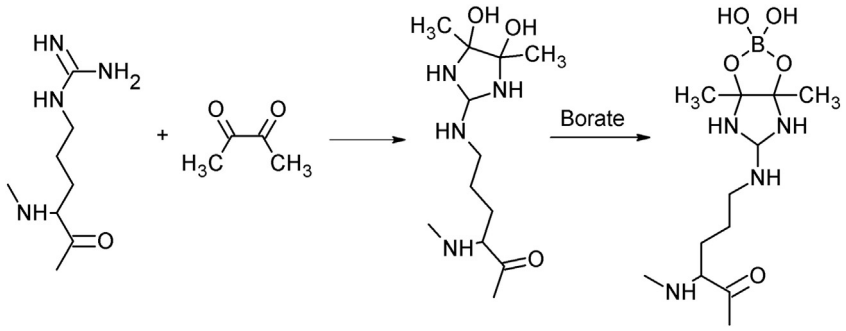
Site-directed mutagenesis was performed according to Axarli et al., [17]. The pairs of oligonucleotide primers used in the PCR reactions were as follows for the Arg111Ala, 5′-GATCTTGGAGCGAAGATTTGGACA-3′ and 5′-TGTCCAAATCTTCGCTCCAAGATC-3′. The mutation was verified by DNA sequencing. The mutant enzyme was expressed and purified as described for the wild-type *Gm*GSTU4-4 enzyme according to [18].

3.4. Viscosity dependence of kinetic parameters

The effect of viscosity on k_{cat} was studied in 0.1 M potassium phosphate buffer, pH 6.5, containing variable glycerol concentrations according to [13,17,18,25]. Viscosity values (η) were calculated as described in Wolf et al. 1985 [26]. Glycerol does not have any inhibitory effect on *Gm*GSTU4-4 catalysis [17,18].

3.5. Enzyme inactivation studies by BTD

Inactivation of *Gm*GSTU4-4 or mutant Arg111Ala was performed in 1 mL of incubation mixture containing borate buffer at pH 8.0 (100 mM), BTD (10–200.0 mM) and enzyme, (0.1 units). The rate of inactivation was followed by periodically removing samples (5–20 μL) for assay of enzymatic activity [20,21]. Inactivation studies of *Gm*GSTU4-4 by BTD in the presence of S-nitrobenzyl-GSH were performed in 1 mL of incubation mixture containing borate buffer at pH 8.0 (100 mM), BTD (70.0 mM), enzyme, (0.1 units) and S-nitrobenzyl-GSH (1 mM) or S-methyl-GSH (1 mM). Chemical modification reaction were carried



Scheme 1. The reaction of BTD with an arginine in proteins. Borate can stabilize the adduct that is formed by arginine with BTD.

out in the dark to avoid possible photoactivation of BTD, which could enhance nonspecific reactions with groups other than arginine [27].

3.6. Determination of enzyme total arginine residues

Determination of enzyme total arginine residues in native *Gm*GSTU4-4 as well as in the BTD-modified enzyme was carried out according to [28]. Control incubation in the absence of *Gm*GSTU4-4 was taken to correct the above determinations.

3.7. Biocomputing analysis

Biocomputing analysis was carried out as described in [12]. The programs PyMOL (DeLano Scientific) and UCSF Chimera were used for the analysis of structural models.

4. Results and discussion

4.1. Kinetics of reaction of BTD with GmGSTU4-4

The modification of arginine residues in proteins is typically based on the reaction of vicinal dicarbonyl compounds to form cyclic adducts [19,29–30]. When *Gm*GSTU4-4 was incubated with BTD, at pH 8.0 and 25 °C, the enzyme was progressively inactivated (Fig. 1A), whereas in the absence of BTD, virtually no change in activity was observed when the enzyme was incubated under identical conditions. The observed rate of inactivation was dependent upon BTD concentration as shown in Fig. 1 (A & B). This indicated that the reaction obeyed pseudo-first order saturation kinetics and was consistent with a Kitz & Wilson model [31] for reversible binding of reagent (BTD), prior to covalent modification according to equation [20,21,31-33]:

$$E + BTD \overset{K_D}{\rightleftarrows} E : BTD \overset{k_3}{\rightarrow} E\text{-}BTD$$

where E represents the free enzyme; E:BTD is the reversible complex and E-BTD is the covalent product. The steady-state rate equation for the interaction is [31–33]:

$$k_{obs} = k_3\,[BTD]/(K_D + [BTD])$$

Table 1
Total arginine determination in the unmodified and BTD-modified *Gm*GSTU4-4. The analysis was performed in three separate experiments. According to enzyme's primary structure, *Gm*GSTU4-4 has nine arginine residues.

Unmodified enzyme (mol of Arg residue/mol enzyme subunit)	Modified enzyme (mol of Arg residue/mol enzyme subunit)	Difference
9.14	8.24	0.90
8.82	7.89	0.93
9.33	8.42	0.91

where k_{obs} is the observed rate of enzyme inactivation for a given concentration of BTD, k_3 is the maximal rate of inactivation (min^{-1}), and K_D is the apparent dissociation constant of the E:BTD complex. From the data shown in Fig. 1B, a K_D value of 81.2 ± 9.2 mM and an apparent maximal rate constant k_3 of 0.18 ± 0.01 min^{-1} were determined. These results are in agreement with the view that BTD forms a reversible complex GST:BTD and that formation of covalent complex is rate limiting [21,21,31–33].

The inactivation of *Gm*GSTU4-4 is irreversible and almost complete restoration of activity was obtained when a sample of BTD-inactivated enzyme was freed of BTD and borate ions using extensive dialysis or after gel-filtration chromatography on Sephadex G-25 column. For example, inactivated *Gm*GSTU4-4 (<5% remaining activity) after extensive dialysis (24 h) against 20 mM potassium phosphate buffer pH 7, restores its activity (~96% reactivation). The same behaviour (~97% reactivation) was observed when the enzyme was subjected to gel-filtration chromatography on Sephadex G-25 column using as elution buffer 20 mM potassium phosphate buffer pH 7. These findings confirm that only arginyl groups are modified in *Gm*GSTU4-4 by BTD [34,35]. The modification of arginine residues by dicarbonyl reagents (e.g. phenylglyoxal, p-hydroxyphenylglyoxal, BTD, 1,2-cyclohexanedione, methylglyoxal) is achieved by the reaction of the dicarbonyl group to form cyclic adducts [23,29,30]. Studies have shown that phenylglyoxal and 1,2-cyclohexanedione can have significant side reactions with amino groups in the absence of borate buffers [34]. The reactions of BTD with lysine and histidine residues are minimal especially in borate

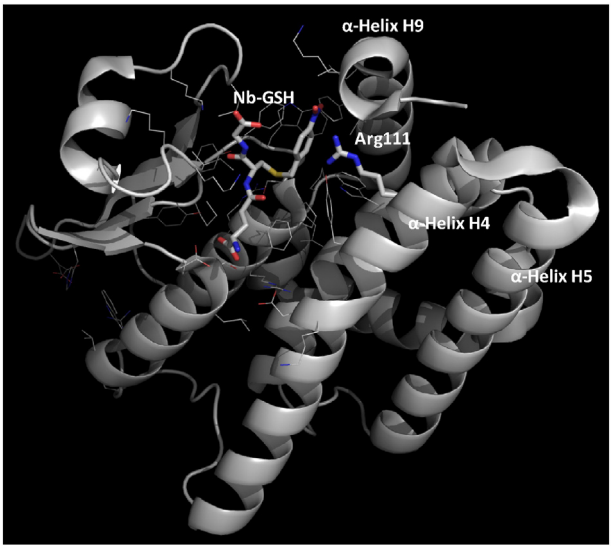


Fig. 2. Structural representation depicting Arg111 and other important residues of *Gm*GSTU4-4. The bound ligand (S-nitrobenzyl-GSH, Nb-GSH) and Arg111 are colored according to atom type and represented as thick sticks. Other active site side chains are represented as thin sticks. S-nitrobenzyl-GSH and Arg111 are labelled.

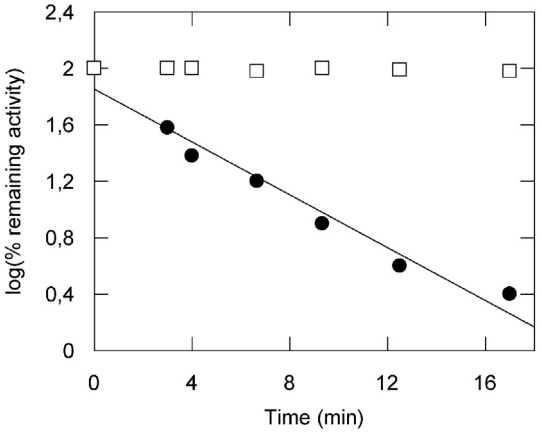


Fig. 3. Time course of inactivation of the wild-type *Gm*GSTU4-4 and mutant Arg111Ala by BTD. Wild type (●) and mutant Arg111Ala (□) were incubated in the presence of 100 mM BTD at pH 8.0 and 25 °C. At the times indicated, aliquots were withdrawn and assayed for enzymatic activity.

buffers [35]. As shown in Scheme 1, borate anions can form complex with the diol group and therefore stabilize the initial adduct and therefore prevent the regeneration of arginine [35]. The specificity of the BTD interaction with *Gm*GSTU4-4 is evidenced from the stoichiometry of incorporation. In order to identify the amino acid residue modified, amino acid analysis was employed. Total arginine determination was carried out for the modified as well as for the unmodified enzyme. The results (Table 1) from a total arginine determination indicated that the BTD-modified enzyme shows loss of 0.91 ± 0.17 mol of Arg/mol enzyme subunit, which is close to unity, suggesting that BTD reacts with a single Arg residue in each enzyme subunit.

The ability of specific ligands (e.g. substrates and inhibitors) to prevent enzyme inactivation by an irreversible inhibitor is the usual criterion used in arguing for active site-directed modification [20,21,32,33]. The inactivation of *Gm*GSTU4-4 was reduced significantly by the presence of S-nitrobenzyl-GSH as illustrated in Fig. 1C. In the presence of S-methyl-GSH or S-nitrobenzyl-GSH the enzyme showed about 30% and 65%, lower inactivation rates, respectively, compared to that in the absence of the inhibitors. Considering the structures of S-nitrobenzyl-GSH and S-methyl-GSH it is reasonable to assume that the target arginine residue is located at the H-site and protected more efficiently by large xenobiotic compounds (e.g. nitrobenzyl group) compared to the smaller structures (e.g. methyl group).

4.2. Identification of the Arg residue modified by BTD

From the analysis of the crystal structure of the enzyme [17,18] it is evident that one arginine residue (Arg111) is located on the topmost region of α-helix H4 and projects into the large H-site (Fig. 2). Its side chain is accessible to the solvent and therefore is accessible for covalent modification by BTD. Although arginines are almost always found on protein surfaces in large numbers, their reactions with dicarbonyl compounds seem to be low. This is due to their ability to interact with aspartic and glutamic acid residues and form salt bridges which reduces their reactivity [34,35].

To provide further experimental evidence and establish the involvement or not of Arg111 in the reaction with BTD, site-directed mutagenesis of Arg111 was employed. Arg111 was mutated to Ala and the mutant enzyme was expressed, purified and subjected to chemical modification by BTD, under exactly the same conditions to those used for the wild-type enzyme. The results showed that the Arg111Ala mutant enzyme is resistant to inactivation by BTD and retains all of its activity (Fig. 3). Comparison of the far UV difference spectra of native and mutated enzyme (data not shown) indicated the absence of any structural perturbation caused by the mutation. This rules out the possibility that the resistance to inactivation of the Arg111Ala-mutated enzyme is due to conformational changes in the structure of the enzyme.

4.3. The role of Arg111 in substrate binding and catalysis

Aminoacid sequence alignments of tau class GSTs [18] suggest that Arg111 is not conserved among other tau class GSTs, indicating a specific role of this residue in *Gm*GSTU4-4. Kinetic analysis of Arg111Ala mutant enzyme using the model substrates CDNB and cumene hydroperoxide was carried out and the results are listed in Tables 2 & 3. The results showed that the mutation does not appreciably alter the affinity of the enzyme for GSH. On the other hand, the mutant enzyme displays increased K_m values for CDNB and cumene hydroperoxide although its catatalytic efficiency (k_{cat}/K_m) towards CDNB and cumene hydroperoxide remained essentially unaltered. Interestingly, more profound effect of the mutation was observed on k_{cat} value. The mutant enzyme exhibits about four times higher k_{cat} value (Tables 2 & 3), compared to the wild type enzyme, suggesting the involvement of this amino acid to the catalytic mechanism.

*Gm*GSTU4-4 catalyzes a broad range of reactions [18] and therefore to evaluate the contribution of the mutation on the enzyme's activity towards different electrophile substrates, a broad range of substrates was examined and the results are listed in Table 4. The wild-type and mutant enzymes were assayed for activities as a glutathione transferase and as a glutathione peroxidase. The results indicate that the contribution of Arg111 depends on the electrophile substrate. In particular, with all aromatic substrates tested (p-nitrobenzyl chloride, atrazine, alachlor, ethacrynic acid, phenethyl isothiocyanate) the enzyme displays increase specific activity, compared to the wild-type enzyme. On the other hand using smaller aliphatic substrates, such as *tert*-butyl hydroperoxide, allyl isothiocyanate and *trans*-2-nonenal, the enzyme exhibits lower or unchanged specific activity, compared to the wild-type enzyme. This observation suggests a specific role of Arg111 in binding large aromatic substrates.

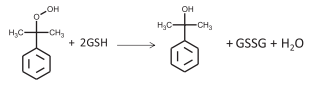
In order to gain a deeper insight into the role of Arg111 in the catalytic mechanism of *Gm*GSTU4-4, analysis of the available crystal structures of the enzyme [17,18] in complex with S-nitrobenzyl-GSH (reaction product analogue; PDB ID: 4VO4) and GSH (substrate; PDB ID: 4TOP) was undertaken. A striking difference between the structure of the enzyme·S-nitrobenzyl-GSH complex and the structure enzyme·GSH is the movement of the side chain of Arg111. In the enzyme·S-nitrobenzyl-GSH structure, the guanidine group of this residue is in hydrogen-bonding

Table 2
Steady-state kinetic parameters of *Gm*GSTU4-4 and Arg111Ala mutant enzyme for the CDNB/GSH reaction.

Enzyme	K_m, (mM) (GSH)	K_m, (mM) (CDNB)	k_{cat} (s^{-1})	k_{cat}/K_m^{CDNB} (s^{-1} mM^{-1})	k_{cat}/K_m^{GSH} (s^{-1} mM^{-1})
Wild-type[a]	0.159 ± 0.019	0.15 ± 80.0316	2.48 ± 0.31	15.7	15.6
Arg111Ala	0.137 ± 0.073	0.40 ± 0.039	8.04 ± 0.18	20.1	58.7

[a] The kinetic parameters for the wild-type enzyme were taken from reference [18] and included for comparison.

Table 3
Steady-state kinetic parameters of *Gm*GSTU4-4 and Arg111Ala mutant enzyme for the cumene hydroperoxide/GSH reaction.

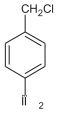
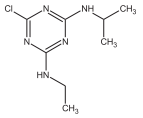
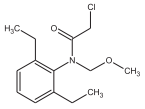
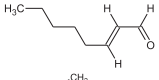
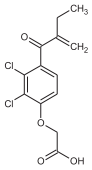
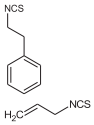
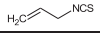


Enzyme	K_m, (mM) (GSH)	K_m, (mM) (CuOOH)	k_{cat} (s^{-1})	k_{cat}/K_m^{CuOOH} ($s^{-1}\,mM^{-1}$)	k_{cat}/K_m^{GSH} ($s^{-1}\,mM^{-1}$)
Wild-type[a]	0.036 ± 0.008	0.45 ± 0.084	0.24 ± 0.01	0.53	6.7
Arg111Ala	0.048 ± 0.01	0.82 ± 0.07	0.62 ± 0.02	0.76	12.9

[a] The kinetic parameters for the wild-type enzyme were taken from reference [18] and included for comparison.

distance (2.7 Å) with the hydroxyl group of Tyr107, whereas in the GSH-complexed enzyme, the distance is about 5 Å (Fig. 4A). The hydroxyl group of Tyr107 makes an on-face hydrogen bond with the π-electron cloud of the benzyl group of the bound S-nitrobenzyl-GSH. This interaction may stabilize aromatic substrates at their productive orientation. The loss of side-chain of Arg in the Arg111Ala mutant enzyme may influence the position of Tyr107 that may lead to higher K_m for aromatic substrates (CDNB, cumene hydroperoxide, Tables 2 & 3). Fig. 4 shows the *in silico* structural study of the wild type (left column) and the Arg111Ala mutant enzymes (right column). In particular, Fig 4C, left, shows that there is no direct interaction between Arg111 and GSH. The interaction energy of the ligand in the wild-type enzyme (PDB ID: 4VO4) was calculated 101.175 kcal/mol^2. In the mutant enzyme the free interaction energy of the ligand was found to be the same. This interaction energy-based comparison confirms the findings from the kinetic analysis (Tables 2 and 3), which showed that the K_m for GSH remained unchanged in the mutant enzyme. However, looking closer into the dynamics of the protein and the route that the ligand has to follow in order to occupy its position in the active site we can suggest that the bulkier side-chain of Arg111 residue, compared to that of Ala residue in the mutant enzyme controls the entrance of the active site. However, as soon as the ligand positions itself in the active site of the wild-type enzyme, the side-chain of Arg111 residue re-occupies its space with total energy of −38.516 kcal/mol^2. Negative interaction energies are indicative of stable molecular systems. On the contrary, upon the Arg111Ala mutation the smaller Ala residue displayed total energy of 16.245 kcal/mol^2, which indicates that the Ala residue is unstable and the adaptation of the position of the original side-chain of Arg residue is unfavourable. This can be explained by Fig 4C, where the interaction between the Ala111 residue with other neighbour residues are depicted. A new pi-stacking interaction has been established with Trp114 (Fig 4C, right), which is clearly pushing and locking the Ala111 residue in a single

Table 4
Substrate specificity for *Gm*GSTU4-4 and Arg111Ala mutant enzyme. Enzyme assays were carried out under standard conditions as described in the Methods section. The specific activity for the wild-type enzyme was taken from reference [18] and included for comparison. Results represent the means of triplicate determinations, with variation <5% in all cases.

Substrate	Structure	*Gm*GSTU4-4 Specific activity (Units/mg protein) ($\times 10^{-1}$)	Arg111Ala mutant Specific activity (Units/mg protein) ($\times 10^{-1}$)
p-Nitrobenzyl chloride		2.2	4.2
Atrazine		0.02	0.04
Alachlor		0.04	0.09
tert-Butyl hydroperoxide		0.85	0.21
trans-2-Nonenal		0.93	0.81
2,3-Dichloro-4-[2-methylene-butyryl]phenoxy acetic acid (ethacrynic acid)		0.53	3.4
Phenethyl isothiocyanate		1.84	2.9
Allyl isothiocyanate		0.63	0.62

high-energy trap position. Fig. 4D shows a structural superposition of the introduced Ala residue (in ball and stick representation) in the 3D conformational space that Arg111 originally occupied (in 3D cloud/surface representation). It is clear that the small side chain of the alanine residue is no substitute for the bulky Arg side chain. The entrance to the active site remains constantly open and the ligand can move freely in and out and as a consequently, the regulation of k_{cat} is expected to be observed in this molecular system (Tables 2 and 3).

Recent studies on the catalytic mechanism of *Gm*GSTU4-4 with CDNB as co-substrate have established that the rate-limiting step in the enzyme is not dependent on a diffusional barrier (e.g. product release) [18]. Instead, other viscosity-dependent motions or conformational changes of the protein, contribute to the rate-limiting step [18]. As illustrated in Fig. 5, the mutant Arg111Ala, exhibits different dependence on viscosity, compared to the wild-type enzyme, suggesting that the mutation affects the rate-limiting step of the catalytic reaction. Structural comparison (Fig. 4A) between the GSH-bound (enzyme-substrate complex) [17] and S-nitrobenzyl-GSH bound enzyme (enzyme-product complex) [18] revealed that large conformational changes occurs in the upper part of α-helix H4 (where Arg111 is located), in the C-terminal region (α-helix H9) as well as in the loop the connects α-helices H4 and H5. These structural transitions as well as dynamic issues may contribute to the observed differences in the rate-limiting step of the mutant enzyme.

4.4. Biological relevance

Dicarbonyl compounds such as BTD, methylglyoxal are cytotoxic by-products that are formed endogenously via different enzymatic and non-enzymatic reactions [36–40]. It is well established that dicarbonyl compounds initiate stress-induced signalling cascade via reactive oxygen species, resulting in the modifications of proteins involved in various signal transduction pathways, that eventually promotes in cell death or growth arrest [38]. The concentration of dicarbonyl compounds is varies in different plant species and increases in response to abiotic stress such as salinity, drought, and cold stress conditions [36]. We have recently showed that *Gm*GSTU4 gene expression displays a specific induction patterns. In particular, *Gm*GSTU4 gene is over expressed (approximately 153-fold) at RNA level under salinity stress caused by NaCl [10]. Considering the specificity of chemical modification of Arg111 as well as the highly specific stress-inducible nature of this enzyme under conditions which promote the elevation of dicarbonyl compounds levels (e.g. salt stress) [36], it is conceivable to assume an *in vivo* relevant functional role of *Gm*GSTU4-4/dicarbonyl interaction and enzyme inactivation.

5. Conclusion

In the present work we presented a combination of protein chemistry and protein engineering studies to characterize an important amino acid residue in *Gm*GSTU4-4. The inactivation of *Gm*GSTU4-4 by BTD exhibited pseudo-first order saturation kinetics and about 1 mol of Arg/mol of enzyme subunit is modified upon complete inactivation. In addition, the active-site inhibitors S-nitrobenzyl-GSH and S-methyl-GSH provided protection against inactivation and the Arg111Ala mutant enzyme is resistant to inactivation. All these findings point to the conclusion that Arg111 is the target for modification by BTD. Kinetic analysis established that Arg111 contributes to xenobiotic substrate binding and catalysis by influencing the K_m and k_{cat} values. These findings

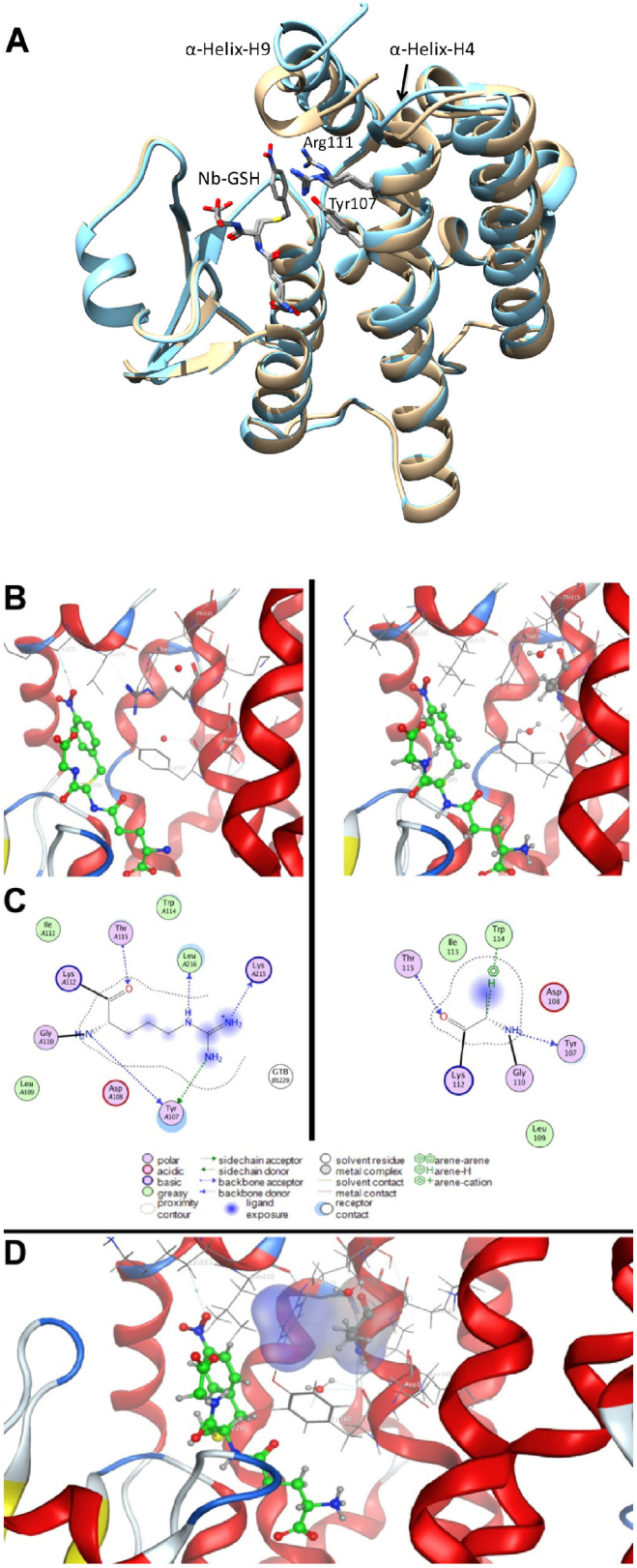


Fig. 4. A. Structural rearrangements in *Gm*GSTU4-4. Superposition of *Gm*GSTU4-4 structure in complex with S-nitrobenzyl-GSH (Nb-GSH, light brown) and GSH (light blue). The bound ligands (S-nitrobenzyl-GSH, and GSH) are colored according to atom type. Arg111 and Tyr107 are represented as sticks. S-nitrobenzyl-GSH, Arg111 and Tyr107 and the α-Helices H4 and H9 are labelled. (B). Molecular modelling study of the wild-type enzyme (left column) and the Arg111Ala mutant enzyme (right column). A zoomed-in view of the area in the proximity of position 111 and the ligand. (C). A schematic 2D interaction plot for both the wild-type enzyme and the mutant Arg111Ala residues. (D) A structural superposition of the wild-type and mutant Arg111Ala 3D structures. The Ala residue is depicted in ball and stick representation, the GSH ligand is colored green and the original 3D conformational space of the Arg111 residue is defined by a 3D electrostatic cloud/surface representation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

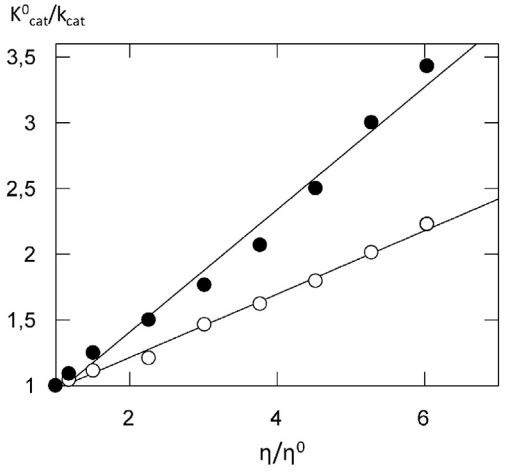


Fig. 5. The effect of viscosity on k_{cat}. The effect of viscosity on k_{cat} for the CDNB/GSH reaction catalyzed by *Gm*GSTU4-4 and its Arg111Ala mutant. Plot of the reciprocal of the relative turnover number (k^{o}_{cat}/k_{cat}) as a function of relative viscosity (η/η^{o}) with glycerol as cosolvent for the wild-type, (○); and for the Arg111Ala (●) mutant enzyme. Lines were calculated by least-squares regression analysis.

have relevance for future efforts aiming to redesign xenobiotic substrate specificity. In addition, the association of dicarbonyl compounds with plant stress response may indicate an *in planta* role of *Gm*GSTU4-4/dicarbonyl interaction.

Transparency document

The Transparency document associated with this article can be found, in online version.

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