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ORIGINAL ARTICLE

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Kinetic analysis of maize glutathione *S*-transferase I catalysing the detoxification from chloroacetanilide herbicides

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Abstract Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of multi-functional enzymes involved in biodegradation of several herbicide classes. The ability of the maize isoenzyme GST I to detoxify from the acetanilide herbicide alachlor was investigated by steady-state kinetics and site-directed mutagenesis studies. Steady-state kinetics fit well to a rapid equilibrium random sequential bi-bi mechanism with intrasubunit modulation between GSH binding site (G-site) and electrophile binding site (H-site). The rate-limiting step of the reaction is viscosity-dependent and thermodynamic data suggest that product release is rate-limiting. Three residues of GST I (Trp12, Phe35 and Ile118), which build up the xenobiotic binding site, were mutated and their functional and structural roles during alachlor conjugation were investigated. These residues are not conserved, hence may affect substrate specificity and/or product dissociation. The work showed that the key amino acid residue Phe35 modulates xenobiotic substrate binding and specificity, and participates in k_{cat} regulation by affecting the rate-limiting step of the catalytic reaction. Trp12 and Ile118 do not seem to carry out such functions but instead, regulate the K_m for alachlor by contributing to its productive orientation in the H-site. The results of the present work have practical significance since this may provide the basis for the rational design of new engineered GSTs with altered substrate specificity towards herbicides and may facilitate the design of new, more selective herbicides.

Keywords Alachlor · Herbicide detoxification · Protein engineering · Xenobiotics

Abbreviations *Alachlor*: 2-chloro-N-[2,6-diethylphenyl]-N-[methoxymethyl]acetamide · *atrazine*: 2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine · *GSH*: glutathione · *GST*: glutathione *S*-transferase

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Introduction

Glutathione S-transferases comprise a large family of ubiquitous detoxifying enzymes that catalyse the nucleophilic attack of the sulphur atom of the tripeptide glutathione (GSH, γ-glu-cys-gly) on electrophilic groups of a variety of hydrophobic compounds (Dixon et al. 1998; Edwards et al. 2000; Sheehan et al. 2001). The resulting GSH conjugates are generally less toxic and more water soluble than the original herbicide molecules (Marrs 1996).

Plant GSTs gained particular attention with respect to the detoxification from herbicides that belong mainly to two classes, chloroacetanilides and triazines (Marrs 1996; Rossini et al. 1997; Noctor et al. 1997; Cummins et al. 1999). These herbicides are widely used for the control of annual grasses and broad-leaf weeds in a variety of major crops such as maize and soybeans (Sharp 1988). Alachlor is a preemergence herbicide and is applied to young plants. It is absorbed through the roots and transferred to the upper parts of the plant through the apoplast, repressing the elongation of the root system and the development of the shoots of young plants.

Plant GSTs are also involved in the response to different biotic and abiotic stresses, and can be specifically induced in response to a variety of stimuli, such as pathogens and chemicals. Also, a number of GSTs, additionally, exhibit GSH peroxidase activity, playing a counteracting role in oxidative stress (Roxas et al. 1997; McGonigle et al. 2000). In addition to the catalytic properties, certain GSTs also feature non-enzymatic ligand-binding functions, serving as carrier proteins for the intracellular transport of hydrophobic molecules (Dixon et al. 1998; Sheehan et al. 2001; Axarli et al. 2004). In maize (*Zea mays* L.) 42 GSTs have been identified (McGonigle et al. 2000), of which some have been characterized in detail (McGonigle et al. 2000; Labrou et al. 2001a, 2004b).

The isozyme GST I from maize (ZmGSTF1, Edwards et al. 2000) has been the major focus of interest as enzyme model for herbicide detoxification (McGonigle et al. 2000; Labrou et al. 2001b, 2004a). GST I is a homodimer protein of 214 amino acids. It does not exhibit GSH peroxidase activity, and shows constitutive expression in maize seedlings (McGonigle et al. 2000). Each subunit features a catalytic site composed of two components (Neuefeind et al. 1997; Prade et al. 1998; Sheehan et al. 2001; Dixon et al., 2002; Thom et al. 2002). The first is a binding site specific for GSH (the G-site) formed of a conserved group of amino acid residues in the amino-terminal domain of the polypeptide. The second component is a site that binds the hydrophobic substrate (the H-site), which is structurally variable and is formed of residues in the carboxyl-terminal domain.

Despite the agronomic potential of plant GSTs, this family of GSTs is poorly characterised, compared to other GSTs families. Kinetic or mutagenesis studies concerning the catalytic mechanism of plant GSTs toward herbicides have not been reported so far. Thus, detailed study of catalytic mechanism operated by this family of enzymes is of academic interest and practical importance.

Materials and methods

Materials

DNTPs were from Roche, UK. Restriction enzymes were obtained from New England Biolabs, USA. Reduced GSH was obtained from Sigma, USA. Alachlor and atrazine were obtained from Rieden-deHaen (Germany). DNA purification kits were obtained from Qiagen (Germany). *Pfu* DNA polymerase and all other molecular biology reagents were obtained from Promega (UK).

Methods

Cloning, expression and purification of maize GST I

Cloning of maize GST I into a pQE70 expression vector to yield the pQEGST expression plasmid was described by Labrou et al. (2001b). Expression and purification of GST I were performed according to Labrou et al. (2001b).

Site-directed mutagenesis

Site-Directed Mutagenesis was performed according to Weiner et al. (1994). The couple of oligonucleotide primers used in the PCR reactions was as follows: for the Ile118Phe mutation, 5′-CCC ATC AAC *CTC* GCC ACC GCC-3′ and 5′-CAC GAT CTC GTA GTC GGA GCC-3′. For the Trp12Ile mutation:

5′-ATGTCGATCAACGTGACGAGGTGC-3′ and

5′-CACCGCCCCGTACAGCTTCATCGG-3′. For the Phe35Leu mutation:

5′-ATCAACCTCGCCACCGCCGAGCAC-3′ and

5′-GGGCACGATCTCGTAGTCGGAGCC-3′. All mutations were verified by DNA sequencing on Applied Biosystems Sequencer 373A with the DyeDeoxy Terminator Cycle sequencing kit. The expression construct pQEGST encoding maize GST I was used as template DNA in all mutagenesis reactions. Expression and purification of mutated forms of GST I were performed as described by Labrou et al. (2001b).

Assay of enzyme activity and protein

Routine enzyme assays were performed by monitoring the formation of the conjugate of CDNB (1 mM) and GSH (2.5 mM) at 340 nm ($\epsilon = 9.6\ mM^{-1}\ cm^{-1}$) at 30°C according to a published method (Labrou et al. 2001a).

Enzyme assays for the alachlor conjugation reactions were performed at 30°C using the reverse phase HPLC method (Skipsey et al. 1997). Separation of the products from the alachlor–glutathione conjugation reaction was achieved on a C18 S5 ODS2 Spherisorb silica column (250×4.6 mm I.D.) with a H_2O/acetonitrile linear gradient containing 0.1% trifluoroacetic acid at a flow rate 0.5 ml/min. The effluents were monitored at 260 nm. The conjugate S-alachlor-GSH was used as a standard in HPLC analysis. This was synthesised by substitution of the chlorine atom of alachlor by reduced GSH as follows: to a solution of alachlor (0.2 mmol, dissolved in 2 ml acetone) was slowly added aqueous GSH solution (0.5 mmol dissolved in 50 mM acetate buffer, pH 5.0; 2 ml). The pH was adjusted throughout the reaction to 5.0 with NaOH (0.1 M). After the reaction was completed (3 hr, 70°C, as judged by TLC) the mixture was extracted five-times with $CHCl_3$ (5×50 ml) and the aqueous phase was collected and concentrated on a rotary evaporator until a solid powder appeared. The solid powder was stored desiccated at −20°C. Product was purified by ascending TLC on silica gel 60 plates using the solvent system: butanol-2/AcOH/water (4/1/1). The product was negative to 5,5-dithio-bis-(−2-nitrobenzoic) acid test for free -SH group and positive to ninhidrin and 2,4,6-trinitrobenzenesulfonic acid tests for primary amine. The product exhibits an ultraviolet

absorption spectrum with a peak at 260 nm, which is not present in the spectrum of the precursor GSH.

Observed reaction velocities were corrected for spontaneous reaction rates when necessary. All enzyme velocities were determined in triplicate in buffers equilibrated at constant temperature. Turnover numbers were calculated on the basis of one active site per subunit. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (fraction V) as standard.

Kinetic analysis

Steady-state kinetic measurements were performed at 30°C in 0.1 M potassium phosphate buffer, pH 6.5. Initial velocities were determined in the presence of GSH (1–2 mM) and alachlor was used in the concentration range of 0.056–0.186 mM. Initial velocities were also determined in the presence of alachlor (0.05–0.2 mM) and GSH (0.4–3.6 mM). Solutions of GSH or analogues were prepared fresh each day and stored on ice under N_2. All initial velocities were determined in triplicate. The apparent kinetic parameters k_{cat}, and K_m were determined at a fixed GSH concentration with various alachlor concentrations, by fitting the collected steady-state data to the Michaelis–Menten equation by non-linear regression analysis using the GraFit (Erithacus Software, Ltd.) computer program. Enzyme assays and kinetic analysis for the atrazine conjugation reaction were performed at 30°C as described above for alachlor.

Viscosity dependence of kinetic parameters

The effect of viscosity on k_{cat} was assayed at 30°C in 0.1 M potassium phosphate buffer, pH 6.5, containing variable glycerol concentrations. Viscosity values (η) at 30°C were calculated as described in Wolf et al. (1985).

Results and discussion

Selection of residues for mutagenesis

The interest in plant GSTs is derived from their agronomic value. It has been demonstrated that GSH conjugation for a variety of herbicides is the major resistance and selectivity factor in plants and provides a tool to control weeds in agronomic crops (Marrs 1996; Rossini et al. 1997; Noctor et al. 1997; Cummins et al. 1999). The phi class isoenzyme GST I from maize has been the subject of intense research because of its ability to detoxify a wide range of xenobiotics (McGonigle et al. 2000). On the basis of the available GST I-S-atrazine-GSH conjugate crystal structure (Prade et al. 1998) three residues (Trp12, Phe35, Ile118, Fig. 1) which build up the H-site, were selected for the assessment of their contribution to alachlor binding and catalysis. These residues were individually mutated to the equivalent residues found in Arabidopsis GST (Prade et al. 1998), which also belongs to the phi class but exhibits different substrate specificity. In particular, Trp12, Phe35 and Ile118 were mutated to Ile, Leu, and Phe, respectively. These residues have proposed to modulate substrate recognition by affecting the size and shape of the H-site (Prade et al. 1998). Analysis of the GST I-S-atrazine-GSH conjugate complex reveals that these residues come from three different parts of the protein. Phe35 comes from helix α2, Trp12 comes from a loop between strand β1 and helix α1, and Ile118 is located at the loop between helices α4 and α5. Xenobiotic substrate is bound in a hydrophobic cleft bearing on one side Phe35 and on the other Trp12 and Ile118. The aromatic ring of Phe35 stacks flat with the aromatic ring of the substrate, whereas Trp12 is more perpendicular.

Kinetic analysis of the GSH conjugation to alachlor with the wild type and mutated enzymes

CDNB is generally considered to be the classical GST substrate because most GST isoenzymes display high activity with it. Although the kinetic mechanism of GSH conjugation to CDNB has been investigated for GST I (Labrou et al. 2001a, 2004b), kinetic analysis for GSH conjugation to herbicides has not been studied so far. When GSH was the variable substrate with several fixed concentrations of alachlor, an intersecting pattern of Lineweaver–Burk plot was obtained, and with alachlor as the variable substrate at fixed concentrations of GSH, an intersecting pattern was again obtained. These data are in agreement with the rapid equilibrium random sequential bi-bi mechanism with intrasubunit modulation between GSH binding site (G-site) and electrophile binding site (H-site), operating by the enzyme when CDND is used as substrate (Labrou et al. 2001a). Addition of chloride ions (KCl) at concentrations to 100 mM gave no significant inhibition of initial rates when GSH and alachlor were fixed at 2.5 mM and 0.2 mM, respectively. The lack of inhibition by chloride ions indicates that little specificity exists for the leaving group from the electrophile. This is consistent with the broad specificity of GSTs, resulting in a variety of leaving groups (Sheehan et al. 2001).

The kinetic constants k_{cat} and K_m for the wild type and mutated (Trp12Ile, Phe35Leu and Ile118Phe) enzyme forms were determined by steady-state analysis and the results are listed in Table 1. All the mutants show differences in K_m values for alachlor, when compared to the wild-type enzyme. The mutant Phe35Leu shows a dramatic increase in the K_m value for alachlor. The Phe35Leu mutation may have two semi-independent consequences: the loss of a π–π aromatic interaction of the Phe side chain with alachlor, and the introduction of a cavity. The cavity could either be filled by new water molecules or by a rearranged local protein structure. Either the loss of the aromatic interaction or

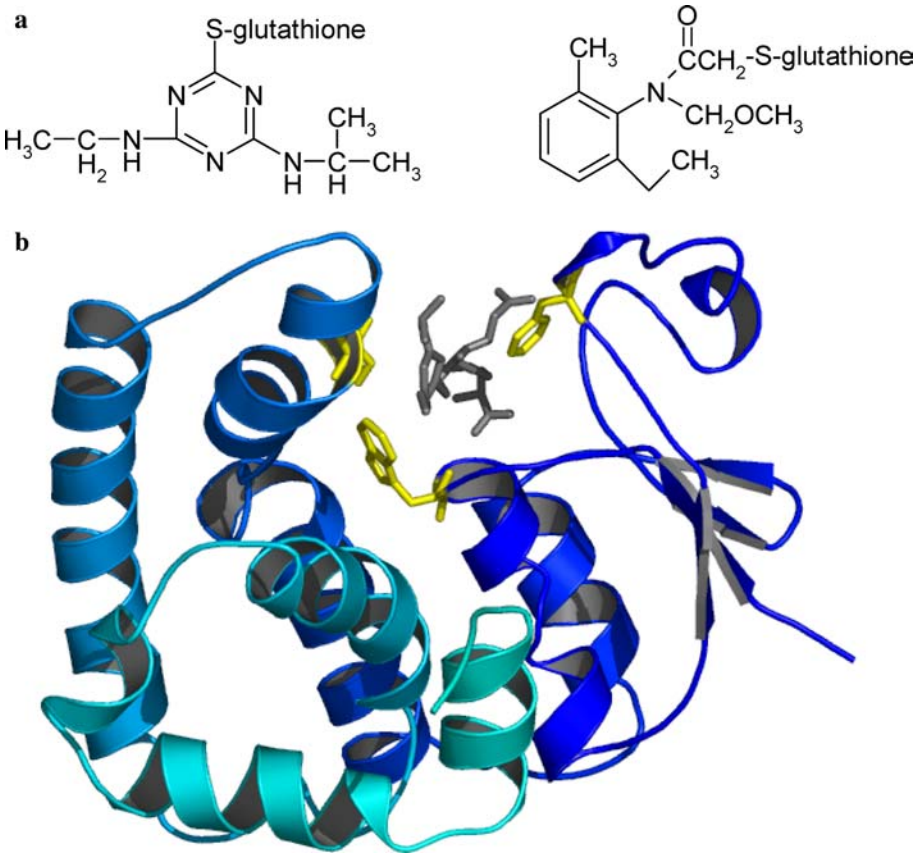

Fig. 1 Chemical formulae for the herbicide-GSH conjugates and diagram depicting mutated residues of maize GST I. **a**: *Left*, S-atrazine-GSH conjugate; *Right*: S-alachlor-GSH conjugate. **b**: Bound S-atrazine-glutathione conjugate is shown in grew. Residues of the H-site (Trp12, Phe35 and Ile118) are shown in yellow. The figure was produced based on the crystal structure of GST I- S-atrazine-glutathione complex (1BYE PDB code, Prade et al. 1998)

the rearrangement of helix α2 may affect alachlor binding and lead to reduced affinity. In addition, the mutant Phe35Leu showed increased K_m value for the GSH (Table 1). The contribution of Phe35 to K_m value for GSH may be relevant to the indirect effect of this residue on the G-site architecture. Phe35 is located in the flexible segment of residues 36–45. In the same segment there are also located residues His40 and Lys41, which make salt bridges with the Gly carboxylate of GSH. In the mutated enzyme, the rearrangement of the segment could affect His40 and Lys41 conformations which affect GSH binding.

The Ile118Phe mutant exhibits the higher affinity for alachlor (Table 1). The mutation may have, as a consequence the development of a new π–π aromatic interaction with the aromatic ring of alachlor, which may lead to the formation of a 'sandwich' complex. This may explain the increased affinity and improved specificity of the mutant exhibited for alachlor. A similar complex was also observed in the pi class GST from human placenta, which exhibits high affinity for aromatic substrates (Oakley et al. 1997). Trp12 is involved in a weak hydrophobic interaction with the aromatic ring of the substrate. In the mutant Trp12Ile the side-chain of Ile may provide more room, compared to Trp that may lead to unproductive binding of alachlor (Piervincenzi and Chilkoti 2004).

The effect of viscosity on the kinetic parameters was measured in order to analyse the rate-limiting step of the catalytic reaction. A decrease of k_{cat} by increasing the medium viscosity indicates that the rate-limiting step of the reaction is related to the product release or to diffusion-controlled structural transitions of the protein (Johnson et al. 1993). A plot of the inverse relative rate constant $k_{cat}\,°/k_{cat}$, ($k_{cat}\,°$ is determined at viscosity $\eta\,°$) versus the relative viscosity, $\eta/\eta\,°$, should be linear, with a slope equal to unity when the product release is limited by a strictly diffusional barrier or a slope approaching zero if chemistry or another non-diffusional barrier is rate-limiting (Sampson and

Table 1 Steady-state kinetic parameters of wild-type and mutants of maize GST I for the alachlor conjugation reaction at pH 6.5 and 30°C

Enzyme	K_m, (mM) (alachlor)	K_m, (mM) (GSH)	k_{cat}, (min^{-1}) ($\times 10^{-3}$)	k_{cat}/K_m(%)
Wild type	0.18 ± 0.04	0.83 ± 0.1	28.7 ± 1.5	100
Trp12Ile	0.82 ± 0.03	0.73 ± 0.1	6.5 ± 0.5	4.96
Phe35Leu	7.23 ± 0.15	3.92 ± 0.3	11.5 ± 1.1	0.997
Ile118Phe	0.042 ± 0.007	0.76 ± 0.1	27.4 ± 2.1	408.4

Knowles 1992). As illustrated in Fig. 2, the inverse relative rate constant $k_{cat}\,^{\circ}/k_{cat}$ for the enzyme catalysed reaction shows linear dependence on the relative viscosity with a slope (0.95 ± 0.1) very close to unity, whereas the uncatalysed reaction is fully viscosity-independent. In contrast, the mutant Phe35Leu exhibits k_{cat} values with different degree of viscosity dependence, compared to the wild type enzyme (slope 0.25 ± 0.05). The mutants Trp12Ile and Ile118Phe show no appreciable difference compared to the wild-type enzyme (data not shown). It is important to note that glycerol does not induce changes in the enzyme structure as detected by far-UV difference spectroscopy (spectra not shown). Furthermore, glycerol does not have any inhibitory effect on catalysis.

The intermediate values of the slope ($0 < \text{slope} < 1$) observed in the mutant Phe35Leu indicate that the rate-limiting step in this mutant is not strictly dependent on a diffusional barrier and other viscosity-dependent motions or conformational changes of the mutated protein contribute to the rate-limiting step of the catalytic reaction (Johnson et al. 1993; Igarashi et al. 2004). Probably, the structural integrity or flexibility of the 3_{10}-helical segment (residues 35–46), where Phe35 is located, of helix a2 has been altered in the mutated form of GST I. We have recently shown that the 3_{10}-helical segment is a flexible region that undergoes the largest C_a fluctuations and influence the rate-limiting step of the catalytic reaction (Labrou et al. 2001a). The perturbation of 3_{10}-helical segment's flexibility in the mutant Phe35Leu may be due to the loss of direct amino-aromatic interaction of Phe35 with His40. This is one of the main interactions that contribute to the structural stability of GST I

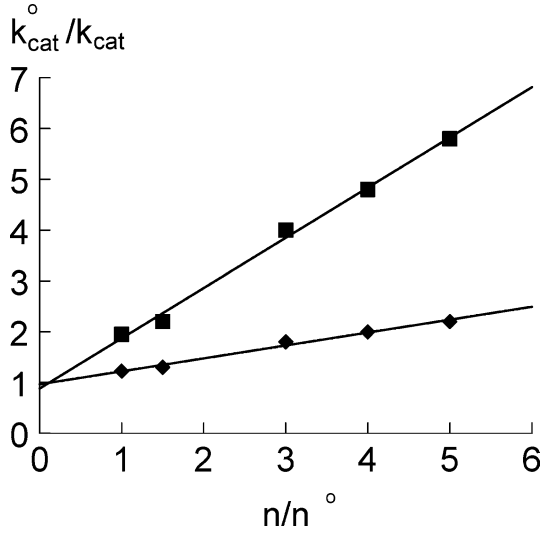

Fig. 2 The effect of viscosity on kinetic parameters. Plot of the reciprocal of the relative turnover number ($k_{cat}\,^{\circ}/k_{cat}$) as a function of relative viscosity ($\eta/\eta\,^{\circ}$) with glycerol as cosolvent for the wild-type, (*filled square*); and for the mutant Phe35Leu (*filled diamond*). Experiments were performed in triplicate and lines were calculated by least-squares regression analysis

(Labrou et al. 2001a). The loss of this important interaction may lead to structural perturbation of helix α2 with concomitant effect of the dramatic reduction in k_{cat} and the adaptation of a new rate-limiting step by the enzyme.

Several pieces of evidence indicate that GSTs utilise structural flexibility for attainment efficient catalysis. A plot of the crystallographic B-factors along the polypeptide chain can give an indication of the relative flexibility of the protein portions (Ricci et al. 2003; Yuan et al. 2003; Kotzia and Labrou 2004). GST I displays a well-defined flexibility pattern (Fig. 3). Several important regions, which include the helix α2 and helix α3‴, undergo large conformational changes. All plant-soluble dimeric GSTs may be grouped into four classes, which are termed phi, zeta, tau and theta. Zeta and theta GSTs are found in both animals and plants, but the tau and phi classes are plant-specific. Phylogenetic analysis suggested that all GSTs have arisen from an ancient progenitor gene (Dixon et al. 2002). Comparison of the mobility profile of phi class GST I from maize and *Ara*GST from *Arabidopsis thaliana* with that of zeta and tau, whose three-dimensional structure are available, showed that every class exhibits a completely different pattern of flexibility (Fig. 3). For example, in class phi several highly mobile regions can be identified throughout the entire sequence, separated by a number of segments with low mobility. In class zeta the numbers of regions with high mobility as well as the overall flexibility has been reduced. In class tau only two well-defined regions exhibit high mobility whereas the mobility of other regions has been restricted. The mobility profile shown in Fig. 3 seems to correlate with the evolution progress of each class, e.g. in the younger tau the mobility is restricted to specific regions that are involved in the formation of H-site, compared to the older phi, which exhibits high overall mobility throughout the entire sequence. The class zeta exhibits a pattern that may be considered as between phi and tau class. These observations suggest that probably during evolution the highly flexible GSTs have restricted their flexibility only to those regions that contribute to substrate binding and catalysis. In this case, the evolution pressure has developed GSTs with lower energetic cost catalysis.

An other possible factor that could explain the reduced k_{cat} in the mutated enzymes is the shift in the pK_a of the -SH group. However, this does not seem to be likely, since the side chains of mutated residues are located away (~6Å) from the -SH group of GSH and do not exhibit acid-base properties.

Kinetic analysis of the effect of replacement of H-site residues on atrazine conjugation reactions

To further evaluate the data obtained by investigating the alachlor conjugation reaction, kinetic analysis of the wild-type and mutants was carried out to analyse the

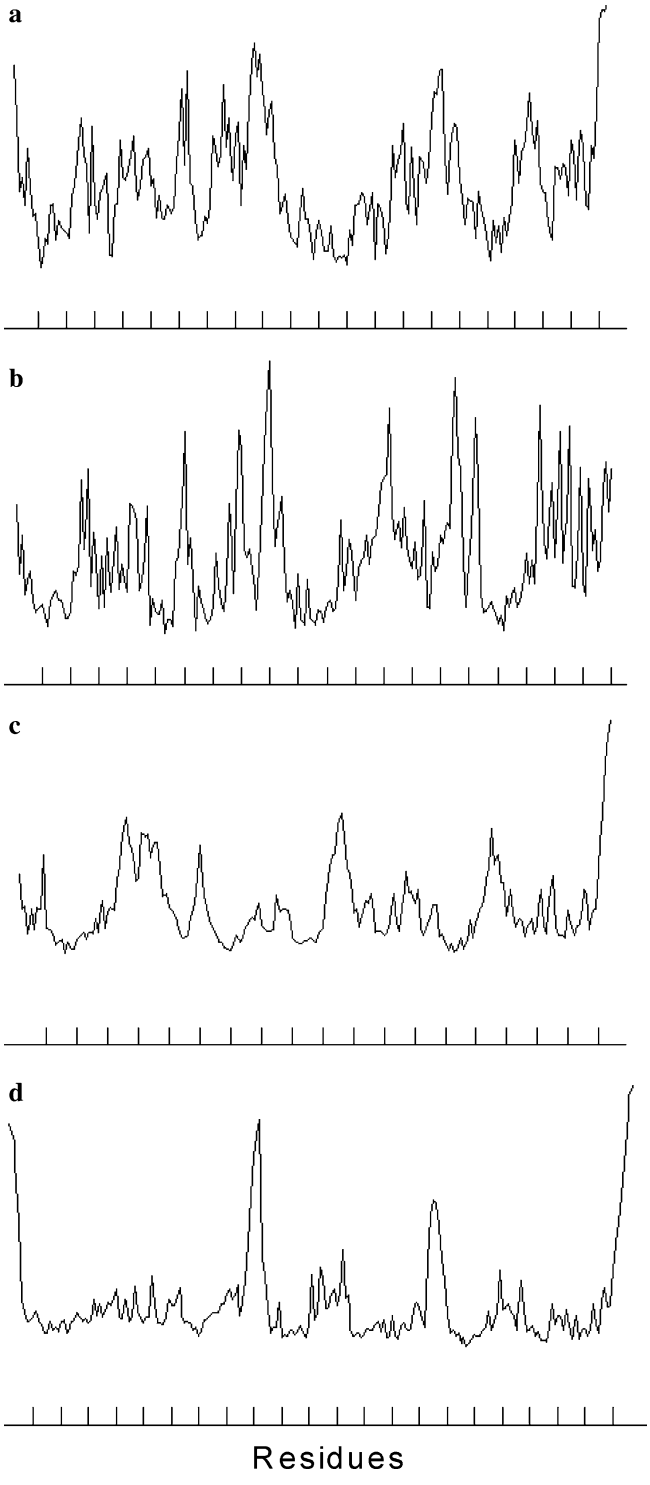

Fig. 3 The flexibility of plant GSTs. Plot of the crystallographic B-factors along the polypeptide chain obtained from the crystal structure of phi class maize GST I (**a**) and *Arabidopsis thaliana* GST (**b**) in complex with atrazine-GSH and FOE-4053-glutathione conjugate, respectively (PDB codes 1BYE and 1GNW, respectively). Plot of the crystallographic B-factors along the polypeptide obtained from the crystal structure of zeta (**c**, PDB code 1E6B) and tau (**d**, PDB code 1OYJ). Similar profiles were obtained employing, in the analysis, all the available plant GSTs' structures (e.g. 1AW9, 1GWC, 1BX9, data not shown). The plots were produced using the WHAT IF software package (Vriend 1990). The height at each residue position indicates the average B-factor of all atoms in the residue

effect of each mutation to the catalytic parameters of the atrazine conjugation reaction. Atrazine is a chlorotriazine herbicide used for selective weed control in agricultural crops and for nonselective weed control on noncropped land.

Atrazine is a known substrate for maize GST I (McGonigle et al. 2000). Atrazine is thought to form a conjugate with GSH via nucleophile addition by GST-driven catalysis. The chlorotriazine moiety is the target for conjugation by GSH (Fig. 1a). The kinetic parameter K_m and k_{cat}, were determined by steady-state kinetic analysis and are listed in Table 2. All mutants show significant kinetic differences when compared to the wild-type enzyme. Comparison of the data presented in Table 1 and 2 points to the conclusion that Trp12 and Phe35 contribute relatively similar towards alachlor and atrazine conjugation reactions. However, Ile118 seems to play a different role in the atrazine conjugation reaction. The mutant Ile118Phe exhibits reduced affinity for atrazine indicative of the fact that Ile118 seems to mainly modulate substrate specificity. Presumably, the bulky isopropyl group of atrazine, compared to the methyl group of alachlor, may be responsible for the above difference (Fig. 1). To this end, a well-known property of GSTs is that catalysis with different substrates involves different residues (Prade et al. 1998; Labrou et al. 2004b).

In conclusion, in this report we addressed questions regarding the functional and structural role of maize GST I. The work showed that the key amino acid residues Trp12, Phe35 and Ile118, that build up the H-site play an important role in substrate binding and catalysis, and leads to a new mutated form (Ile118Phe) that exhibits fourfold improved specificity towards alachlor. The results of the present work will form the basis for a rational design of new engineered GSTs with altered specificity and enhanced catalytic efficiency toward herbicide. In addition, the mutagenesis data could be used as an initial point for analysing the specificity of other plant GSTs of unknown structure.

Table 2 Steady-state kinetic parameters of wild-type and mutants of maize GST I for the atrazine conjugation reaction at pH 6.5 and 30°C

Enzyme	K_m, (mM) (atrazine)	k_{cat}, (min^{-1}) ($\times 10^{-3}$)	k_{cat}/K_m(%)
Wild type	0.07 ± 0.03	0.27 ± 0.03	100
Trp12Ile	0.52 ± 0.1	0.24 ± 0.02	12.3
Phe35Leu	0.2 ± 0.15	0.48 ± 0.04	62.1
Leu118Phe	0.18 ± 0.06	0.26 ± 0.03	36.9

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