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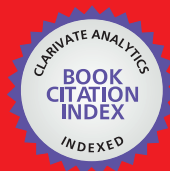
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Molecular Docking for Detoxifying Enzyme Studies

Rafael Trindade Maia and Vinícius Costa Amador

Additional information is available at the end of the chapter

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Abstract

In this chapter, we pointed some relevant results obtained by protein-ligand docking simulations in the context of insecticide and herbicide resistance performed by glutathione S-transferases (GSTs), a detoxifying superfamily enzyme. We present here some in silico evidences of GST binding against chemical insecticides in the malaria and dengue vectors (*Anopheles gambiae* and *Aedes aegypti* mosquitoes) and against chemical herbicides used on rice (*Oryza sativa*) culture. Our findings suggest that some members from epsilon class (GSTE2, GSTE5) can metabolize some insecticide compounds and that a tau class member (GSTU4) can metabolize some herbicides. The results reinforce the importance of docking studies for enzyme activity comprehension. These information can allow in the future the implementation of new strategies for mosquito control and herbicide management on rice culture through biotechnological improvements designed to specific GST targets. Induced mutations on catalytic binding sites of GSTU4 could improve rice herbicide resistance and minimize produce damage, while rational compounds can be designed to inhibit GSTE members to decline insecticide resistance on mosquito control. In both cases, biotechnological tools could be developed focusing on GSTs that would reduce environmental impact by the use of insecticide and herbicide.

Keywords: GSTs, insecticide resistance, herbicide resistance, AutoDock, detoxifying enzymes, mosquito control, rice culture, bioinformatics

1. Introduction

Mechanisms of resistance to chemical insecticides include the pathways of metabolization of toxic compounds, because of overexpression of detoxification enzymes or structural modifications in these enzymes. Glutathione S-transferases (GSTs) are one of the most important groups of enzymes involved in this type of resistance and comprise enzymes that catalyze reactions that transform various xenobiotic compounds into soluble products [1].

In eukaryotic organisms, these enzymes are classified into cytosolic GSTs, microsomal GSTs (associated with membranes), and mitochondrial GSTs [2, 3]. In insects, only two of these classes were found: cytosolic and microsomal [4]. In the present study, we found no GST of the mitochondrial class in insects to date [5, 6]. Microsomal GSTs catalyze reactions very similar to cytosolic ones, with trimeric structure and being associated with plasma membranes, although they have different structures and origins than cytosolic one [7, 8]. However, cytosolic GSTs have already been identified as important for resistance to chemical insecticides [5, 9, 10], while microsomal GSTs have not yet been related to resistance to insecticides [5].

In insects, cytosolic GSTs are represented, at least, by six classes: delta, epsilon, omega, sigma, theta, and zeta [5, 11, 12]. In the present study, it was found that these genes were found to be similar to those of other species, such as the *A. gambiae* malaria vector and the fruit fly *Drosophila melanogaster* [11]. The delta and epsilon classes are arthropod-specific and represent more than 65% of the total cytosolic GSTs found in these organisms [11]. Most GSTs found in insects and involved in the target (omega, sigma, theta, and zeta) have a much broader distribution between taxonomic groups, from bacteria to vertebrates [13, 14].

Members of delta, sigma, and epsilon classes were initially called class I, II, and III, respectively, and later, with the increase in the number of sequences deposited in databases and classification studies, the nomenclature was adopted based on the Greek alphabet in agreement with the system of nomenclature of GSTs of mammals [15].

This classification was supported by phylogenetic analyses in both mammalian and insect GSTs [4, 13]. Currently the nomenclature of insect GSTs consists of three parts: the name of the species of which GST belongs, the specific class of GST, and the number that specifies the order in which the routine was discovered. In this way, the name AgGSTD1 is used to designate a GST of *A. gambiae*, member of delta class, being the first protein of this class to be discovered [12].

Cytosolic GSTs are composed of two subunits of approximately 25 kDa each, which may be homodimeric or heterodimeric. Each subunit has a specific glutathione binding site (G-site), near an electrophilic site (H-site). The G-site is located at the N-terminus of the protein and is a highly conserved region in the GSTs. However, the H-site residues that interact with the hydrophobic substrates are found at the C-terminus. The H-site diversity causes the GSTs to present different specificities in relation to the substrates they metabolize [16, 17]. The GST-catalyzed reaction consists of promoting the conjugation of the reduced glutathione tripeptide (GSH) to a specific and generally cytotoxic compound which, upon binding to such electrophilic grouping, will pass from the reduced state to the oxidized state and form a more soluble compound and easier to excrete from the cell. This phase of conjugation represents phase II of the cellular detoxification process, and the GSTs represent the most important enzymes of this phase, although others are involved. The GST enzymes display a big variety of substrate catalytic reactions. As multispecific and promiscuous proteins, the GSTs represent potential targets of inhibitors selection and design. In *Aedes aegypti*, hematin binds to GSTs resulting in activity inhibition [18].

Molecular docking is a computational technique that aims to predict the best orientation between two molecules. Usually, one of the compounds is small compound that is bounded to a macromolecule (protein). This powerful approach is an excellent tool that helps to understand relevant physiological processes in a wide range of organisms and systems, such as

insecticide and herbicide resistance. Molecular docking is based on molecular recognition and often is referred as a “lock-and-key” problem. In general, the best-fit orientation is obtained by shape complementarity and a score function based on binding energy affinity. In protein-ligand simulations, dockings generally are applied in a stochastic search algorithm to achieve the best binding complexes, and the energy can be estimated by molecular mechanic force fields.

2. Molecular docking between mosquitoes' GSTs and chemical insecticides

The atomic coordinates of AgGSTE2 and AgGSTE5 were from their respective PDB files, as well as their ligand, the tripeptide glutathione, or GSH (C10 H17 N3 O6 S). The geometry of the ligand was obtained from the PDB database.

An isoform of AgGSTE2 (AgGSTE2mut) with two mutations, I114T and F120L (isoleucine for threonine at residue 114, phenylalanine for leucine at amino acid 120) was also submitted to the simulations. The three proteins (AgGSTE2, AgGSTE2mut, and GSTE5) were simulated with and without the GSH linker. For the construction of the mutant (AgGSTE2mut), the nonmutant protein geometries (AgGSTE2) were used, and the residues in the PDB file were replaced manually in the two subunits.

The receptors used in the docking analyses were the crystallographic structure of AgGSTE2 and its mutant (AgGSTE2mut) and the structure of the model constructed for AgGSTE5. The ligands used were the insecticides DDT, carbaryl, cypermethrin, and malathion, being all these synthetic and commercially used organic insecticides normally used to control Culicidae vectors (**Table 1**). The atomic coordinates of the compounds were obtained from the ZINC database (<http://zinc.docking.org/>).

Molecular docking is a computational technique that aims to calculate atomic interactions between a small binding molecule and a macromolecule in search of the lower energy conformation. The AutoDock 4.2.2 program [18] was used to convert the files into PDB format for the form *pdbqt*, which is the file format used by AutoDock. The ligands were marked with *Gasteiger* load parameters and only the nonpolar hydrogens explicitly represented. The *Gasteiger* charge parameters provide charges properties of each atom, by the *SetPartialCharge* method, an algorithm that includes partial charges. In this algorithm, it

Singlet	Name	Access number
DDT	Dichlorodiphenyltrichloroethane	ZINC01530011
Carbaryl	1-Naphthyl methylcarbamate	ZINC00001090
Cypermethrin	Cypermethrin	ZINC71789490
Malathion	Malathion	ZINC1530800

Table 1. Compounds used as ligands for the calculation of docking.

is admitted that all hydrogens are explicitly represented and based on electronegativity equilibration. The *Kollman* set parameters were used to assign the receptor molecules. This force field uses values for each amino acid that was derived from the corresponding electrostatic potential. The simulations were performed with the Lamarckian genetic algorithm (LGA). The box was set in the 126×126×126 dimensions centered on the ligand and the active site, and the LGA was subjected to calculations of 10,000 replicates with populations of 150 individuals to a maximum of 27,000 generations and crossover mutation rates of 0.02 and 0.08, respectively.

The binding energies between the three proteins and the five different compounds studied were calculated and are available in **Table 2**. The lower energy conformations of each complex were visually analyzed (VMD, visual molecule dynamics) and was listed all residues in radius of 4.0 Å of the ligand (**Figures 1–4**).

The lowest energy was observed in the AgGSTE2muT-DDT complex, indicating a greater affinity between this enzyme and this insecticide. The observed distance between DDT and GSH (<4 Å) and position shows that this conformer is a potential candidate to metabolize DDT. The binding energy of this complex was the smallest among all comparisons. In the docking with the DDT, we observed a few higher energies for AgGSTE2 and AgGSTE5 when compared with the AgGSTE2mut values, but the values in both were negative. The distances between DDT and GSH in these conformers shows a value which allows for interactions, with AgGSTE5 being the shortest distance (2.91 Å) observed in complexes simulated with DDT. In all three enzymes, an approximation was observed between the trichloromethyl group of DDT and GSH, evidencing the ability of these enzymes to bind to this insecticide.

For carbaryl, the enzyme with the lowest binding energy was AgGSTE5, followed by AgGSTE2mut and AgGSTE2. However, it was the AgGSTE2mut that showed the conformation with the smallest distance between the ligands. The proximity of carbaryl to glutathione suggests that the three systems can form GSH conjugated with this insecticide.

In simulated complexes with cypermethrin that were observed, the lowest energy values were used, except for the AgGSTE2mut whose lowest energy score was for the DDT simulation. In the conformations of AgGSTE2 and AgGSTE2mut, the binding distances between cypermethrin and GSH were 3.39 and 2.74 Å, respectively, showing a potential of these enzymes to metabolize cypermethrin. In AgGSTE5, the distance between the ligands was 4.81 Å, indicating that although the enzyme has insecticide-binding affinity, the likelihood of the glutathione conjugation reaction is low.

Malathion, despite having demonstrated negative values when complexed with enzymes, was the compound that showed the highest energy values for all three systems. In addition, no

	DDT	Carbaryl	Cypermethrin	Malathion
AgGSTE2	-5.13	-5.85	-8.37	-3.37
AgGSTE2mut	-9.16	-6.09	-8.81	-3.67
AgGSTE5	-7.68	-6.42	-8.64	-3.24

Table 2. Binding energies (kcal/mol) for the best conformations of each complex.

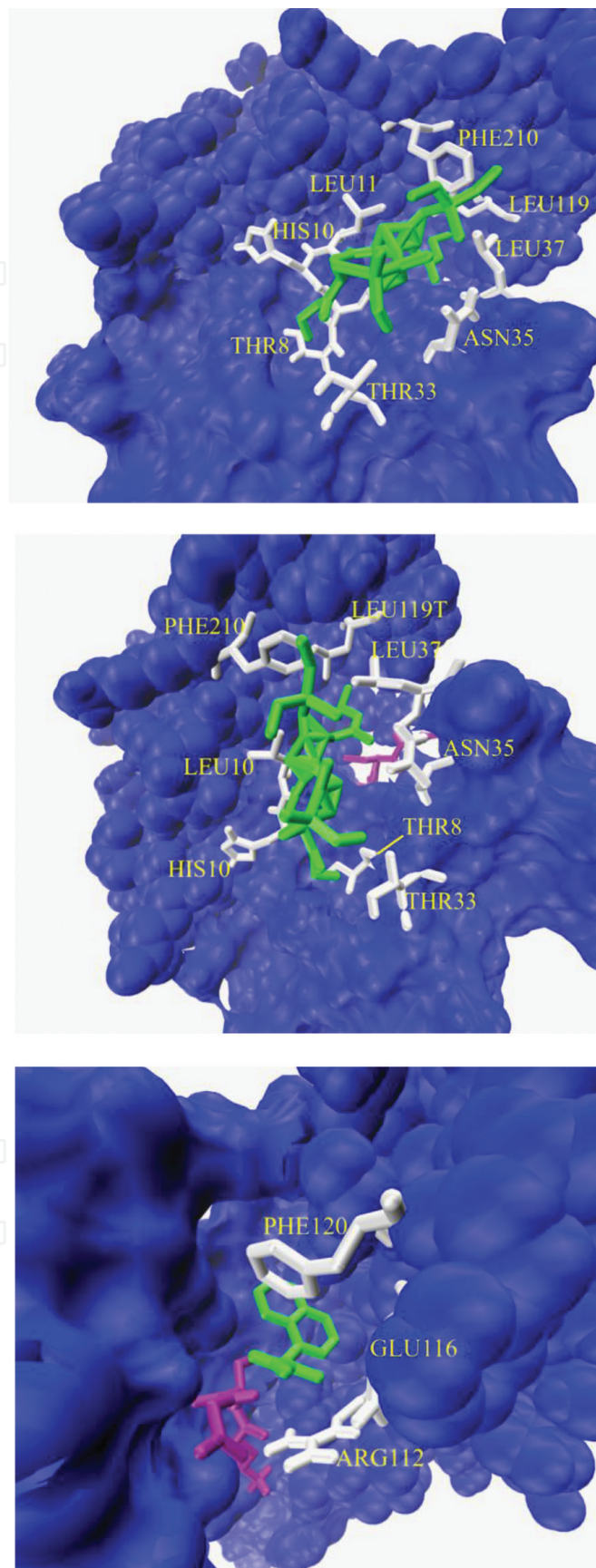


Figure 1. Representation of the best conformation of the AgGSTe2-carbaryl (top), AgGSTe2mut-carbaryl (middle), and AgGSTe5-carbaryl (bottom) complexes. Residues are represented in rods and spheres. The GSH is represented in sticks (purple). In green the carbaryl.

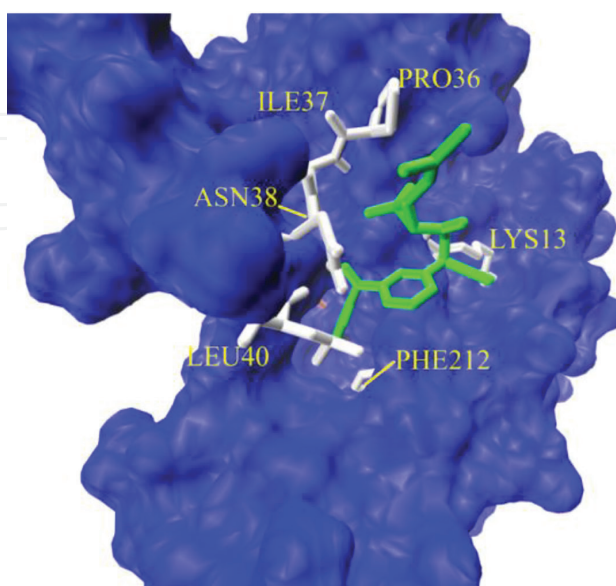
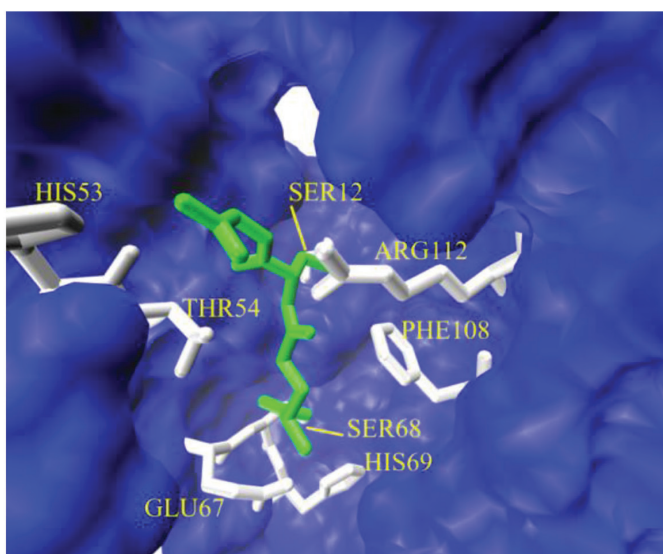
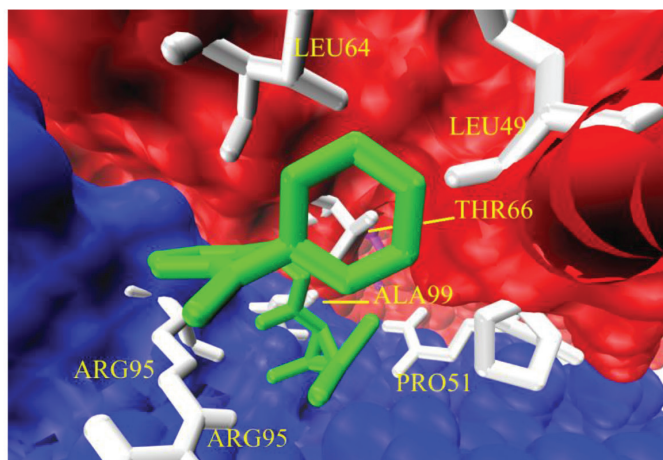


Figure 2. Representation of the best conformation of the AgGSTE2-cypermethrin (top), AgGSTE2mut-cypermethrin (middle), and AgGSTE5-cypermethrin (bottom) complexes. Residues are represented in rods and spheres. The GSH is represented in sticks. In green the cypermethrin.

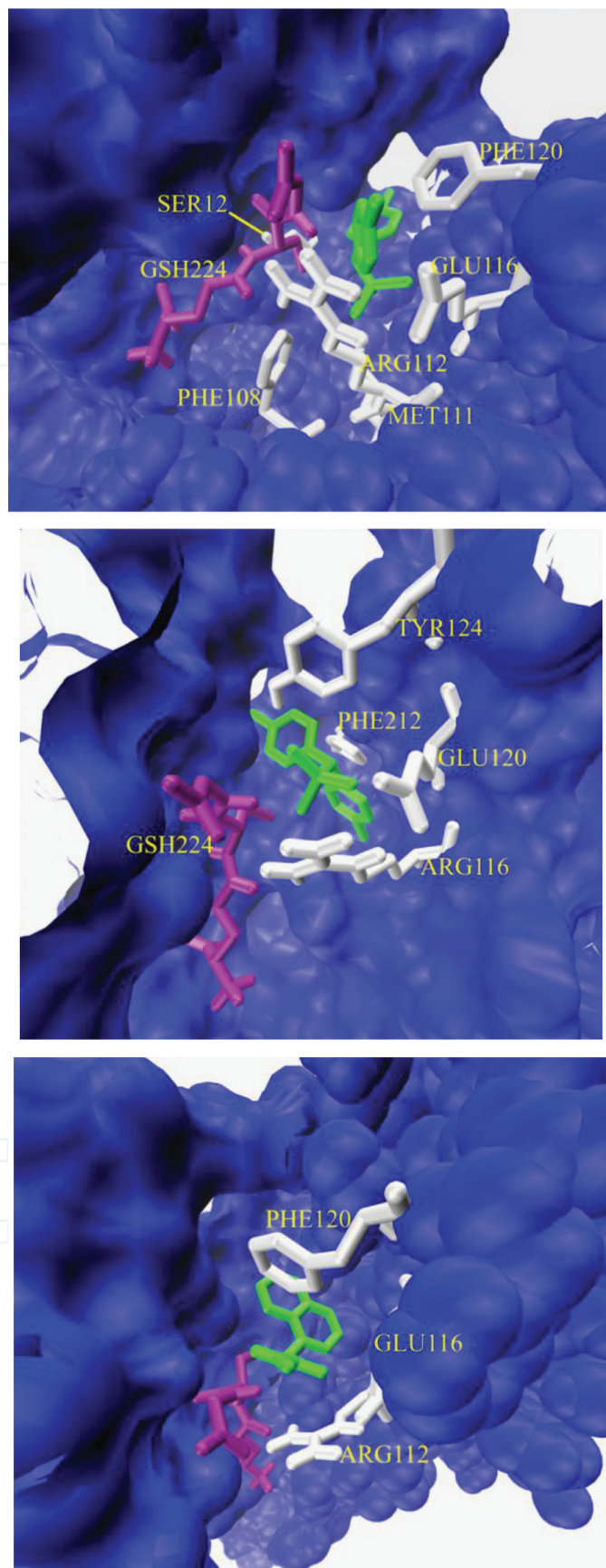


Figure 3. Representation of the best conformation of the AgGSTE2-DDT (top), AgGSTe2mut-DDT (middle), and AgGSTe5-DDT (bottom) complexes. Residues are represented in rods and spheres. The GSH is represented in sticks. In green the cypermethrin.

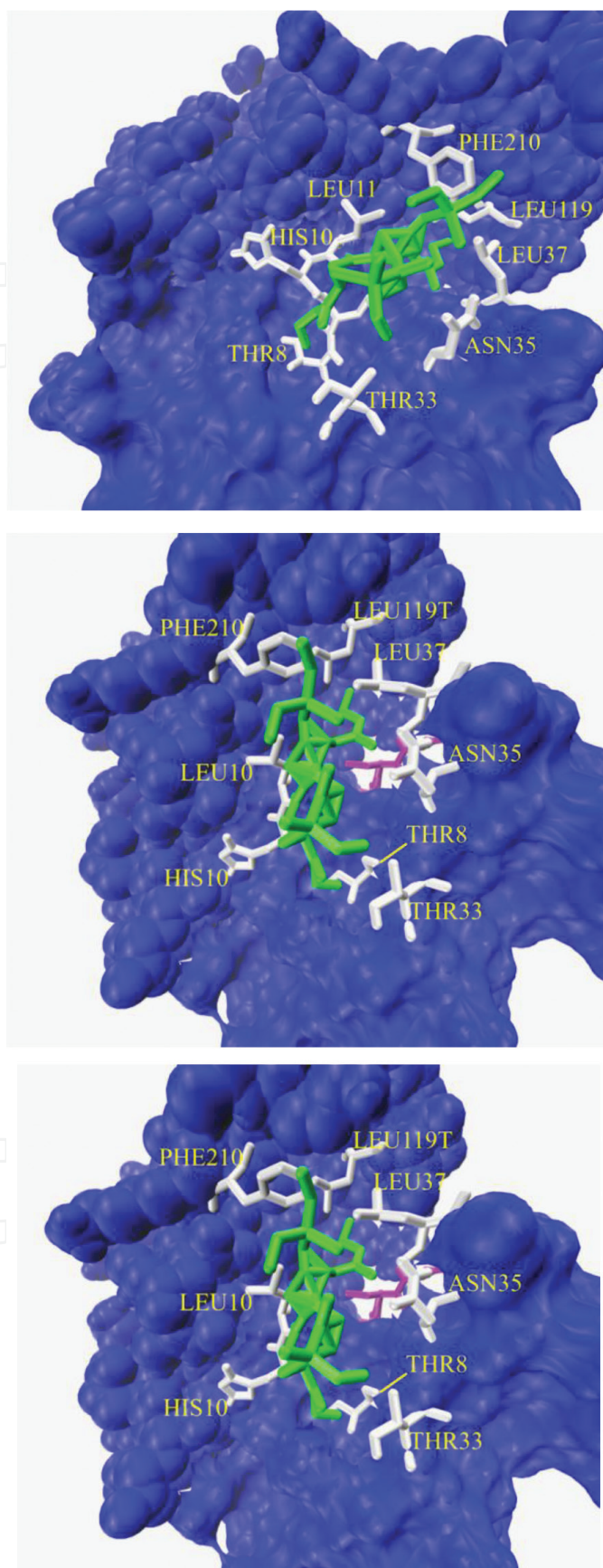


Figure 4. Representation of the best conformation of the AgGSTE2-malathion (top), AgGSTE2mut-malathion (middle), and AgGSTE5-malathion (bottom) complexes. Residues are represented in rods and spheres. The GSH is represented in sticks. In green the cypermethrin.

reasonable proximity of GSH (AgGSTe2 = 8.10 Å; AgGSTe2mut = 9.57 Å; AgGSTe5 = 5.26 Å) was observed in any of the conformers, which rule out the possibility that one of these enzymes could metabolize the malathion.

The docking results showed that the three enzymes have affinity for compounds of different nature. In fact, this represents an *in silico* that these enzymes show a remarkable functional promiscuity, resulting from a multi-specificity to the substrate. Although the AgGSTe2mut presented the lowest values for five of the seven compounds submitted to the docking calculation, the values did not differ much. When comparing the two isoforms, it was observed that for six of the seven compounds tested, the mutant enzyme had slightly more favorable energies than the wild type. The most plausible explanation for this result lies in the fact that AgGSTe2mut has a higher catalytic site resulting from the mutations in this enzyme, which probably allows a better accommodation of the compounds.

The docking results showed that the three enzymes have affinity for compounds of different nature. In fact, this represents *in silico* evidence that these enzymes show remarkable functional promiscuity, resulting from multi-specificity to the substrate. Although the AgGSTe2mut presented the lowest values for five of the seven compounds submitted to the docking calculation, the values did not differ much. When comparing the two isoforms, it was observed that for six of the seven compounds tested, the mutant enzyme had slightly more favorable energies than the wild type. The most plausible explanation for this result is that AgGSTe2mut has a larger catalytic site volume, resulting from the mutations in this enzyme, which probably allows a better accommodation of the compounds.

The multi-specificity presented by these enzymes, especially AgGSTe2mut, may represent an important aspect in the ability of *A. gambiae* to have populations resistant to chemical insecticides. This is a recent concept [19] and should be taken into account in future studies of the molecular evolution of enzyme superfamily. The use of chemical insecticides in this species needs to be rethought and reevaluated as a mode of control. A future perspective may be on the potential of development of specific inhibitors for these enzymes, in an attempt to decrease the response to the insecticides used, especially DDT. Another aspect that evidences the potential of the epsilon class GSTs as targets for inhibition is the fact that this class of enzymes is specific to arthropods, which enables the further development of inhibitory compounds that do not affect other species, such as mammals. Understanding the mechanisms of evolution and adaptation of these enzymes and details of their dynamics and functioning is indispensable when planning a rational and integrated control of a vector species. Another possible application is to use these enzymes as indicators of resistant populations and refractory to various insecticides and thus to choose the best type of compound to be used for each population.

3. Molecular docking between a rice GST and chemical herbicides

It is known that the superfamily of glutathione S-transferases (GSTs) gives rice (*Oryza sativa*) a catalytic action, protection against biotic and abiotic stress [20, 21]. The inactivation of the toxic effects of herbicides on plants has different defense systems [22]. Another study [23] has shown that the GST enzyme is associated with several crop herbicides' harmful effect tolerance,


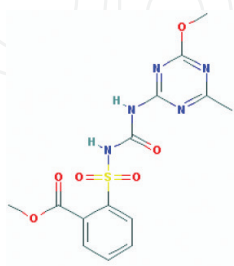
promoting the resistance of grasses to its chemicals substances. In plants GST is also responsible through the metabolism of a huge name of commercial important herbicides [24] reducing damage that could occur through the toxically herbicides' action [25]. The reaction consists of the conjugation of the tripeptide glutathione to a hydrophobic compound, making it more soluble and less toxic [26], maintaining the cellular homeostasis. For this study two herbicides were selected, metsulfuron and bentazon sodium.

The herbicide metsulfuron-methyl belongs to the group of sulfonylureas and acts on the enzyme acetolactate synthase (ALS), consequently inhibiting the synthesis of the amino acids leucine, valine, and isoleucine, interfering in the protein synthesis and inducing the death of the plant by interfering in the cellular division. Among its properties, it is reported that metsulfuron-methyl has a systemic action and is rapidly absorbed by the whole plant, besides presenting selectivity to the crops for which its use is recommended. In susceptible plants, the absorption of this herbicide results initially in growth stoppage; due to the rapid translocation of this group of molecules to the meristems, apices, and later, death is inevitable, considering the impossibility of the essential amino acid biosynthesis to the plant. This mechanism inhibition of ALS was elucidated due to works done and published [27, 28].

Bentazon is a herbicide from the benzothiazinone class, which, after being absorbed, interferes in the photosynthesis process and is therefore a photosystem II photosynthesis inhibitor, affecting the carbohydrate synthesis in leaf areas that have received treatment, occasionally and may occasionally lead the plants to death, especially when they are in the early development stage. The photosynthesis inhibitors mechanism action is the removal or the inactivation of intermediary charge carriers from the electron transport process, and are considered to be inhibitors of electron transport [28]. The inhibitory mechanism of photosynthesis results in the blockade of the electron transport of the compound QB component of the photosynthetic system and, thus, makes impossible the occurrence of electron transport to plastoquinone B [29]. The aforementioned blockade occurs through the binding of the herbicides to the active site of QB in the D1 protein belonging to photosystem II, located on the membranes of the thylakoids of the chloroplasts. This process interrupts the fixation of CO₂ and interferes in the production of essential elements to the plant growth, such as ATP and NADPH₂; however, plant death usually occurs due to other factors. The interruption of the electron flow in photosystem II promotes a significant increase in the energy status of the chlorophyll, resulting in a state called "triplet," which causes an energy overload derived from the attenuation effect of the carotenoid pigments, and this characterizes the peroxidation process. In other study [30], lipid peroxidation due to excess triplet chlorophyll may occur through two mechanisms: direct formation of lipid radicals in unsaturated molecules of fatty acids constituting membranes and production of singlet oxygen through the reaction of chlorophyll triplet with oxygen. In both cases, the peroxidation process will corroborate with damage to cell membranes.

3.1. Molecular docking of rice GST and herbicides

The atomic coordinates of the compounds were obtained from the ZINC database (<http://zinc.docking.org/>) on .mol2 file extension (Table 3).

Herbicide name	Molecular formula	2D structure	Access code
Bentazon-sodium	C ₁₀ H ₁₂ N ₂ O ₃ S		ZINC05442053
Metsulfuron-methyl	C ₁₄ H ₁₅ N ₅ O ₆ S		ZINC01532069

Source: ZINC database (<http://zinc.docking.org/>).

Table 3. Compounds used as ligands for the calculation of docking.

The *.mol2* files were converted to *.pdbqt* in AutoDock 1.5.6 (<https://www.chpc.utah.edu/documentation/software/autodock.php>) and had the polar hydrogens removed, and their molecules were flagged with the Gasteiger parameters [31]. The structure of OsGSTU4 was obtained from a *.pdb* file modeled using homology which was converted to *.pdbqt* file in AutoDock and added hydrogens and Kollman load parameters [32, 33]. For this step, glutathione was treated as a cofactor. The docking calculations were run in AutoDock 1.5.6 program, and the simulations were performed using the Lamarckian genetic algorithm (LGA). In this work, the LGA was used in conjunction with the Goodford method, allowing simultaneous sampling of the ligand configurational space and calculating the receptor and ligand atomic interaction energy [34, 35]. The grid parameters are established in 126×126×126 Å by the program Autogrid (<http://autodock.scripps.edu/wiki/AutoGrid>) and receiver-centered (GST). The parameters used for simulations were as follows: 10,000 replicates, energy analyzes per 1,500,000 and 27,000 generations, population size of 150, and mutation rates and crossing over of 0.02 and 0.08, respectively. Ten conformations were generated that were ranked based on the lowest energy and analyzed in the VMD (<http://www.ks.uiuc.edu/Research/vmd/>).

4. Results

The docking result for the herbicide metsulfuron-methyl, performed in the AutoDock program, ranked ten possible complexes; **Table 4** shows the best possible complex. This procedure is based on intermolecular energy, binding energy, and hydrogen bond scores, showing the atoms (and residues) of the protein and the ligand that present favorable interactions for the model.

Binding energy (kcal/mol)	Intermolecular energy (kcal/mol)	Hydrogen bond
-3.74	-5.53	B: LYS 111 HZ1-O2 C: GTX1226 H11-N3 B: LYS 111 HZ2-O6 C: LYS 56 HZ1-O2

Source: Research data.

Table 4. Results of AutoDock-ranked complexes in the metsulfuron-methyl docking.

In metsulfuron-methyl, binding energies were lower than those of bentazon. The results revealed by the metsulfuron-methyl docking show that some residuals (LYS 111, LYS 56, GTX1226) were extremely favorable, being these possibly anchor residues for the binding, in combination with results evidenced by previous studies. The identification of the GTX1226 molecule as an anchor residue (**Table 5**) is evidence of a possible conjugation process [36] between metsulfuron-methyl and glutathione, evidencing the possibility of detoxification of metsulfuron-methyl by OsGSTU4. The best complex result ranked by the AutoDock for metsulfuron-methyl can be visualized in **Figure 5**. The image shows a zoom in a pocket where probably conjugation occurs by a hydrogen bond between bentazon and glutathione. The complex generated suggests that the OsGSTU4 displays a relevant role on the resistance for this herbicide (**Figure 5**).

The result of the docking performed for the herbicide bentazon sodium, also executed in the AutoDock program, is presented in **Table 6**. This procedure is the same used for metsulfuron-methyl and is also based on intermolecular energy, binding energy, and hydrogen bond scores, showing the atoms (and residues) of the protein and the ligand that present favorable interactions for the mode (**Figure 6**).

The results of **Table 6** also show the identified repeated residue (GLN 75) that presents the lowest binding energy, possibly showing as an anchor residue for the herbicide bentazon sodium, corroborating with the results obtained on previous studies [37].

Near residue atoms	Reference atoms (ligand)	Respective distance (Å)
ASP110: O	<0>:C14	3.43
GLU69:OE2	<0>:C5	2.95
LYS56:HZ1	<0>:O2	1.91
LYS111:HZ1	<0>:O2	1.91
GLN134:OE	<0>:C10	2.87
HIS54:HE2	<0>:C5	3.91

Source: Research data.

Table 5. Representation of the atoms of near residues belonging to metsulfuron-methyl, atoms used as corresponding in the ligand and their respective distances in angstroms in the output.

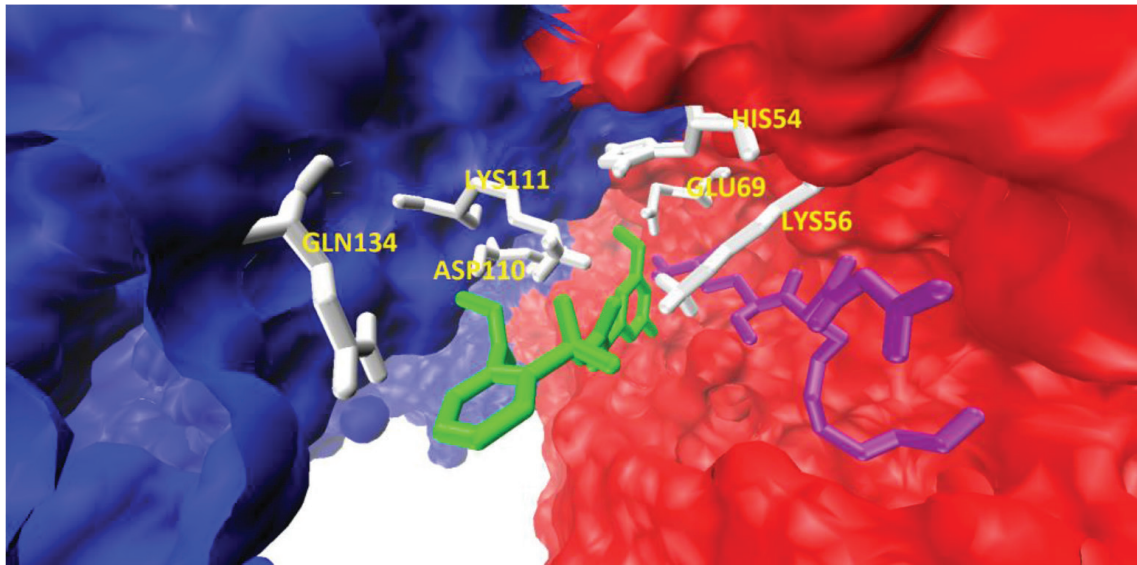


Figure 5. Deep view of catalytic site. In red, the chain A; in blue, the chain B. In green, the metsulfuron. Glutathione (purple) and residues (white) from H-binding-site, an interchain region. Source: Research data.

Binding energy (kcal/mol)	Intermolecular energy (kcal/mol)	Hydrogen bond
-0.86	-1.16	B: GLN 75 HE21-O3

Source: Research data.

Table 6. Results of AutoDock-ranked complexes in the bentazon sodium docking.

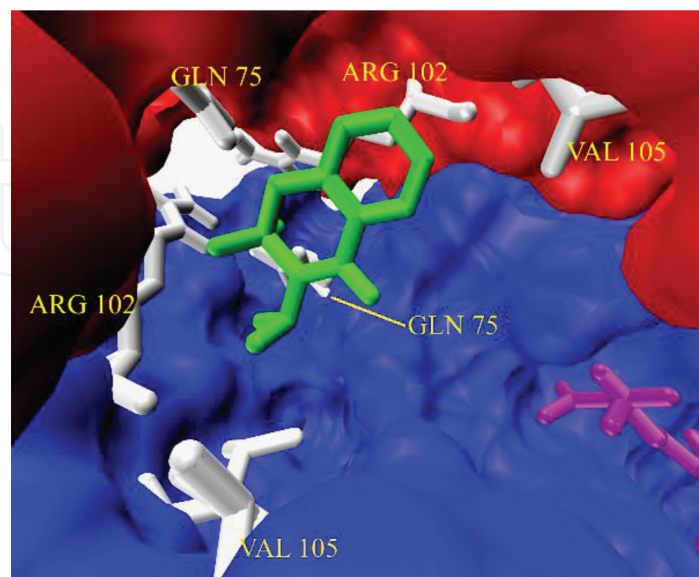


Figure 6. Deep view of catalytic site. In red, the chain A; in blue, the chain B. In green, the bentazon. Glutathione (purple) and residues (white) from H-binding-site, an interchain region. Source: Research data.

Near residue atoms	Reference atoms (ligand)	Respective distance (Å)
Val105:CG'	<0>0:C8	3.38
ALA106:HN	<0>0:C7	3.25
ARG102: O	<0>0:C7	2.79
VAL105:CG'	<0>0:C1	3.58
ALA106:HN	<0>0:C1	3.45
ARG102:HE	<0>0:O2	3.71
GLN75:2HE2	<0>0:N2	2.11
GLN75:1HE2	<0>0:O3	1.78

Table 7. Representation of the atoms of near residues belonging to bentazon sodium, atoms used as corresponding in the ligand and their respective distances in angstroms in the output.

Figure 6 depicts a catalytic cavity where a conjugation with metsulfuron may occur. In the image, the complex with lower binding energy was chosen. The interaction with glutathione is made by a hydrogen bond. This is evidence that OsGSTU4 is able to bind to metsulfuron in order to promote the conjugation reaction. Theoretically, this enzyme plays an important role in the resistance to this herbicide.

Complementing the information in the figure information, **Table 7** shows the atoms of surrounding amino acid residues at distances less than 4 Å and their respective distances to atoms of the ligand.

5. Conclusions

Molecular docking has proved to be an extremely useful technique for studying GSTs, especially in the context of resistance to chemical insecticides and herbicides. The methodology applied in these studies may be excused for other GSTs and other compounds. The complexes obtained provide a better understanding of the detoxification process performed by these enzymes.

However, although we find strong evidence of metabolization of these compounds, experimental studies should be undertaken to validate the *in silico* experiments. Site-directed mutation studies can be extremely providential to complement the information obtained here.

Not surprisingly, we notified that the GSTs here studied showed an affinity for more than one compound. This corroborates with the fact that members of this enzyme family display a multi-specificity on their H-binding-site.

As promiscuous proteins, these GSTs may be involved in metabolization of a wide range of toxic compounds, including other insecticides and herbicides. Further studies must be performed to investigate this.

Once the herbicide and insecticide resistance are multigenic, multi-enzymatic, and multifactorial process, the molecular docking technique can help to elucidate other pathways. Other computational techniques, such as molecular dynamics, can also give more insights about these systems.

Since herbicide and insecticide resistance is one of the major constraints of agriculture and mosquito control, the information from this study may be extremely useful for the development of specific inhibitors for these GSTs, thereby reducing the amount of herbicides and insecticides to be used and consequently reducing the environmental impact and other side effects.

New strategies of control can be applied too. The results point these enzymes as very promising targets for iRNA technique.

The molecular docking is a powerful approach for understanding the interactions of molecules, and it is useful to elucidate biochemical processes. In the field of molecular modeling, this tool is an option of rapid, with low computational requirements, to perform molecular simulations of many systems. Many software, including the commercial ones, have been developed, and new algorithms are quickly incorporated to the packages. In the fields of computational biology and bioinformatics, it has become one of the most popular tools, with a wide range of applications. The diffusion of this amazing technique is a great strategy on the advance of molecular studies and must be applied in many fields of knowledge.

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